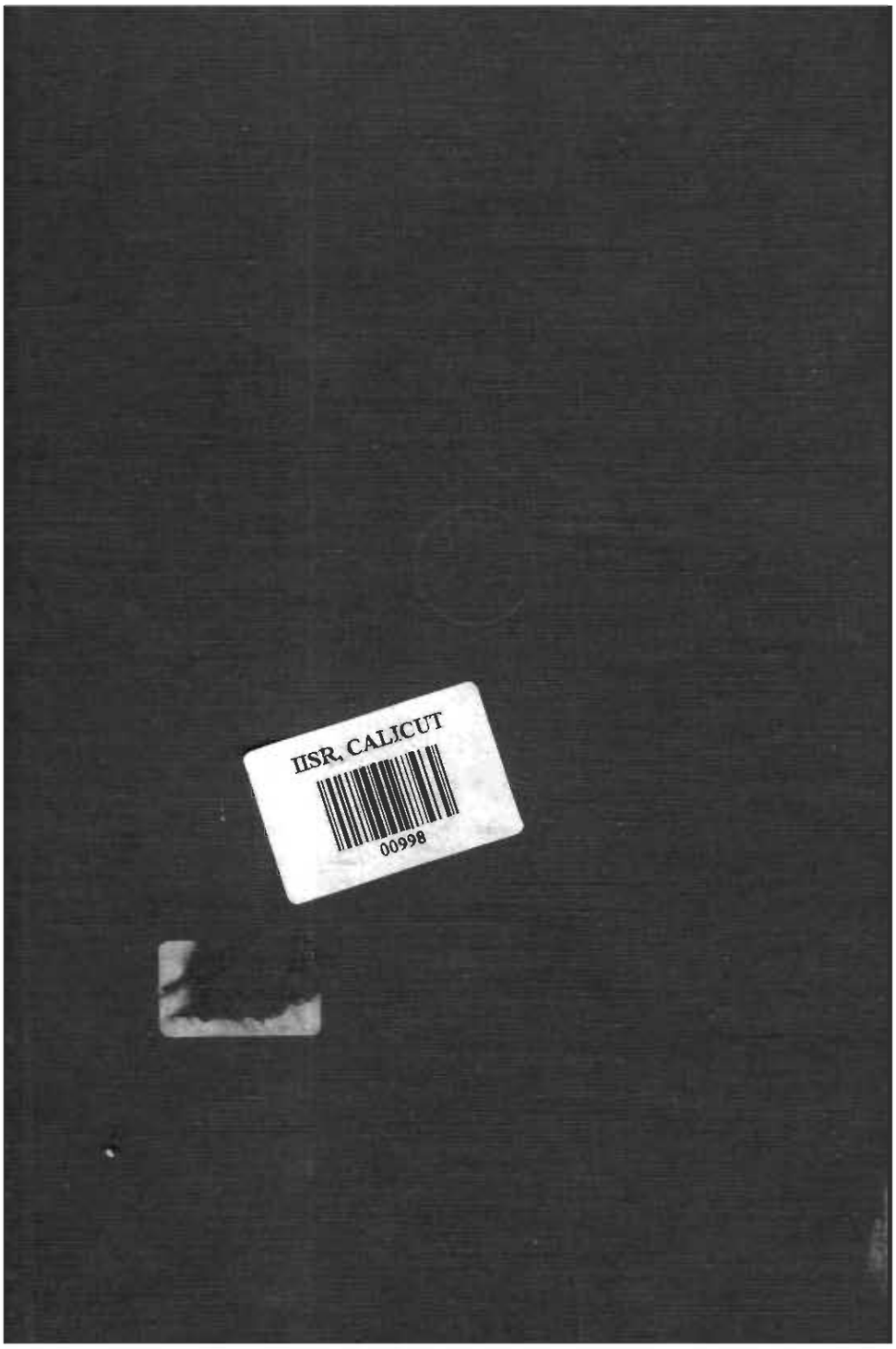

THE FUNGI

VOLUME I

The Fungal Cell

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THE FUNGI

An Advanced Treatise

Edited by

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VOLUME I

The Fungal Cell

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Preface

Fungi are ubiquitous organisms which play an essential part in the conomy of nature and profoundly affect human affairs. Traditionally assified as plants, fungi usually receive scant mention, among lower yptograms, in the average botanical textbook, and in academic texts there a tendency to give merely a detailed taxonomic account of the group in- rspersed with phylogenetic speculation. While there are satisfactory texts n the physiology of fungi, on fungi as pathogens of plants, animals, and an, and on other aspects of pure and applied mycology, there is no satis- ctory modern general treatment, a deficiency which this volume, and its o companions, attempt to correct.

So much has been written about fungi that it is now impossible for a ngle author to give an acceptable comprehensive and balanced account of em in depth. Recourse must be made to a range of specialists and this, owever eminent the specialists, introduces difficulties regarding variation n treatment and integration.

The object of this work is not to supply a series of articles on "recent dvances" on diverse aspects of fungi or to review the major divisions of pplied mycology but rather to summarize what is known about fungi as ungi, with the major exception that in the third volume taxonomic prin- iples only will be treated. This work, as the subtitle implies, is also in- ended as a reference book, and doubtless many users will select such hapters or sections as are relevant to a present need. The work has, how- ver, been planned as a whole and by reading the chapters consecutively he three volumes will, it is hoped, give a logically developed account of ungi as living organisms.

This first volume deals with events at the cellular level; in the two suc- eeding volumes the fungus organism and fungal populations will be treated. A number of topics therefore recur at the different levels of organization, nd these may be traced via the subject indexes and the indexes to Fungi, Lichens, and Actinomycetes which appear in the volumes.

Those who delve into any topic in any of the volumes and who feel a need to refresh their memories on generalities regarding fungi and fungal structure should first read Chapter 2 of this volume.

The editors are most grateful to the many authors whose labors have made this joint effort possible, and hope that the readers will feel the venture to have been worthwhile.

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April, 1965

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NUTRITION AND GROWTH OF CELLS

THE FUNGI

VOLUME I

The Fungal Cell

Introduction

CHAPTER 1

Historical Introduction to Mycology

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Why, it may be asked, should an attempt to survey current knowledge of the fungi begin with a backward glance? Scientists build on foundations laid by their predecessors and, as Aldous Huxley noted when discussing the literary style of his grandfather, they show great reluctance to inspect these foundations. One aspect of the study of history is to elucidate the present, and—owing, at least in part, to the major effects of science throughout the world today—the historian of science is becoming an increasingly fashionable figure. Although at a first reading histories of science may induce complacency in the bench-working scientist who is struck by the failure to accept the obvious and the lack of imagination shown by many of his predecessors, imaginative reflection on the climate of opinion and on the technical limitations under which pioneers work is likely to induce a more humble reaction and the certainty that many research workers today will appear to posterity to have been equally obtuse. If this makes for a greater suspicion of authority and less conformity to prevailing fashions, and if it leads to the doubts and uncertainties that frequently are the first steps to new knowledge—all to the good.

As the history of science cannot be divorced from social and economic studies, so the development of mycology has not been an isolated phenomenon within science. Deeper understanding of fungi has frequently depended on advances in physics or chemistry, and mycology has always been greatly influenced by botany, of which traditionally it has been considered a branch. The history of mycology cannot here be treated comprehensively; at the risk of oversimplification, only a few main themes are traced.

I. FUNGI AND THE CLASSICS

Fungi are of ancient lineage and have a fossil record that extends back to the Devonian and Pre-Cambrian eras. They must, along with plants and animals, have been noticed by primitive man, but during historical times there seems to have been little change in human nature. Man has always been impressed by calamity, and the earliest written records of fungi are not of the fungi themselves, but of their depredations. There are records of plant diseases in the Vedas [1200 B.C.] and of blasting and mildew in the Bible [e.g., Amos, 4, 19] which also [Lev., 14, 34–48] gives detailed, practical instructions to the priest for the treatment of "leprosy in a house" (probably dry rot). From earliest times man must, presumably, have made use of fungi as food; but, again, what is possibly the first written reference to a fungus is the death from fungus poisoning at Icarus on one day of a mother, daughter, and two full-grown sons, an event which Euripides [450–456 B.C.] commemorated by an epigram.

Many references to fungi in the Greek and Roman classics have been conveniently summarized by Buller (1915a). Fungus diseases of plants feature prominently, and the Romans held a spring festival, the Robigalia, to ward off the "rubigo" or rust, "the greatest pest of the crops," according to Pliny [ca. 23–79 A.D.]. There is also mention of the fungi as medicaments—including the greatly esteemed universal remedy "agaricum" (the fruit body of *Fomes officinalis*); but it is to fungi as food to which reference is most frequently made. The Romans considered fungi to be great delicacies and they distinguished a number of different sorts, including "boleti" (*Amanita caesarea*), which were cooked in special vessels or "boletaria," "fungi suilli" (*Boletus edulis*), and truffles (*Tuber spp.*), as well as the common field mushroom (*Agaricus campestris*). They also distinguished edible from poisonous sorts by tests of no greater reliability than those advocated in current folklore.

The Romans are also credited with the first illustration of a fungus. At Pompeii, buried by the eruption of Vesuvius in 79 A.D., excavations have revealed fungi (thought to be *Lactarius deliciosus*) illustrated in some of the frescoes. Like the potato, mushrooms have been the inspiration for pottery and sculpture. A series of ancient stone sculptures based on agaric sporophores, collected by the Wassons (1957) in Guatemala and other Central American countries (where ceremonial use has been, and still is, made of hallucinogenic mushrooms), have been dated 1000 B.C. to 200 A.D. Harshberger (1929) noted a second century A.D. Roman representation of an agaric, probably a species of *Amanita*, as part of an architectural ornament at Timgrad, Algeria. And recently Eichhorn (1962) has identi-

fied as *Amanita ovoidea* certain ancient Egyptian alabaster models of agarics of uncertain provenance now in the Vienna Museum.

II. THE HERBALISTS

The thousand years of the Middle Ages added little to the knowledge of fungi. The advent of printing in the fifteenth century led to the heyday of the printed herbal (Arber, 1938) during 1470–1670, the two centuries that preceded the modern era. The herbalists tended, at least at first, to look back. They were anxious to record in print the classical accounts of plants, particularly of materia medica, for many herbalists were physicians. They did, however, and with increasing frequency, show caution if not scepticism in accepting ancient tenets and at the same time added original observations and attempted fresh schemes of classification.

Many of the well known herbals mention, and not infrequently illustrate, a few fungi, such as the common mushroom, various truffles and "agaricum," but their inclusion is incidental and the treatment slight. In the words of "The Grete Herbal," 1526, "Fungi ben mussherons. They be colde and moyst in the thyrde degre and that is shewed by theyr vyolent moysture. There be two maners of them, one maner is deedly and sleeth them that eateth of them and be called tode stooles, and the other dooth not." Attention was paid also to such curiosities as the 'fungus stone" (*Polyporus tuberaster*) (Ramsbottom, 1932). Ergot of rye was noted by Lonicer as early as 1582, but its connection with ergotism—the St. Anthony's fire of the Middle Ages—was not established until the seventeenth century (Barger, 1931).

A. Clusius

The herbalist of greatest mycological importance is the versatile and much traveled Charles de l'Escluse (Clusius) [1526–1609], who was born at Arras and introduced to botany at the University of Montpellier. Subsequently he made an expedition to Spain and Portugal, lived for about fourteen years in Vienna, and finally became a professor at Leyden, from where he issued in 1601 a reedited and augmented version of earlier writings as "Rariorium Plantarum Historia," a fine folio, well printed by the Plantin Press at Antwerp. This book has a 28-page appendix on "Fungorum in Pannoniis Observatorum Brevis Historia" in which Clusius gives an account of the fungi found during his stay in Vienna in the Roman province of Pannonia, now parts of Austria, Hungary, and Yugoslavia. This first monograph on fungi has thirty-two woodcuts (mostly original) and includes many larger fungi recognizable today. To aid him in his mycological

studies Clusius had a series of watercolor drawings of fungi prepared, and these—the Code de l'Escluse, now preserved at Leyden—were published by Istvánffi (1900) together with a facsimile of the fungal section of the "Rariorum Plantarum Historia."

B. Van Sterbeeck

What may be considered to be the climax on this line of mycological advance, the first book devoted to fungi, was the "Theatrum Fungorum oft het Tooneel der Campernoelien," 1675, by Johannes Franciscus Van Sterbeeck [1630–1693]. Van Sterbeeck, whose family was of noble extraction, was born in Antwerp. In 1655 he was ordained priest, and for the next eight years he suffered from a chronic illness that enforced repose. He thus belonged to a profession which over the centuries has harbored many eminent mycologists, and he was also subject to a period of isolation, which so frequently has seemed to be essential for the maturation of an inquiring mind or a man of genius. During his illness Van Sterbeeck deepened his botanical knowledge and subsequently visited Dutch botanists; in 1663 he was himself visited by John Ray [1627–1705], who admired the rare plants in Van Sterbeeck's garden. From the beginning of his studies Van Sterbeeck paid special attention to fungi, and when in 1672 the Antwerp pharmacist and botanist Adriaan David showed him the celebrated Code de l'Escluse (then the property of Dr. Sijen, professor of botany at Leyden), he copied the designs and used many of them as a basis for the copperplate illustration of his "Theatrum Fungorum," a fact forgotten for some 200 years. Van Sterbeeck's objective in giving an account of the fungi was largely practical. He was anxious that "edible" fungi should be correctly identified and distinguished from the poisonous, and so the "Theatrum Fungorum" was written in Dutch.

III. THE MICROSCOPISTS

The introduction of the microscope, following improvements in lens making by Christiaan Huygens [1629–1695] and others, was a fundamental step in microbiology. The most familiar name in the development of microbiology is that of Antony Van Leeuwenhoek [1632–1723], a minor official of Delft, who himself constructed the microscopes and lenses with which he made the many original observations on microorganisms that he communicated in a series of letters to the Royal Society in London. These discoveries have been given wider currency during recent years by Dobell (1932). Van Leeuwenhoek was the first to see bacteria—in scrapings from the human mouth—and he also observed yeast cells. For the first illustra-

tions of microfungi, however, one must turn to Robert Hooke's [1635–1703] "Micrographia," 1665—"the most ingenious book I ever read in my life" according to Samuel Pepys [1633–1703], and one which kept the diarist out of bed until two in the morning. There, one well-known plate (Schem :xii) shows the sporangia of *Mucor* and the teliospores of rose rust (*Phragmidium mucronatum*). A few years later, Marcello Malpighi [1628–1694] devoted the greater part of a plate of this folio "Anatome Plantarum," 1679, to delineating molds now referable to *Rhizopus, Mucor, Penicillium,* and *Botrytis.* The next step, the exploitation of combined macroscopic and microscopic observations on fungi, was left to the Italian P. A. Micheli, who made observations that underlie several major subdivisions of mycology today.

IV. MICHELI

Pietro Antonio Micheli [1679–1737], according to the Latin description on his memorial in the church of Sante Croce, Florence, "lived 57 years and 22 days, happy though in moderate circumstances, an expert in natural history, a leading botanist of Tuscany, well known everywhere for his researches and writings, and much loved by all the worthy men of his age on account of his sweetness of disposition, and modesty." He came of poor parents, was self-taught, and was appointed by Cosimo III, the Grand Duke of Tuscany, as botanist in charge of the public gardens in Florence, his catalogue for which was published posthumously in 1748.

Micheli's most famous work, and the publication on which his reputation as an outstanding mycologist rests, is the "Nova Genera Plantarum," a quarto with 108 copperplates, published in 1729. Written in Latin, this volume, in which the genera are arranged after the method of J. P. Tournefort [1656–1708], was envisaged as the first part of a larger work, never completed. There are, however, sets of the copperplates illustrating marine algae, etc., for the second volume, in the British Museum and elsewhere, and much unpublished manuscript material is still available in Florence. Of the 1900 plants (1400 observed for the first time) enumerated in the "Nova Genera Plantarum," 900 were fungi; here space allows only some of the more important of Micheli's mycological contributions to be listed.

Among the new genera which he described and named, and illustrated most beautifully, were *Mucor, Aspergillus, Botrytis, Polyporus, Clathrus,* and *Geastrum.* He observed seeds (spores) in all groups of fungi, noted the quaternary arrangement of basidiospores on the gill surface of various dark-spored agarics (Tab. 73; fig. 1H), and was the first to describe asci and ascospores (in lichens) (Tab. 51; 56; fig. 1 A-D), and truffles (Tab. 102). He illustrated the mycelial cords attached to the fruit bodies of

agarics (Tab. 75) and was the first to describe cystidia, on the gill edge (Tab. 73; fig. 1B, C) (which he interpreted as being apetalous flowers), and to recognize that the function of the cystidia projecting from the gill surface of *Coprinus* (Tab. 73; fig. 1, I, K, L) was to keep the gills apart and allow spore dispersal. In a series of "observations" (translated by Buller, 1915b) Micheli described experiments in culturing fungi from spores. In June, 1718, he collected examples of many species of fungi which he had never seen coming up in the woods of the Boboli gardens in Florence, allowed spores from these collections to be deposited separately on the leaves of oak, ash, etc., and then kept the inoculated leaves under observation in various places in the Boboli gardens considered to be suitable for the production of fungi. Toward the end of October, the appropriate sporophores developed from several of these cultures. Such experiments he repeated with similar results, and in addition he sowed spores of *Mucor, Aspergillus,* and *Botrytis* on pieces of melon and noted the development of the characteristic sporulations. Finally he cut triangular pyramids of melon, and truncated pyramids of quince and pear with pentagonal and hexagonal bases (Tab. 95), and inoculated the faces of these with three, five, and six kinds of spores, respectively, which in due course produced "seeds" of their kind. He also recognized the contamination of his cultures by spores fallen on the pieces of melon by chance. These examples are sufficient to show that Micheli was much ahead of his time. Many of his observations were overlooked and had to be made anew up to a century or more later.

V. THE TAXONOMIC APPROACH

Taxonomy is fundamental to all branches of biology that are epitomized by sound "natural" classifications. Such classifications are dependent on an appreciation of the nature of the organisms to be classified and on detailed knowledge of their morphology, physiology, life histories, and ecology; Classifications therefore evolve toward greater stability and become more informative as knowledge of any particular group of organisms deepens.

A. The Nature of Fungi

The origin of fungi was mysterious to the Greeks and Romans (Buller, 1915a). To the physician and poet Nicander [ca. 185 B.C.], fungi were "the evil ferment of the earth;" poisonous kinds originating from the breath of vipers, a traditional association later indicated by P. Mattioli [1501–1577], who included a serpent in the woodcut of toadstools in his "Commentarii," 1560; while truffles, which as Pliny marveled can live without roots, were commonly attributed to the action of lightning. The widespread

classical view that fungi were without seeds and were derived from organic matter had a long currency. Although G. della Porta [ca. 1535–1615] in his "Phytognomonica," 1588, was the first to observe "seeds" (spores) in agarics and truffles, Hooke in 1665 still believed that the rose rust originated from leaf tissue, and L. F. Marsigli [1658–1730] in "Dissertatio de Generatio Fungorum," 1714, expounded and illustrated the view that different types of plant tissue on decomposition became transformed into different sorts of fungi. In spite of Micheli's observations, such views, by becoming involved with the notion of spontaneous generation, persisted for another 150 years. The story, ably summarized by Bulloch (1938, Chapter 4) of the elegant experiments at Pavia with *Rhizopus* and other microorganisms by the Italian Lazzaro Spallanzani [1729–1799], which demonstrated that the origin of every plant must be from some other part of another plant, a conclusion unacceptable to less critical workers until they were finally silenced in the 1870's by John Tyndall [1820–1893] and Louis Pasteur [1822–1895], need not be detailed here. Mention should, however, be made of a final phase when—as one result of the recognition of the pleomorphism in fungi by brothers L. R. and C. Tulasne [1815–1885; 1816–1884], as set out in the three magnificent volumes of their "Selecta Fungorum Carpologia," 1861–1865—workers who overlooked the necessity of ensuring the purity of experimental cultures involved many different species in one life cycle. The climax was the fantasies of Ernst Hallier [1831–1904] (see Bulloch, 1938; Ramsbottom, 1941), an aspect of mycological error that can be exemplified by the diagram in W. Tilbury Fox's [1836–1879] "Skin Diseases of Parasitic Origin," 1863, showing the supposed relationship of a range of molds to "one essential fungus," *Torula* (yeasts such as *Torulopsis* and *Saccharomyces*), a point of view which in the light of current experimental studies on mycelial-yeast transformations may yet include a grain of truth.

B. The Structure of Fungi

Hooke in his "Micrographia" (p. 138) observed that the flesh of mushrooms and of other basidiomycete sporophores consisted of "an infinite company of small filaments," and Micheli saw ascospores within asci, but more critical and convincing elucidation of fungal structures was not made until the turn of the eighteenth century. It was J. Hedwig [1730–1799] in "Descriptio et Adumbratio Microscopico-Analytica Muscorum Frondosorum," 1787–1797, who unequivocally established the existence of asci and ascospores by describing and illustrating these organs for the twenty discomycetes included in his genus *Octosporus*. He designated the structure enclosing the spores a "theca"—the term "ascus" was not introduced

until 1817, by C. G. Nees von Esenbeck [1776–1858]—and shortly afterward coined the term "spore."

Elucidation of basidial structure proved more troublesome. Basidia were interpreted as asci, and for a quarter of a century after H. F. Link [1767–1851] in 1809 endorsed this view, everyone, including Ness von Esenbeck, A. C. J. Corda [1809–1849], and R. K. Greville [1794–1866], who examined the hymenium of agarics described and illustrated the spores in asci. Then suddenly F. M. Ascherson [1798–?] in Germany in 1836, J. H. Léveille [1796–1870] in France and Corda in Prague in 1837, and finally M. J. Berkeley [1803–1889] in England and J. F. Klotsch [1805–1860] and P. Phoebus [1804–1880] in Germany in 1838, by describing what they saw rather than what they expected to see, independently elucidated the structure of the basidium. Léveille's paper (Léveille, 1837) is the best known, for in it he coined the terms "basidium" and "cystidium," but both Berkeley (1838) and Phoebus gave good and critical accounts. It is of interest that Berkeley in "English Fungi" (p. 76) had in 1836 unconsciously correctly noted basidial structure when he wrote of *Agaricus* [*Clitopilus*] *prunulus*, "*Gills* . . . covered with very minute conical papillae, ending in four spiculae. *Sporules* . . . often seated upon the spiculae." The phenomenon of seeing in specimens what is expected from textbook or authority is well known to every teacher, and the simultaneous announcement of the same new discovery is a commonplace in the history of science. Improvements in the compound microscope may be a partial explanation of the recognition of basidial structure, but Berkeley worked with doublets until 1868 when he was given a compound microscope by J. D. Hooker, Director of the Royal Botanic Gardens, Kew.

Earlier C. G. Ehrenberg [1795–1876] in 1828 had described zygospore production in the mucoraceous *Syzgites megalocarpus,* and many new genera of molds had been proposed. Hyphomycetes was introduced by K. F. P. von Martius [1794–1868] in 1817. Additional facts, particularly about larger fungi, were supplied by a series of finely produced works, aimed at the wealthy amateur naturalist, such as P. Bulliard's [1752–1793] "Histoire des Champignons de la France," 1791–1792, and James Sowerby's [1757–1822] 3-volume "Coloured Figures of English Fungi or Mushrooms," 1797–1815, with their beautiful hand-colored plates which have never been surpassed artistically. Data for the delimitation of the main classes of fungi were thus available.

C. The Classification of Fungi

1. First Attempts

Although some groups have always been approached with a certain ambivalence by both botanists and zoologists (e.g., the slime fungi, or Myxomycetes, which have frequently found a place in the animal kingdom as Mycetozoa, fungus animals), fungi have traditionally been considered plants, if for no other reason than it was even more unacceptable to classify them as animals. The early botanists tended to follow the binary system of "The Grete Herbal" already quoted and to classify fungi into those that are "deedly and sleeth" and those which "dooth not." Clusius grouped 21 of his genera as "Fungi esculenti," the other 26 as "Fungi noxii et perniciosi." Similarly, Van Sterbeeck devoted the first two parts of his book to edible and poisonous fungi (the third on "Aerd-buylen," earth bladders, included truffles along with potatoes and other tubers). Although this pragmatic division is still popular, edible and poisonous fungi have no regular taxonomic distribution. Toxicity to man had thus to give place to more fundamental taxonomic criteria.

The most useful of these criteria is morphology, and it was soon found that for fungi as for the flowering plants the sexual organs and types of fructification were of major taxonomic significance. J. D. Gleditsch [1714–1786] in "Methodus Fungorum exhibens genera, species et varietates," 1753, divided the eleven genera that he recognized into two groups according to whether the sporulating surface was exposed or enclosed; if *Clavaria, Boletus,* and *Agaricus* fell into one group, the association of *Mucor, Stemonitis,* and *Lycoperdon* in the other and the separation of *Elvela* [*Helvella*] from *Peziza* were less successful, but the emergence of a sound classification of fungi was dependent on a detailed knowledge of their microscopical structure.

2. Linnaeus

Eighteenth century taxonomy of both plants and animals was dominated by Carl von Linné (Linnaeus) [1707–1778], a Swede, whose influence still persists. Although Linnaeus was a versatile taxonomist of genius, he had certain weaknesses. One was his treatment of fungi, the ordering of which he found "a scandal to art, no botanist knowing what is a Species and what a Variety" (which, alas, has been a perennial dilemma); finally a residue of the few fungi which he compiled were classified among the Vermes (worms) in his genus *Chaos.* He also used classical terms in new senses for some of his generic names for fungi (e.g., *Agaricus* and *Boletus,* cf. Section 1), but mycology like other branches of biology benefited

greatly from his introduction of binomial nomenclature. For example, the fungus designated "Fungus ramosus niger compressus parvus, apicibus albidis" by the pre-Linnaean John Ray became "*Xylaria hypoxylon*" for the post-Linnaean Greville.

3. *Persoon and Fries*

One recognition of the debt of biologists to Linnaeus is that the starting points for the nomenclature of both animals and plants under the International Codes are two of his major publications. The nomenclature of lichens also starts from Linnaeus, but for fungi two later works were chosen, the "Synopsis Methodica Fungorum," 1801, by C. H. Persoon [1761–1836] and the "Systema Mycologicum," 1821–1832, by E. M. Fries [1794–1878]. Whether the selection of these two works was in the best interests of mycology is a matter of argument, but mycologists are unanimous in recognizing the outstanding contributions made by Persoon and Fries to the systematics of fungi.

Persoon, of German and Dutch parents, was born at the Cape of Good Hope, educated in Germany, and from 1803 lived in Paris. He was somewhat eccentric, held no official appointments, and in 1825, owing to poverty, made over to the Dutch government, his herbarium (now in the Rijksherbarium, Leyden) in return for a pension (Ainsworth, 1962). Fries held academic posts and, like his fellow countryman Linnaeus before him, became professor of botany at the University of Uppsala. Fries specialized mainly on agarics. Persoon, although he made little use of the microscope, accurately described many microscopic fungi, both saprophytes and parasites. Between them they laid a firm foundation on which others could build. Their classifications, though archaic today, brought together many related forms, and their writings did much to systematize and encourage the study of fungi.

4. *De Bary*

Following the publication of the "Origin of Species" in 1859, evolution became accepted as a fact and systems of classifications of both plants and animals were given a phylogenetic slant. It was the German Heinrich Anton De Bary [1831–1888], professor of botany at Halle and later at Strasbourg and noted for his many and diverse important publications on fungi, who in his well-known textbook "Morphologie und Biologie der Pilze, Flechten, und Myxomyceten," 1866 (2nd ed., 1884; English translation, 1887), recast the broad classification of fungi in what approaches the modern pattern, and in doing so was the first to delimit and designate the Phycomycetes.

5. *Since De Bary*

During the rest of the nineteenth century and since, the papers, monographs, and books on the taxonomy of fungi have steadily increased in number, diversity, and quality. A selection from this large output could in the present context only be arbitary, but three outstanding compilations must be mentioned. First, here is the "Sylloge Fungorum Omnium hucusque cognitorum," 1882–1925, by P. A. Saccardo [1845–1920], professor of botany at Padua, who systematically compiled all the hitherto described genera and species of fungi, giving for each one he accepted a short Latin description. This involved many taxonomic decisions, resolving questions of synonymy, and the proposal of many new names and taxa, a task for which he had to enlist collaborators. One of Saccardo's best-known innovations was his system of "spore groups," a code for the classification and retrieval of many ascomycetes and fungi imperfecti.

The twenty-five volumes of the "Sylloge," still invaluable for systematists, are frequently supplemented by two other works, C. J. Oudeman's [1825–1906] posthumous "Enumeratio Systematica Fungorum," 5 volumes, 1919–1924, and G. Lindau [1866–1923] and P. Sydow's [1851–1925] "Thesaurus Litteraturae Mycologicae et Lichenologicae," 5 volumes, 1908–1918. The first is an elaborate plant host index of parasitic (and many saprophytic) fungi which supplements the host-parasite indexes of the "Sylloge"; the second is a virtually complete list of the taxonomic literature of mycology up to about 1910, arranged under authors and variously indexed.

VI. THE IMPACT OF PATHOGENICITY

Although advances in scientific knowledge may in part be attributed to man's curiosity and desire for explanation, more practical ends and economic drives have also given impetus to the advance of knowledge in most branches of science. What navigation was to horology and cartography, disease in man, animals, and, particularly, plants has been to mycology. As already noted (Section I), there have been references to fungal diseases of crop plants since very early times. Ringworm (favus) in man was known to Celsus [ca. 30A.D.]. In spite of this, understanding of infectious disease was a late development. Less than a century ago it was still possible for some diehards to doubt whether the microorganisms associated with certain diseases were the cause rather than results of the disorders, but by then the majority had been convinced of the reality of pathogenicity.

A. Fungal Diseases of Plants

One of two lines which converged in the fourth and fifth decades of the nineteenth century concerned fungal diseases of plants. This development began in 1775 with the experimental proof by Mathieu Tillet [1714–1791] Director of the Mint at Troyes, France, that bunt of wheat (caused by the smut fungus later named in his honor *Tilletia caries*) was contagious. He also showed that it can be controlled at least in part, by seed treatment, a fact known to Jethro Tull [1674–1741], an English farmer, who in the third edition of his "Horse-Hoeing Husbandry," 1751, wrote of smuttiness of wheat: "Whatever the Cause be, there are but Two Remedies proposed; and these are Brining and Change of Seed."

In 1767 Felice Fontana [1730–1805] and Giovanni Targioni-Tozzetti [1712–1783] in Italy independently concluded that cereal rust diseases are caused by microscopic parasitic plants, but proof of this proposition had to await the conclusive experimental findings of I. B. Prevost [1755–1819] in his classical monograph "Mémoire sur la cause immédiate de la carie ou charbon des blés, et de plusiers autres maladies des plantes, et sur les préservatifs de la carie," 1807, in which he describes experimental smut infections, the germination of smut spores (which he clearly illustrated), and the control by steeping seed in copper sulfate solution. His conclusions proved unacceptable to his contemporaries, and it needed the devastations of potato blight which led to the Irish famine in the 1840's to refocus attention on plant disease. This time, after much wild etiological speculation it was left to an English country clergyman, the Reverend Miles Joseph Berkeley, to conclude in his paper "Observations botanical and physiological on the potato murrain," 1846, that the fungus *Botrytis* (now *Phytophthora*) *infestans* was the cause, not the result, of the disease. The conclusion paralleled the then recently established view that fungi were able to cause disease in animals and in man, a second line of advance going back to the mid-eighteenth century.

B. Mycoses of Animals and Man

In 1748 what was clearly a fatal *Saprolegnia* infection in a roach was described in the *Transactions of the Royal Society*. Avian aspergillosis was also early observed, and in 1832 the zoologist Richard Owen [1804–1892] after finding a green mold in the lungs of a flamingo concluded that there were "Entophyta" as well as "Entozoa." It was, however, Agostino Bassi [1773–1856] of Lodi near Milan (Ainsworth, 1956) who by prolonged and careful experimentation was able to prove to the satisfaction of him-

elf and others (he finally performed key experiments before a Commission f Professors appointed by the University of Pavia) that the muscardine isease of silkworms which for many years had ravaged the silkworm in- ustry of France and Northern Italy was caused by the fungus subsequently amed *Beauveria bassiana*. He made public the results of his investigations 1 two monographs published in 1837[1] and 1839. Bassi's findings were onfirmed by others and accepted by some, and they must have influenced he views both of those investigating diseases of plants and of those study- ng diseases of man. Mycotic disease in man was firmly established by David Gruby [1810–1898], a Hungarian Jew working in Paris, who in a rilliant series of short papers published during 1841–1844 (Zakon and Benedek, 1944) described the fungi associated with four of the common ypes of ringworm in man. Also in the same year, F. T. Berg [1806–1887] of Stockholm independently recognized the fungus (*Candida albicans*) causing thrush. Studies on fungi pathogenic for man and animals and for plants then sharply diverged. After a brief and rather overenthusiastic reception, fungi were overshadowed first by bacteria and then by viruses as pathogens of man, and medical mycology was almost completely neglected until the last decade of the century when R. Sabouraud [1864–1938] had to rediscover and confirm findings made by Gruby fifty years earlier. Medical men interested in mycology had few contacts with nonmedical mycologists, who in their turn made rare excursions into the medical field. Medical mycology became very confused.

In contrast, students of plant disease, having usually had a botanical training, tended to concentrate on the mycology of fungal infections of plants, and in doing so they made many important contributions to syste- matics and to the life histories of fungi. Identification of plant pathogenic fungi became very accurate but tended to be an end in itself. Only in recent years, with the development of national plant pathological services, has it been generally realized that recognition of the pathogen is only the first step toward controlling a plant disease. On the other hand, as a consequence of following the example of the United States in employing mycologists to collaborate with medical men, there has been a worldwide increased in- terest in mycoses of man and the realization that the correct identification of the fungus involved is frequently the first step to rational therapy.

[1] English translations of Bassi's first monograph, Berkeley's potato blight paper, the monograph by Prevost and the papers by Fontana, Targioni-Tozzetti, and Tillet have been published by the American Phytopathological Society as "Phytopathological Classics," Nos. 10, 8, 2, 9, 5, respectively.

VII. BIOCHEMICAL ASPECTS

Another aspect of fungal activity which has given impetus to studies on fungi is the biochemical. From time immemorial man has consoled himself with diverse alcoholic beverages and practiced the leavening of bread. The nature of the fermentation underlying such ameliorating procedures was not, however, elucidated until the nineteenth century.

A. Fermentation

As already mentioned, Leeuwenhoek (see Bulloch, 1938, Chapter 3) observed the cellular nature of yeast which he described as being composed of aggregates of "globules," but he was mystified by fermentation. A more precise description of yeast cells was given by J. B. H. J. Desmazières [1767–1862] of Lille in 1826, when he distinguished five species of *Mycoderma* including *M. cerevisiae* (beer yeast), but no relationship of yeast to fermentation was claimed. Then, within a couple of years the nature of yeast associated with alcoholic fermentation was independently announced by the French physicist C. Cagniard de la Tour [1777–1859] in 1836 and by the physiologist Theodor Schwann [1810–1882] and the algologist F. T. Kützing [1807–1893], both Germans, in 1837. Cagniard de la Tour believed that fermentation of sugar was due to the vital activity of the yeast; Schwann recognized the fungal nature of yeast (it is from his name "Zuckerpilz" that the generic name *Saccharomyces* was derived); and if Kützing's views on fermentation were more muddled, he did suggest that different fermentations were caused by physiologically different organisms. These findings were received with scepticism by many and were never accepted by the influential German chemist J. von Liebig [1803–1873] in spite of the conclusive confirmation of the vital theory by the experimentation of Pasteur in the 1860's and 1870's. Another critical stage was reached in 1897 when the chemist E. Buchner [1860–1907] of Tübigen, demonstrated the action of "zymase" in cell-free yeast extracts on sugar, while the development of pure culture techniques brought many empirical brewing and wine-making operations under control.

B. Fungi as Chemical Tools

Another aspect of the biochemical deployment of fungi can be traced to the early experiments of Pasteur in which in addition to differentiating the two optically active forms of tartaric acid he demonstrated the differential utilization of the two forms by *"Penicillium glaucum,"* by the action of

which an optically inactive racemic mixture became levorotatory. The use of fungi in chemical analysis is now a commonplace. *Aspergillus niger* is used to determine traces of iron and copper in soils, and vitamin B can be assayed by means of *Ashbya gossypii.*

Fungi are also widely used for the synthesis of complex compounds from simpler material. One familiar example is the production of citric acid from sugar by strains of *Aspergillus niger,* a conversion which underlies an industry which has outmoded the production of citric acid from the juice of citrus fruit, at one time its only commercial source. Similarly gluconic acid and, in times of emergency such as war, fats and glycerol can be synthesized by fungi, on a commercial scale, from simple carbohydrates. But it is the production of antibiotics by fungi which attracts most attention in this field today.

C. Antibiotics

The recent recognition of "antibiotics" is now a familiar story (see Raper, 1952). It began with the discovery of penicillin by Alexander Fleming [1881–1955] in 1928, a discovery which biochemists and clinicians failed to exploit until 1940 when the work of Chain, Florey, and Heatley at the University of Oxford made the large-scale production and the use of penicillin a matter of urgency. This development, which, owing to the pressures and exigencies of war, was rapidly undertaken, mainly in the United States, gave rise in postwar years to a new major branch of the pharmaceutical industry.

The greatest novelty in the concept of antibiotics was perhaps in the name: antibiotic was first used as a name in the current sense by Waksman in 1942 (see Waksman, 1947). There were many chemotherapeutants before penicillin, and antagonism between fungi and between fungi and other microorganisms was a familiar phenomenon with a large literature. One early attempt to utilize an antibiotic for practical ends resulted from Weindling's studies in the 1920's on antagonism between soil fungi when trials were made to control soil-inhabiting plant pathogenic fungi such as *Corticium solani* by the use of gliotoxin produced by *Gliocladium virens.* But the history of antagonism extends far back, and even Fleming's observation of the antagonistic effect of *Penicillium* on bacteria had been made before. Tyndall in 1876 is frequently credited with having been the first to observe the action of penicillin, but even earlier William Roberts [1830–1899], a Manchester physician, in studies in 1872–1873 on spontaneous generation observed interactions between *Penicillium glaucum* and bacteria and between bacteria; these he interpreted in Darwinian language as a struggle for existence and a survival of the fittest, and he was the first to

use "antagonism" in this connection (Roberts, 1874). These early observations were of course unknown to Fleming, who appears neither to have been influenced by nor to have currently influenced students of antagonism between microorganisms. As with other notable discoveries, an acute, suitably experienced observer made an observation and saw its relevance and potentialities at a time when there was a need for its application and there were the techniques available for its exploitation. As a side effect, interest in fungi was further intensified.

VIII. RECENT ADVANCES

The present is too close at hand for "recent advances" to be seen in perspective. Current fashions can so easily be given too great a significance. One point that is quite clear is that today more fungi are being described more competently than ever before. Another is that there are still many fungi awaiting description. The advent of the electron microscope is, by revealing fine structure, having an effect comparable to that which followed the perfection of the light microscope in the seventeenth century, while refined biochemical techniques are giving much new information about the bricks from which fungi are built and methods of their assembly. Geneticists have taken advantage of the short life cycle of fungi to use *Neurospora* and *Saccharomyces,* as they have used the fruit fly *Drosophila,* to elucidate problems of formal genetics, and in doing so have discovered such novelties as the parasexual cycle. Ecologists are elucidating the role of fungi in diverse habitats and communities. These are but some of the growing points of mycological research; the chapters that follow fill in much detail.

REFERENCES

During the preparation of this review most of the classical mycological publications mentioned in the text have been inspected; but, because bibliographical details of these often rare works are easily obtained from standard bibliographies (such as Lindau and Sydow, 1908–1918) and from library catalogues, they have been excluded from the References, which are mainly to secondary and usually more easily accessible sources.

Particularly useful are Paulet (1790), which summarizes in French the contents of much early mycological writing, the histories of Richon and Roze (1888) and of Lütjeharms (1936), the taxonomic survey by Vuillemin (1912), and the informative and stimulating essay by Ramsbottom (1941). Reed (1942), Morandi and Baldacci (1954), and Ramsbottom (1953) also provide background detail.

The history of phytopathology has frequently been treated and Section VI may be supplemented by the broader, well-documented reviews by Whetzel (1918), Large (1940), and Keitt (1959).

Ainsworth, G. C. (1956). Agostino Bassi, 1773–1856. *Nature* **177**:255–257.
Ainsworth, G. C. (1962). Christiaan Hendrik Persoon (1761–1836). *Nature* **193**:22–23.
Arber, A. (1938). "Herbals, Their Origin and Evolution," 2nd ed., 326 pp. Cambridge Univ. Press, London and New York.
Barger, G. (1931). "Ergot and Ergotism," 279 pp. Gurney & Jackson, London and Edinburgh.
Berkeley, M. J. (1838). On the fructifications of the pileate and clavate tribes of Hymenomyetous fungi. *Ann. Mag. Nat. Hist.* **1**:81–101.
Buller, A. H. R. (1915a). The fungus lore of the Greeks and Romans. *Brit. Mycol. Soc. Trans.* **5**:21–66.
Buller, A. H. R. (1915b). Presidential address. Micheli and the discovery of reproduction in fungi. *Trans. Roy. Soc. Can.* [3] **9**:1–25.
Bulloch, W. (1938). "The History of Bacteriology," 422 pp. Oxford Univ. Press, London and New York. (Reprinted 1960.)
Dobell, C. (1932). "Antony Van Leeuwenhoek and His Little Animals," 435 pp. London. (Reissued in reduced facsimile, Dover, New York, 1960.)
Eichhorn, O. (1962). *Amanita ovidea* (Bull.) Quél. als Pharaonenpilz. *Sydowia Ann. Mycol.* **15**:218–220.
Harshberger, J. W. (1929). An ancient Roman toadstool carved in stone. *Mycologia* **21**:143–144.
Istvánffi, G. (1900). "Etudes et commentaires sur le code de l'Escluse," 287 pp. Budapest. [Includes a facsimile of Clusius, "Fungorum in Pannoniis Observatorum Brevis Historia" and biographical details of Clusius and Sterbeeck.]
Keitt, G. W. (1959). History of plant pathology. In "Plant Pathology" (J. G. Horsfall and A. E. Dimond, eds.), Vol. I, pp. 61–97. Academic Press, New York.
Large, E. C. (1940). "Advance of the Fungi," 488 pp. Cape, London.
Léveille, J. H. (1837). Recherches sur l'hymenium des champignons. *Ann. Sci. Nat. Paris* [2] **8**:321–338.
Lindau, G., and P. Sydow. (1908–1918). "Thesaurus Litteraturae Mycologicae et Lichenologicae," 5 vols. Borntraeger, Leipzig.
Lütjeharms, W. J. (1936). Zur Geschichte der Mykologie das xviii. Jahrhundert. *Mededel. Ned. Ver. Mycol.* **23**: 262 pp.
Morandi, L., and E. Baldacci. (1954). "I Funghi. Vita, Storia, Leggende," 633 pp. Garzanti, Milan.
Paulet, J. J. (1790). "Traité des champignons," Vol. I, 629 pp. L'Imprimeric Royale, Paris.
Ramsbottom, J. (1932). The fungus stone, *Polyporus tuberaster*. *Proc. Linnean Soc. London* **1931–32**:76–79.
Ramsbottom, J. (1941). The expanding knowledge of mycology since Linnaeus. *Proc. Linnean Soc. London* **151**:280–367.
Ramsbottom, J. (1953). "Mushrooms and Toadstools," 306 pp. Collins, London.
Raper, K. B. (1952). A decade of antibiotics in America. *Mycologia* **44**:1–59.
Reed, H. S. (1942). "A Short History of the Plant Sciences," Chapters 18–19. Chronica Botanica, Waltham, Massachusetts.
Richon, C., and E. Roze. (1888). "Atlas des Champignons," Chapter 1, pp. i–lxxiii. Doin, Paris.
Roberts, W. (1874). Studies in biogenesis. *Phil. Trans. Roy. Soc. London* **B164**:457–494.

Vuillemin, P. (1912). "Les champignons. Essai de classification," 425 pp. Doin, Paris.
Waksman, S. A. (1947). What is an antibiotic or an antibiotic substance? *Mycologia* 39:565–569.
Wasson, V. P., and R. G. Wasson. (1957). "Mushrooms, Russia, and History," 2 vols. Pantheon Books, New York.
Whetzel, H. H. (1918). "An Outline History of Phytopathology," 130 pp. Saunders, Philadelphia, Pennsylvania.
Zakon, S. J., and T. Benedek. (1944). David Gruby and the centenary of medical mycology, 1841–1941. *Bull. Hist. Med.* 16:155–168.

CHAPTER 2

Fungal Structure and Organization

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I. INTRODUCTION. RELATIONSHIP WITH OTHER ORGANISMS

The fungi comprise a large, heterogeneous, and ubiquitous group of heterotrophic organisms living as saprophytes or parasites, or associating with other organisms as symbionts. They are characterized by a distinctive, filamentous, multinucleate vegetative structure known as the *mycelium*. It consists of a branching system of walled tubes, the *hyphae*, which contain protoplasm and continually extend by apical growth and lateral branching. Food material is absorbed over the whole surface of the mycelium, and it forms a highly effective system for exploiting the resources of the substratum. After a period of growth reproduction occurs with the formation of *spores*, uni- or multicellular bodies that become detached from the parent and give rise to new individuals (see p. 33).

In the traditional division of living organisms into the Plant and Animal Kingdoms, a division based on the differences between higher plants and higher animals, fungi have been classified as plants. This means no more than that they have been regarded as being more like plants than like animals. Though they differ fundamentally from plants in being non-photosynthetic, fungi have been grouped with them because of their, largely, nonmotile habit, the presence of cell walls, and absorption of food materials in solution; and because of the theory, no longer generally held, that they have evolved from algae.

The problem of relationship with traditional groups is one that exists not

[1] No illustrations are provided in this review because of limitations of space. Therefore, the reader is referred to books by Alexopoulos (1962) and Gäumann and Dodge (1928), in which the morphology and anatomy of fungi are illustrated.

only for fungi but for microorganisms in general, and during the last century various solutions, involving the estab'ishment of one or more new kingdoms (Whittaker, 1959), have been offered for the problem of how to classify organisms lacking the characteristics of higher plants and animals. The best known of these schemes, that of Haeckel (1866), grouped simple organisms, lacking tissues, in a third kingdom, the Protista. Neither Haeckel's proposal, as first put forward or as later modified (1878, 1894), nor those of his contemporaries were generally adopted. In recent years renewed interest has been shown in this problem. It is now widely accepted that the two-kingdom system allows no place for organisms of simple structure which are neither "plant" nor "animal," or which possess characters of both. The case for primary division on the basis of levels of biological organization has been cogently presented by Stanier *et al.* (1963), and schemes of classification removing from the plant and animal kingdoms organisms with relatively simple organization, unicellular, coenocytic, or multicellular without tissue differentiation, have been put forward by these and other authors recently (e.g., Weisz, 1961; Weisz and Fuller, 1962).

At the cellular level a most important contribution to present-day conceptions of interrelationships between organisms has resulted from electron microscope studies of the fine details of cell structure. Recent observations have established the existence of two fundamentally different types of cells, *prokaryotic* and *eukaryotic,* representing different levels of cell evolution, simple and advanced, respectively.

The main features distinguishing the prokaryotic from the eukaryotic cell (Stanier and van Niel, 1962; Stanier *et al.*, 1963) are absence of a nuclear membrane, absence of chloroplasts (in the photosynthetic forms), absence of mitochondria, nuclear division other than by mitosis, presence of a specific mucopeptide as the strengthening element of the cell wall, and, lastly, relatively simple structure of locomotor organelles where these occur. Those of prokaryotic cells consist of a single fibril (eubacteria) or of an axial filament, equivalent to a bundle of fibrils (spirochetes). Those of eukaryotic cells conform to a common pattern consisting of a regularly arranged group of eleven fibrils, a central pair surrounded by the other nine, the whole being enclosed by a membrane continuous with that of the cell itself (Sleigh, 1962).

Within the simply organized eukaryotic forms, the algae are distinguished physiologically by possession of chlorophyll whereas the protozoa are basically unicellular, motile organisms without cell walls. The fungi are functionally coenocytic and nonmotile. The essential character of the coenocytic condition is that during growth nuclear division occurs without formation of new cells leading to the development of a large mass of cytoplasm containing many nuclei. Fungal coenocytes are spatially limited

in their extension by the wall material of their hyphae. Contrasted with this is the coenocytic condition as seen in slime molds, a fourth group of simple eukaryotic organisms that in certain characteristics resemble the fungi (Martin, 1960). Here the coenocyte is not bounded by a cell wall and flows over the substratum in an amoeboid fashion as a sheet of protoplasm, the plasmodium (see p. 39).

II. THE FUNGUS THALLUS

A. Nonmycelial Fungi

Though the hypha is the characteristic unit of structure in fungi, it does not occur in all members of the group. In the most simple fungi (certain phycomycetes) the vegetative body, the *thallus,* is a single microscopic cell, spherical, ellipsoidal, tubular, or irregular in shape, ranging from a few to a few hundred microns in largest dimension (Sparrow, 1960). At maturity it becomes wholly transformed either into a single reproductive organ (Olpidiaceae, Anisolpidiaceae, Olpidiopsidaceae) or into several (Synchytriaceae, Achlyogetonaceae, Sirolpidiaceae, Lagenidiaceae, Ectrogellaceae). This type of thallus is described as *holocarpic.* In contrast, the thallus of most other fungi is differentiated into vegetative and reproductive portions. It is *eucarpic.* This differentiation is seen at different levels within the Phycomycetes. In the Phlyctidiaceae, Rhizidiaceae, and Chytridiaceae the mature thallus consists of a reproductive organ and a system of vegetative branches known as *rhizoids.* These are very narrow, delicate, rootlike structures, tapering toward the tip. Depending on the species, they are unbranched or show repeated dichotomous branching and form a well-developed absorbing system. Exceptionally rhizoids are not formed and the vegetative system is bulbous or coralloid, or consists of single or branched, blunt cylindrical elements, or is distinctly hypha-like. This type of thallus, with one center of reproduction, is described as *monocentric.* More complex is the *polycentric* thallus of the Cladochytriaceae and Megachytriaceae, composed of a richly branched, very extensive system of rhizoidal filaments bearing a number of reproductive organs.

More highly differentiated thalli, larger and visible to the naked eye, though still relatively limited in extent, occur in the Blastocladiales, Monoblepharidales, and Leptomitales. In *Blastocladia* and *Rhipidium,* for example, the thallus is characteristically arborescent. A large, trunk-like basal cell is anchored to the substratum by a series of strongly tapering, branched, chytrid-like rhizoids which provide, in addition, an effective absorbing system. At its distal end it bears a number of reproductive organs. In *Allomyces* and *Leptomitus,* on the other hand, the rhizoidal system bears a

relatively slender basal cell which gives rise distally to branched hyphae of seemingly unlimited extent while in the Monoblepharidales a well-developed mycelium of hyphae arises directly from the anchoring rhizoids.

B. The Mycelium

The thallus of most fungi consists of a mycelium of hyphae. As already mentioned, hyphae are cylindrical tubes whose walls enclose multinucleate protoplasm that continuously lays down new wall material at the tips.

1. Aseptate Hyphae

In the widely spreading mycelia found in the Saprolegniales, Pythiaceae, and Mucoraceae of the Phycomycetes, as seen in agar culture, hyphae of the younger parts of the colony are characteristically aseptate, i.e., without cross walls. As the hyphae grow in length, branches arise behind the tip, and the protoplasm continuously moves forward to occupy the young, actively extending regions, leaving the other parts in the rear empty. These empty lengths are cut off by *septa* that arise as ingrowths of the hyphal wall. Delimitation of successively emptied lengths of hyphae in this way results in the occurrence of a series of septa in the hyphae of old colonies. Septa are also formed in Phycomycetes to delimit reproductive organs and to cut off the damaged ends of broken hyphae. A striking demonstration of the capacity of aseptate mycelium to heal itself in this way was provided by Davies (1959), who found homogenized mycelium of *Phytophthora fragariae* (and of related fungi) very satisfactory as inoculum for liquid cultures. The minimum volume of protoplasm that will form a viable unit after such fragmentation is indicated by his unpublished observation that lengths of hypha less than approximately 100 μ would not survive.

Although septa occur there is little difficulty in recognizing the aseptate, uninterrupted nature of the active mycelium in the phycomycetes referred to above. However, in other phycomycetes the hyphae are regularly interrupted along their length by septa. True septa occur very frequently in members of the Entomophthorales, e.g., in *Basidiobolus* (Robinow, 1963), and there is a tendency in the majority of forms in this order for the hyphae to fragment into their constituent cells, these being termed *hyphal bodies*. In other groups *pseudosepta* are formed. In *Allomyces* irregularly perforated, sieve-like pseudosepta occur at intervals along the hyphae. In *Gonapodya* and in members of the Leptomitaceae, e.g., *Leptomitus*, *Apodachlya*, the hyphae are conspicuously constricted at regular intervals and at these points become sometimes plugged with granules giving the appearance of septa.

2. Septate Hyphae

The hyphae in Ascomycetes, Basidiomycetes, and Fungi Imperfecti are regularly septate. In some species septa occur very near together, in others widely separated. Septa are not formed close to the tip of the hypha, and the apical segment is thus much longer than those behind it (Smith, 1923; Plomley, 1959). Branches may develop close behind the growing apex or farther back, behind this apical segment, and in some species they are associated with septum formation. In contrast to those of Phycomycetes the septa of these other groups are incomplete, having a minute central pore through which protoplasmic continuity is maintained throughout the length of the hypha. Thus the hyphae are not divided into a series of independent cells, but are coenocytic with incomplete septa.

Recent electron microscope studies of the septum in fungi indicate that in the ascomycetes examined (Shatkin and Tatum, 1959; Moore and McAlear, 1962) and Uredinales (Ehrlich and Ehrlich, 1963) it is a simple poroid disk whereas in other basidiomycetes there is an annular thickening of the septum around the edge of the pore, producing what Moore and McAlear have termed the *dolipore septum* (cf. Chapter 5). In their studies with *Auricularia auricula, Dacrymyces* spp., *Merulius tremellosus,* and *Polyporus betulinus,* using material from fruit bodies and "presumed, therefore, to be dikaryotic," they observed a dome-shaped structure, consisting of a double membrane similar to the endoplasmic reticulum, on each side of the pore. They suggested that these structures, which they named *parenthesomes,* would prevent nuclear migration while allowing cytoplasmic continuity through the septum, and they put forward the hypothesis that in this way these highly advanced fungi have achieved functional diploidism. Later studies by Bracker and Butler (1963) with *Rhizoctonia* [*Corticium*] *solani* confirmed the general structure of the dolipore septum but clearly showed that the dome-shaped structure, which they called the septal pore cap, is porous in the area of the septal pore and, therefore, unlikely to prohibit the passage of nuclei through it. It would be difficult to explain on any other basis the passage of nuclei through a basidiomycete mycelium as described by Buller (1933).

As in phycomycetes, old or damaged parts of a septate hypha are cut off from living parts, and this is accomplished by the formation of a plug which blocks the pore in the septum separating the two regions.

a. Dimorphism. In yeasts true hyphae are never found. These fungi occur as uninucleate single cells that multiply by budding or by fission. In a budding yeast, such as *Saccharomyces,* the parent cell develops a small projection which enlarges with growth until it is approximately the same

size as the parent. Nuclear division takes place, and the new cell becomes separated by a constriction at its point of origin. A single cell may give rise to one or several buds. In *Schizosaccharomyces*, a fission yeast, growth and nuclear division is followed by formation of a transverse septum dividing the parent into two, approximately equal-sized, daughter cells. In actively growing yeast colonies the cells remain loosely attached to one another, forming a sprout mycelium or *pseudomycelium*.

When members of the closely related Endomycetaceae are grown in concentrated sugar solutions, the hyphae readily break up into their constituent cells and become yeast-like. After transfer back to an ordinary agar medium the mycelial habit is regained. A similar reversible transformation from a mycelial to a unicellular growth form occurs among several fungi pathogenic to man (Ainsworth, 1952). In infected tissues they occur as single, yeast-like cells that multiply by budding, but their saprophytic growth in culture is mycelial. This dimorphism appears to be an inherent characteristic of a number of fungi. In addition to those already mentioned it is seen in members of the Taphrinales and Ustilaginales, which are mycelial in their plant hosts but yeast-like in artificial culture. It also occurs among species of *Mucor*, e.g., *M. rouxii* (Nickerson, 1959).

With regard to the mechanism of the yeast-mycelial interconversion, work with bacteria and yeasts during the past few years indicates that growth and cell division are not always interdependent processes. Cell division, by which the unicellular condition is maintained, can be prevented without affecting growth, and this results in the filamentous or mycelial condition.

Factors that favor the nonmycelial phase are listed by Cochrane (1958), and the mechanism of the reversible transformation of the yeast phase to the mycelium is discussed in Volume II.

3. Modifications of Hyphal Structure

The hyphae of most fungi measure 5–10 μ across. In culture there is little variation in diameter along the length of hyphae, but in their plant hosts hyphae of parasitic species tend to be somewhat irregular as they conform to the variation in size of the intercellular spaces. Over the group as a whole, hyphal diameter varies from as little as 1–2 μ, almost as narrow as many bacterial cells (some of which, e.g., *Streptomyces*, are arranged in hyphalike filaments), to 25 μ or more. In a few groups the hyphae taper toward the tip, notably in the Saprolegniaceae. Some of these, e.g., *Achlya conspicua*, possess gigantic hyphae measuring between 160 and 170 μ across at the base (Coker, 1923; Johnson, 1956).

a. Chlamydospores. Irregularly swollen hyphae are not uncommon in certain fungi, e.g., in species of *Phytophthora;* they may bear hyphal swellings

of various shapes and sizes as in *P. cinnamomi* (Blackwell, 1949), where they are so characteristic as to aid identification. Groups of hyphal swellings may also occur on otherwise fairly regular hyphae as in *P. cryptogea.* It is not known whether the swellings have any special function in this species, but in *P. cinnamomi* they become thick walled and are cut off from the hyphae as resistant vegetative cells or chlamydospores. Chlamydospores develop in the older hyphae of many fungi. In aseptate hyphae a portion of the protoplasm accumulates food reserves and secretes a thick wall cutting it off completely from the hypha on each side. In septate mycelia "cells" of the hyphae and, in some species, of the spores also, e.g., in *Fusarium,* become walled off in the same way, to remain as viable vegetative units when the rest of the mycelium dies. Among mycorrhizal fungi sac-like swellings occur along the length, or at the tip, of the hyphae of the phycomycetous vesicular–arbuscular endophytes (Mosse, 1963), and similar storage and reproductive functions have been ascribed to them.

b. Strands and rhizomorphs. In many root-invading or wood-destroying fungi hyphae are aggregated longitudinally in varying degrees of complexity to form organs of mycelial migration and food transport. Some authors (Townsend, 1954) describe as rhizomorphs all levels of aggregation, from the simple bundle of interweaving hyphae to the very complex, root-like organ. Garrett (1956, 1963), on the other hand, distinguishes between the simply organized mycelial strand, described by Butler (1957) as "a loose federation of individual hyphae," and the rhizomorph proper, a complex, internally differentiated and highly coordinated, autonomous organ consisting of thousands of hyphae and elongating from an apical meristem like a root. Between these two extremes, represented by *Hymenogaster luteus* and *Armillaria mellea,* respectively, are intermediate forms. Garrett has concluded that compared with individual hyphae, the significance of mycelial aggregation lies in the pooling of the resources of many hyphae. This is biologically advantageous in that it provides a greater inoculum potential whether this is used for the colonization of a substrate, as in lignin- and cellulose-decomposing saprophytes, or for infection of a host, as in parasites such as *A. mellea.*

c. Sclerotia. Among fungi possessing septate mycelium are species in which the hyphae become aggregated to form bodies known as sclerotia. These consist typically of a rind composed of relatively small, thick-walled, and usually darkly pigmented cells that surround a mass of thinner-walled, closely interwoven hyphae. Sclerotia originate as localized areas of increased branching and septation of vegetative hyphae. Three distinct types of development have been recognized (Townsend and Willetts, 1954). In *Rhizoctonia* [*Corticium*] *solani* there is no definite pattern of organization of the hyphae, and the mature sclerotium is very loosely constructed, with-

out well-defined zones or a thickened rind. In *Botrytis cinerea, B. allii,* and *Sclerotium cepivorum* the sclerotium develops terminally by repeated dichotomous branching of a hyphal tip followed by septation and differentiation to form three layers: rind, cortex of pseudoparenchyma, and medulla of loosely arranged, filamentous hyphae. In the strand type of development which occurs in *Sclerotinia gladioli, Sclerotium rolfsii,* and *Phymatotrichum omnivorum,* sclerotia originate in the production of numerous side branches along one or more main hyphae or a strand of hyphae, followed by differentiation as in the terminal type.

Sclerotia vary in size from less than a millimeter as in *S. cepivorum* to approximately a foot across in *Polyporus mylittae.* Many are irregular, e.g., those of *B. cinerea* and *Phymatotrichum omnivorum,* and some are spherical, e.g., *S. rolfsii.* Some are composed partly of hyphae and partly of host tissue, and they resemble in shape the structure that is replaced, e.g., the ergots of *Claviceps purpurea.*

Sclerotia are formed by a number of plant-parasitic fungi. They are very resistant to unfavorable conditions and ensure survival for long periods in the absence of host plants (Garrett, 1956). In general it appears that the sclerotium population declines with time through spontaneous germination, but, in contrast, germination of the sclerotia of *S. cepivorum* in soil is dependent entirely on stimulation from roots of the host (Coley Smith and Hickman, 1957; Coley Smith, 1960).

d. Appressoria. Further modifications of hyphal structure and organization are found in relation to special functions. Thus the hyphae of many plant parasites develop attachment organs known as appressoria. These are localized swellings of the tips of germ tubes or of older hyphae that develop in response to contact with the host. They are enveloped in a mucilaginous secretion and adhere firmly to the host surface over a relatively wide area, furnishing a broad base of attachment for the thrust of the extremely fine, needle-like infection hypha that forces its way through the cuticle.

e. Haustoria. Most obligate, and some facultative (Blackwell, 1953), parasites of plants possess haustoria, lateral outgrowths of intercellular hyphae (or superfical hyphae in the powdery mildews) specially modified for absorption of nutrients. They enter the cells as fine hyphae, resembling the infection hyphae arising from appressoria, and are variously shaped: minute, knob-shaped in *Albugo candida;* large and irregularly swollen, almost filling the host cell in *Peronospora parasitica;* branched, hypha-like, in *Puccinia menthae.* Haustoria provide intimate contact between fungus and host, and particular interest attaches to the physical relationships that they establish with their host cells which, despite invasion and some redirection of metabolism (Allen, 1959), continue to live for long periods, sometimes as long as the plant itself.

Observations with the light microscope indicate that though there is very close contact between haustorium and host protoplast, the plasma membrane of the host cell is not penetrated by the haustorium, but merely invaginated by it. This observation has been confirmed by plasmolysis experiments (Fraymouth, 1956) in which host protoplasts contracted away from haustoria, leaving them free within the cell cavities. There is general reference, also, to the secretion by the host protoplasm, from an early stage, of a layer of material continuous with the host cell wall that sheaths the haustorium.

From studies of the fine structure of the haustoria of *Puccinia graminis* f. sp. *tritici,* Ehrlich and Ehrlich (1963) concluded that the actual interface between host and pathogen is not plasma membrane and haustorium wall, but a sheath of material (an "encapsulation zone") similar to the cytoplasm of the haustorium and continuous with it through pores in the haustorium wall. No such discontinuity of the haustorium wall was observed by Peyton and Bowen (1963) in *Peronospora manshurica* and though they recognized a distinct layer, a "zone of apposition," between host and haustorium, this was not comparable with the encapsulation zone. Evidence that a specific secretory process is induced in parasitized cells was drawn from the existence in the host cytoplasm near haustoria of spherical "secretory bodies" and their apparent discharge through the host plasma membrane into the zone of apposition.

f. Traps. Highly effective modifications for the capture of their prey are possessed by the predacious fungi (Drechsler, 1941; Duddington, 1962), a taxonomically mixed group of fungi that capture small animals, protozoa, rotifers, and, mainly, nematodes. Some of these fungi are phycomycetes (Zoopagales), but most are fungi imperfecti.

Two types of predacious activity are recognized, capture by adhesion and by mechanical traps. Among the fungi of the first group are those which possess no special modification of structure, but rely on the production of a sticky substance by which the victims are held on contact. These include *Stylopage grandis,* in which the mycelium as a whole is sticky, and also fungi that possess sticky spores, e.g., species of *Harposporium, Meria,* and *Nematoctonus.* Some species of the latter genus, e.g., *N. concurrens,* bear, in addition, adhesive branches along their hyphae. Lateral adhesive branches are found in other genera including *Dactylella* and, among aquatic fungi, in *Sommerstoffia spinosa* and *Zoophagus insidians,* which capture rotifers (Prowse, 1954). The most common trap among eelworm-catching hyphomycetes is the adhesive network found in *Arthrobotrys oligospora.* This arises by the formation of short lateral branches that curve round and anastomose with the parent hyphae or with neighboring branches, forming complex three-dimensional networks in which eelworms

become entangled and held by a sticky secretion produced by the cells of the loops. Evidence of a toxin which paralyzes and kills the nematodes after capture by this fungus has been obtained by Olthof and Estey (1963).

The mechanical traps consist of a ring of three cells borne on a lateral branch. In the nonconstricting ring, seen in the well-known *Dactylaria candida,* eelworms attempting to thrust their way through become firmly wedged. Most remarkable of all is the constricting ring system seen, e.g., in *D. gracilis.* Here the three cells of the ring swell up very rapidly to about three times their original volume after stimulation of the inner surfaces of their walls, with the result that the space within the ring is occluded. Any eelworm that has entered the ring and has triggered off this mechanism is thus held in an unbreakable, strangling grip. The mechanism of ring constriction has been discussed recently by Muller (1958).

4. Internal Structure and Coordination

The young parts of hyphae contain dense, homogeneous protoplasm with many nuclei and mitochondria while older parts become conspicuously vacuolated. Cytochemical tests (Zalokar, 1959) show in addition marked biochemical differentiation along hyphae, associated in particular with the growing tip region. Zalokar has compared the morphological changes between young and older hyphae with those taking place in the differentiation of a newly formed parenchyma cell. Interesting though this analogy is in principle, it is doubtful if it can be pursued in detail, for Robertson's observations on the origin and nature of fungus vacuoles (1961) suggest that they may be quite unrelated to those in parenchyma cells.

Although the cytoplasm of fungal hyphae resembles that of other eukaryotic cells in the occurrence of such characteristic organelles as mitochondria and endoplasmic reticulum, it appears to be unique in the occurrence, associated with the plasma membrane, of bodies of unknown function named *lomasomes* (Moore and McAlear, 1961). These were noticed first by Girbardt (1958, 1961) and more recently by Peyton and Bowen (1963), who interpreted their structure as an elaboration of the plasma membrane in the form of a "system of unit membrane tubules and vesicles." These bodies have not been reported in any cells but those of fungi and certain algae (see p. 111), though they are apparently not present in all fungi (Hawker and McV. Abbott, 1963).

In aseptate hyphae, nuclei occur throughout the cytoplasm. In septate hyphae, the segments contain, in different species, one, two or a number of nuclei, up to approximately 100 in *Neurospora crassa* (Fincham and Day, 1963). It is generally accepted (Olive, 1953; Ward and Ciurysek, 1962) that somatic nuclei divide by mitosis although this is not always of the conventional form (Robinow, 1962; see Chapter 6, this volume).

In discussing fungus structure and organization it is important to consider the hypha not merely as an individual element, but as one of many similar and functionally interrelated elements which comprise the mycelium.

Structurally, hyphae of many fungi, especially higher fungi, become interconnected from an early stage by the formation of numerous anastomoses which permit functional interrelationship through the passage of cytoplasm, nuclei, and food materials from one part of the mycelium to another. Functional interrelationship within the mycelium is also shown by the regularity of growth of hyphae in a colony, a very characteristic feature of fungi in culture, especially on solid media. In *Neurospora,* Ryan *et al.* (1943) showed that individual hyphae branch profusely and that the branches are oriented more or less at random, whereas associated in a colony the hyphae of the growing edge have relatively few branches and their growth is mainly parallel with the parent hyphae. At a certain density of hyphae, branching is suppressed and, bearing in mind the inhibition of conidium germination by hyphae of this fungus (Backus, 1939), the conclusion is drawn that branching within a colony is controlled in a similar manner. In *Coprinus disseminatus* Butler (1961) has described a regular hierarchy of hyphae, consisting of main, primary, and secondary branches, which is maintained by differences in extension rates in the proportions, respectively, of *100:66:18*. Considering main and branch hyphae separately, extension rate was correlated with hyphal diameter; but compared with main hyphae of equal width, primary branch hyphae had a significantly lower extension rate. Whether this was an effect of an internal or an external factor has not been determined.

One of the most obvious and long-recognized features of actively growing hyphae, aseptate and septate alike, is streaming of cytoplasm toward the growing regions. Buller (1933) made the interesting observation that the rate of streaming, as measured by the rate of movement of vacuoles, is similar in both types of hyphae, indicating that the perforate septum offers little resistance to the flow of protoplasm through septate hyphae. Though the direction of streaming is generally toward the apex from older parts that have ceased to grow, simultaneous currents, moving in opposite directions, have been reported in members of the Mucoraceae, a forward movement in the center of the hypha and a return flow in a thin peripheral layer (Schröter, 1905; confirmed by Buller). Some light has been thrown on this phenomenon by Hawker and McV. Abbott (1963), whose studies of fine structure of *Rhizopus* hyphae revealed in the apical regions a "cortical membrane" separating an outer zone, containing numerous mitochondria, from an inner zone that contained nuclei and a few mitochondria. These authors concluded that food materials and nuclei move up the center of the young hyphae and that mitochondria accumulate at the tip

and then move slowly back in the peripheral layer, where they may contribute to the biochemical reactions involved in wall formation.

Working with species of *Rhizopus*, *Aspergillus*, and *Penicillium*, Schütte (1956) confirmed earlier conclusions relating streaming to food translocation. He demonstrated rapid accumulation of nutrients at the tips of hyphae at a distance from the source in fungi exhibiting streaming, but not in those where streaming was absent. The presence among the latter group of "vigorous and rapidly growing species" underlines the need for further work on streaming and transport in fungi, as does the observation by Robertson (1961) that severance of the leading hyphae in a young culture of *Fusarium oxysporum* has no effect on their growth rate (cf. Chapter 26 of this volume).

With the aid of dyes, Schütte demonstrated translocation zones defined physiologically but not morphologically, in mature agaric fructifications. Movement of dye was increased by conditions favoring loss of water from the fructification, but both translocation and loss of water occurred at appreciable and similar rates under conditions of complete saturation indicating the operation of some protoplasmic mechanism in translocation through the fructification. Mycelium in the soil was found to translocate in the same manner. Evidence of a similar "vital" component of translocation was found by Plunkett (1958) in the fructifications of *Polyporus brumalis* although the movement there was due mainly to evaporation.

III. REPRODUCTIVE STRUCTURES

A. Life Cycles

Like other sexually reproducing organisms many fungi show an alternation between haploid and diploid phases in their life cycles, the former beginning with the completion of meiosis, the latter with fusion of nuclei in sexual reproduction. In many fungi, particularly basidiomycetes, a third phase is interposed between haploid and diploid phases. The nuclei that are brought together in the preliminaries to sexual reproduction do not immediately fuse. Instead they associate in pairs known as *dikaryons* (cf. Volume II) which persist for a short or long period through conjugate division of the component nuclei and separation of the products, one nucleus of each sort, into each of the new cells. The dikaryon is thus a delayed step in the process of sexual reproduction. Biologically it serves to increase the number of sexual fusions that eventually take place and subsequent genetic recombinations. Although dikaryons composed of genetically unlike nuclei are often included as a form of heterokaryon, biologically they are quite distinct, as Jinks (1952) has pointed out.

Heterokaryosis is association in somatic cells of nuclei of unlike genetic stitution. *Heterokaryons* can originate by mutation in a *homokaryon* by the migration via hyphal anastomoses of nuclei of one mycelium) another. They are characteried by variability in the proportions of two sets of nuclei that exist side by side and provide a unique system omatic variation.

With respect to the mating system which brings together the nuclei in ual reproduction, fungi can be divided into two groups, *homothallic* . *heterothallic*. In fungi of the former group, sexual reproduction is omplished by union of nuclei from one and the same thallus, a process : leads to inbreeding. In the heterothallic group, sexual reproduction es place between nuclei from thalli of different mating types. Heterolism is an outbreeding device (Mather, 1942) preventing fusion of ıetes from one individual. (For a discussion of inbreeding and outeding, see Volume II.) Two forms of heterothallism occur, isolation gametes in different individuals and operation of incompatibility es that allow mating only between nuclei carrying unlike allelo-phs. These have been distinguished by Whitehouse (1949 as morphocal and physiological heterothallism, respectively. The interacting thalli norphologically heterothallic forms bear either male or female reprotive organs, while those of physiologically heterothallic forms differ only nating type. At one extreme, some of these latter lack sex organs comely, at the other, both strains possess both male and female organs. fe cycles and basic patterns of sexuality are reviewed in Volume II.) As a group, fungi display a number of different life cycles (Raper, 1954) tering around sexual reproduction. Differing from sexual reproduction *arasexuality*, demonstrated by Pontecorvo (1956) in members of the ıgi Imperfecti and since found also in other groups (Bradley, 1962). : parasexual cycle involves fusion of haploid nuclei in a heteroyon, mitotic crossing over, and haploidization. Qualitatively it is equiva- to the sexual cycle but differs in the absence of a precise time uence for its stages. For heterokaryotic organisms, with their capacity rapid adjustment to changing conditions through alteration in the ance of the different types of nuclei in the heterokaryon, parasexuality vides in addition the advantages of true sexual reproduction—gene ombination.

B. Spores

Reproduction in fungi results in the formation of enormous numbers of res, uni- or multicellular, microscopic propagules containing one or re nuclei, which are liberated from the parent thallus passively or by

active discharge (Ingold, 1953). Most are dispersed by wind, often fo very long distances (Gregory, 1961), or by water, insects, and other ani mals. Spores are extremely efficient as agents of dispersal and are encountered, in a viable condition, in every conceivable habitat.

Typically both asexual and sexual spores are formed, and the corresponding stages of the life cycle are often described as imperfect anc perfect, respectively. Asexual reproduction occurs usually when conditions are favorable to growth, and several generations may follow one another in one season. Asexual spores are capable of immediate germination and bring about rapid increase in numbers and spread of the organism under favorable conditions. Many types of spore formed as the products of sexual reproduction, on the other hand, are thick-walled, resistant structures and serve for survival.

1. Asexual Spores

a. Sporangiospores. Asexual spores are of two main types, sporangiospores formed within sporangia, and conidia formed externally on hyphae usually morphologically differentiated as *conidiophores*. Sporangia are characteristic of phycomycetes. In holocarpic forms the mature thallus becomes a sporangium. In mycelial forms sporangia may be indistinguishable from hyphae as in *Aphanomyces* and some species of *Pythium;* or, typically, develop as swellings, usually at the tips of hyphae, quite distinct both in shape (spherical, pyriform, irregular) and in size. They are essentially multinucleate segments of the thallus delimited by septa whose contents form spores. In the most advanced members of the group the hyphae bearing sporangia are morphologically distinguishable as *sporangiophores*.

In aquatic phycomycetes and their relatives, the protoplasm of the sporangia differentiates at maturity to form *zoospores,* which are naked, motile spores containing usually one nucleus and possessing one or two flagella by which they are propelled. The zoospores emerge through one or more exit pores and, after a period of motility, encyst. The flagella are lost, and each spore develops a cell wall. After a short time, in favorable conditions, the cysts germinate with the formation of germ tubes that develop into hyphae.

Several types of zoospore, distinguished by number and structure of flagella and by internal structure, have been recognized among aquatic phycomycetes, reflecting origins from distinct ancestral lines. Other characters have been found to correlate with divisions based on zoospore morphology and it has become accepted as the basis for primary classification within this group (Sparrow, 1958; Sparrow, 1960; Waterhouse, 1962).

Studies of soil-borne phycomycete plant pathogens of the genus *Phy-*

tophthora show that zoospores carried by moving water, draining through soil after heavy rain, or following irrigation, are effective dispersal units (Hickman, 1958). In species that attack aerial parts, however, the major role in dissemination is played by the sporangium itself, which, becoming detached, is borne aloft as part of the air spora. This biological adaptation to an aerial environment has evolved further in the most advanced group of the zoosporic series, the Peronosporaceae. In this family sporangia are without exception caducous and wind borne. In most genera germination in moist conditions is by zoospores, but in drier surroundings the sporangium puts out a germ tube, i.e., it functions as a spore. In *Peronospora* germination is exclusively by germ tube and the sporangium is indistinguishable from a conidium.

Nonmotile spores (*aplanospores*) are characteristic of zygomycetes, a group of largely soil-inhabiting phycomycetes. The contents of the terminal sporangia differentiate into walled spores containing several nuclei, and these are released by breakdown of the sporangium wall or by discharge of the sporangium as a whole (Ingold, 1953). As in the Peronosporales, evolution to the conidial condition has taken place and species can be arranged in a series supporting the theory that this may have occurred by reduction in size of the sporangium to a monosporus condition, the walls of spore and sporangium becoming fused together.

b. Conidia. Asexual reproduction by conidia is highly developed in ascomycetes and occurs in a few basidiomycetes and exclusively in fungi imperfecti. Conidia are borne on conidiophores which (1) are grouped in a saucer-shaped fructification known as an *acervulus,* or (2) are enclosed within a *pycnidium,* a globose or flask-shaped structure from which the spores emerge through a pore, the ostiole, in a mucilaginous tendril, or (3) arise directly from the mycelium. These differences in conidiophore arrangement form the basis of the first and only complete scheme of classification of the Fungi Imperfecti (Saccardo, 1880, 1884, 1886). In passing it should be emphasized that this classification is not equivalent to that in the other classes of the fungi, which is based on the perfect state, and does not imply close relationship between the forms that are grouped together. Fungi imperfecti are continually being linked up with sexually reproducing species, most of them ascomycetes. It is known that one ascomycete genus may include species with widely different imperfect fructifications and, conversely, the conidial forms of species belonging to quite distinct genera may be similar. Saccardo's classification is thus artificial, its main purpose being to facilitate identification.

With regard to the conidia themselves, which exist in an immense variety of form and size, Vuillemin's (1910, 1912) system of classification of imperfect fungi drew attention to the widely different developmen-

tal origin of the spores described as conidia. He recognized two main types, *thallospores* and *conidiospores,* each including several distinct forms. The former arise by transformation of preexisting elements of the thallus, i.e., of vegetative hyphae, and are not readily detachable whereas conidiospores are formed as new structures on the thallus and are caducous. Our understanding of *conidiophore* structure, and of the formation and type of conidium, has been broadened further by the contributions of Mason (1933, 1937, 1941), Hughes (1953), and Tubaki (1958). Emphasizing ecology rather than morphology, Mason (1937) pointed out that these fungi can be divided into two biologically natural groups, one with moist or slime spores and the other with dry spores, these being dispersed by water and by wind, respectively. This distinction, adopted by some authors, e.g., Wakefield and Bisby (1941), has been criticized by Hughes on two grounds: one, that it is imprecise; the other, that it is uncorrelated with methods of conidial development.

2. Sexual Spores

Fungi show many variations in the pattern of their sexual cycles, in the degree of differentiation of sex organs, and in the type of spores formed as a result of sexual reproduction. Three types of spores are formed, thick-walled resting spores, ascospores, and basidiospores. The characteristics of the sexual spore stage, together with the nature of the mycelium, provide the basis for recognition of the classes Phycomycetes, Ascomycetes, and Basidiomycetes.

a. Resting spores. The resting spore of phycomycetes contains one or more diploid nuclei. Apart from *Allomyces,* which is unique in possessing distinct haploid and diploid generations, and, possibly, the Plasmodiophorales, in which a diploid plasmodium is believed to arise by growth and nuclear division of a zygote, the resting spore has been regarded until recently as the only diploid structure in these fungi (Olive, 1953). This opinion has now been challenged by Sansome (1963), who claims that oomycetes are "most probably diploid throughout their life history, meiosis occurring immediately before karyogamy."

Among phycomycetes sexual reproduction is accomplished in several ways: (1) fusion of motile gametes, isogamous (e.g., *Olpidium*) or anisogamous (*Allomyces*), the zygote being motile for a time by means of the persistent flagella; (2) copulation of thalli (*Rhizophydium*) or parts of thalli (*Polyphagus*); (3) fertilization of an *oosphere,* a large, nonmotile female gamete, enclosed within an *oogonium,* by a motile male gamete which enters through a pore in the oogonial wall (*Monoblepharis*) to give an *oospore;* (4) fertilization of an oosphere by the contents of an *anthe-*

ridium which enter the oogonium through a fertilization tube (*Pythium*); (5) copulation of gametangia (*Mucor*) to give a typical *zygospore*.

With few exceptions (*Allomyces*, Plasmodiophorales) each zygote gives rise to a single spore. It may be freed from the thallus and/or host tissue by decay or may germinate *in situ*. Though field observations (Garrett, 1956) suggest that the resting spores of many phycomycete plant parasites remain dormant for long periods, they are not unique among fungi in this respect (Gottlieb, 1950), nor do they all remain dormant for long periods. Germination of resting spores of *Rhizophydium* has been reported within 2–5 days of their formation (Couch, 1935; Karling, 1939), and of *Phytophthora* oospores (Legge, 1953) from a week to a year after burial in soil. The significance of such physiological heterogeneity in relation to survival has been discussed by Garrett.

b. Ascospores. In ascomycetes fusion of nuclei in sexual reproduction occurs in a cell known as the *ascus mother cell* and, with the exception of some yeasts, is followed immediately by meiosis and mitosis with the formation of eight haploid nuclei around each of which an ascospore is formed. Meanwhile the ascus mother cell has enlarged to form the *ascus* from which the ascospores are liberated by active discharge or by breakdown of the ascus wall (Ingold, 1953).

Sexual reproduction takes place (1) by copulation of simple gametangia. These may be alike (e.g., *Eremascus*) or show slight morphological differentiation (*Dipodascus*); (2) by means of well-defined male and female sex organs, *ascogonia* and antheridia, passage of male nuclei taking place via a pore at the point of contact between the walls or through an appendage of the ascogonium, the *trichogyne* (*Pyronema*); (3) in other species antheridia are not formed and fertilization is accomplished via minute, uninucleate cells, either *spermatia*, formed within pycnidium-like structures known as *spermogonia* (*Mycosphaerella*), or *microconidia* (*Sclerotinia, Neurospora*); and, in *Neurospora* by the conidia themselves. Association of nuclei may also be brought about in such fungi as *Neurospora* by hyphal anastomosis, or, as in *Ascobolus stercorarius*, by means of *oidia*, minute spores formed by fragmentation of short aerial hyphae, which fuse with hyphae, or give rise to hyphae that anastomose. In such cases the introduced nucleus travels through the hypha to the developing ascogonium. In some species no sex organs whatever are formed, and nuclei are brought together (*Taphrina, Saccharomyces*) by fusion of cells arising from ascospores by budding, or by fusion of the ascospores themselves.

A feature of many ascomycetes is the association in pairs of sexually differentiated nuclei and their multiplication by mitosis in a dikaryon phase. In such species as *Pyronema* this takes place in *ascogenous hyphae*

which grow out from the wall of the ascogonium. Ultimately these give rise to a number of binucleate cells, the ascus mother cells. This process thus leads to multiplication of the number of zygote nuclei that arise from one ascogonium. Species of *Taphrina* are dikaryotic throughout their vegetative phase following association of nuclei soon after ascospore formation. Here ascus formation is initiated by fusion of pairs of nuclei in differentiated ascogenous cells, each of which gives rise to one ascus.

In ascomycetes that possess ascogenous hyphae, vegetative hyphae envelop the developing sex organs to form fructifications known as *ascocarps*. These may be (1) *cleistocarps,* completely closed structures from which the ascospores are liberated by decay or rupture of the wall; (2) *perithecia,* globose or flask-shaped ascocarps, with an apical opening, the ostiole, through which the spores are discharged; (3) *apothecia,* cup- or saucer-shaped ascocarps. Many modifications of these basic ascocarp types occur, and in many species the ascocarps are formed in groups within a *stroma,* a pseudoparenchymatous tissue of hyphae having some resemblance to a sclerotium. Other members of the group, ascostromatic ascomycetes, form their asci directly within cavities, locules, in stromata.

c. Basidiospores. Sexual reproduction in basidiomycetes culminates in the fusion of a pair of haploid nuclei in a cell known as the *basidium.* The diploid, zygote nucleus undergoes meiosis and four haploid nuclei pass one into each of four thin-walled *basidiospores,* which are borne externally on the basidium and in the majority of species forcibly discharged (Ingold, 1953) from it. The basidium is essentially the terminal cell of a long series of dikaryon cells, for a large part of the life cycle in this group is spent in the dikaryon condition. No sex organs are formed. Dikaryons may be established directly after meiosis, within the basidium itself, or by copulation of basidiospores, as in some of the Ustilaginales, or later, in the haploid mycelium arising on germination of the basidiospores. In these hyphae, dikaryons are initiated by anastomosis or through the agency of oidia or spermatia.

In the Uredinales a succession of dikaryon spores, *aecidiospores, uredospores* and *teleutospores,** provides for increase and spread of the species, and the final, or teleutospore stage, also serves for survival. In the Ustilaginales the brand spore also serves for spread and survival. In both of these groups these thick-walled, resistant spores give rise to the basidia. In the other members of the class the basidia are associated in large numbers in highly organized, often structurally complex, fruit bodies —the mushrooms, bracket fungi, puff balls, stinkhorns, bird's-nest fungi, etc.

* *aeciospores, urediospores,* and *teliospores* in Arthur's terminology

IV. VEGETATIVE AND REPRODUCTIVE STRUCTURES IN MYXOMYCOTA

The limitations of a system of classification that recognizes only plants and animals are nowhere more clearly exposed than by consideration of the slime molds, for they exhibit characteristics of both kingdoms. The vegetative phase of these organisms, which is a naked, creeping mass of protoplasm that takes in food particles by ingestion, is definitely animal-like. On the other hand, their reproduction by spores resembles that in fungi. Originally described as fungi by Persoon (1801), slime molds have since been included by botanists in that group, as Myxomycetes, and by zoologists in the Protozoa, as Mycetozoa. Discussing the systematic position of these organisms, Martin (1960) has suggested recently that they can best be regarded as an offshoot from the main line of evolution of fungi from colorless flagellates, a view that has received support from another prominent student of slime molds (Alexopoulos, 1962).

Several other groups have been associated with the Myxomycetes. In possessing a plasmodium-like thallus and in the character of the zoospores, the Plasmodiophorales resemble the Myxomycetes and have often been classified with them. Mainly on account of the recognition of phycomycetous, thin-walled sporangia, this group is now included in the Phycomycetes. Two other groups, the Acrasiales and the Labyrinthulales, in which vegetative cells aggregate, giving a superficial resemblance to plasmodia, have also been classified alongside the Myxomycetes. These, however, are basically unicellular, uninucleate, amoeboid organisms, and they are now believed to be more closely related to the Protozoa than to other forms. Discussing these four groups, Bonner (1959) concludes that they comprise primitive colonial organisms with both fungal and animal characters, but with probably little interrelationship.

A. Myxomycetes

Myxomycetes are common saprophytes of damp woodlands, growing on decaying logs, leaves, and other organic matter. The vegetative structure is a *plasmodium,* which is a multinucleate mass of protoplasm not bounded by a cell wall. Its nuclei are diploid. Often brightly colored, the plasmodium flows over the substratum as a sheet of protoplasm, up to a square foot or more in area in very large specimens, continually changing shape and engulfing particles of organic matter from the substratum. In forms such as the well-known *Physarum polycephalum,* the actively moving plasmodium

is roughly fan-shaped, with a continuous mass of protoplasm in front connected with a reticulum of protoplasmic strands at the rear. Protoplasmic streaming is a characteristic feature of plasmodia, as of the hyphae of many fungi, and occurs along differentiated cytoplasmic channels known as veins. Under unfavorable conditions the plasmodium contracts to form an immobile, hard, irregular resting body, or sclerotium, inside which groups of nuclei and surrounding cytoplasm are enclosed by membranes to form *macrocysts.* With the return of favorable conditions, the sclerotium softens and a new plasmodium emerges.

As long as conditions are favorable the plasmodium continues to grow and its nuclei increase in number by repeated mitotic division. At a particular stage in development it is converted into one or more fruiting bodies. With the exception of the genus *Ceratiomyxa,* in which the spores are borne externally on a sporophore, the spores are formed within sporangia. These are of several types: (1) individual, stalked or sessile sporangia; (2) an *aethalium,* representing a group of fused sporangia; (3) a *plasmodiocarp,* a sessile, sporangium-like, branched structure formed around some of the veins of the plasmodium. Within the sporangia of many species *capillitial threads* are formed, and lime granules are frequently deposited on these and on the sporangial wall.

Immediately before spore formation the nuclei of the sporangium undergo meiosis, and haploid, uninucleate, thick-walled spores are formed. These may retain their viability for long periods. On germination, from one to four microscopic, uninucleate, haploid naked cells are released from each spore. These may be amoeboid *myxamoebae,* or *swarm cells,* motile by means of one or, usually, two unequal flagella attached at the anterior end. Myxamoebae may also develop flagella, becoming swarm cells, soon after release; the two forms appear to be readily interconvertible, depending on the conditions. Myxamoebae and swarm cells feed in a typically amoeboid manner, ingesting particles of food. In this respect the swarm cells differ conspicuously from the zoospores of fungi. Under favorable conditions myxamoebae divide repeatedly to give large populations of haploid cells. After a period of feeding and multiplication, fusion occurs between pairs of swarm cells or myxamoebae. The zygotes thus formed grow, with successive mitotic division of their diploid nuclei, to form naked, amoeboid plasmodia. In some species, in addition, plasmodia arise by coalescing of many zygotes.

B. Acrasiales

The members of this group are abundant and widespread in soils, where they feed on bacteria. In the vegetative stage they exist as microscopic,

ıninucleate, haploid, naked amoebae which multiply by fission and are in-listinguishable from amoeboid protozoa. No flagellated cells are formed. Their distinguishing feature is the aggregation of amoebae to form a *ıseudoplasmodium* (Bonner, 1959) in which the amoebae do not coalesce ıut remain as individuals, hence the name cellular, or communal slime nolds.

Aggregation of amoebae is a prelude to sporulation. It is followed by lifferentiation of the cells of the pseudoplasmodium into two types. Those .t the anterior end begin to form a stalk. It increases in height by movement ›f cells up to the apex. There they are converted into large, pithlike stalk ells which become enclosed within a cylinder of cellulose that is deposited ıround them. The posterior, sporogenous cells rise to the top of the stalk, vhere each becomes surrounded by a cellulose wall. A mass of spores is hus formed, held together by slime but not enclosed within a common nembrane. Present evidence regarding sexuality in the group is conflicting. The existence of seven chromosomes during vegetative division suggests hat the amoebae are haploid.

C. Labyrinthulales

The Labyrinthulales comprise a small group of marine and freshwater ›arasites of algae and higher plants. The unit of vegetative structure is ıgain a naked, uninucleate cell. It is spindle shaped and secretes from each ɔnd a long, slimy filament. These link up with those of other cells, forming a network over which the cells glide. The network increases in size by secretion of new filaments by groups of cells at the margin, and the cells themselves multiply by division. At a certain stage in the development of some species, many cells mass together; they become surrounded by a membrane, and each cell forms a wall. These spores are eventually lib-erated and elongate to form vegetative cells. In one species, *Labyrinthula algeriensis,* cells aggregate within the slime filaments. Each then enlarges, surrounds itself with a mucilaginous envelope, and gives rise to several heterokont, biflagellate zoospores which initiate new colonies. Sexual re-production has not been observed among these organisms.

REFERENCES

Ainsworth, G. C. (1952). "Medical Mycology. An Introduction to its Problems," 105 pp. Pitman, New York.

Alexopoulos, C. J. (1962). "Introductory Mycology," 2nd ed., 613 pp. Wiley, New York.

Allen, P. J. (1959). Metabolic considerations of obligate parasitism. *In* "Plant Pathology. Problems and Progress" (C. S. Holton ed.), pp. 119–129. Univ. of Wisconsin Press, Madison, Wisconsin.

Backus, M. P. (1939). The mechanics of conidial fertilization in *Neurospora sitophila*. *Bull. Torrey Botan. Club* **66**:63–76.

Blackwell, E. (1949). Terminology in *Phytophthora*. *Mycol. Papers No.* **30**.

Blackwell, E. (1953). Haustoria of *Phytophthora infestans* and some other species. *Brit. Mycol. Soc. Trans.* **36**:138–158.

Bonner, J. T. (1959). "The Cellular Slime Moulds," 150 pp. Princeton Univ. Press, Princeton, New Jersey.

Bracker, C. E., and E. E. Butler. (1963). The ultrastructure and development of septa in hyphae of *Rhizoctonia solani*. *Mycologia* **55**:35–58.

Bradley, S. G. (1962). Parasexual phenomena in microorganisms. *Ann. Rev. Microbiol.* **16**:35–52.

Buller, A. H. R. (1933). "Researches on Fungi," Vol. V, 416 pp. Longmans, Green, New York.

Butler, G. M. (1957). The development and behaviour of mycelial strands in *Merulius lacrymans* (Wulf.). Fr. *Ann. Botany (London)* [N. S.] **21**:523–537.

Butler, G. M. (1961). Growth of hyphal branching systems in *Coprinus disseminatus*. *Ann. Botany (London)* [N. S.] **25**:341–352.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York; Chapman & Hall, London.

Coker, W. C. (1923). "The Saprolegniaceae with Notes on Other Water Molds," 201 pp. Univ. of North Carolina Press, Chapel Hill, North Carolina.

Coley Smith, J. R. (1960). Studies of the biology of *Sclerotium cepivorum* Berk. IV. Germination of sclerotia. *Ann. Appl. Biol.* **48**:8–18.

Coley Smith, J. R., and C. J. Hickman. (1957). Stimulation of sclerotium germination in *Sclerotium cepivorum* Berk. *Nature* **180**:445.

Couch, J. N. (1935). New or little known Chytridiales. *Mycologia* **27**:160–175.

Davies, M. E. (1959). The nutrition of *Phytophthora fragariae*. *Brit. Mycol. Soc. Trans.* **42**:193–200.

Drechsler, L. (1941). Predacious fungi. *Biol. Rev. Cambridge Phil. Soc.* **16**:265–290.

Duddington, C. L. (1962). Predacious fungi and the control of eelworms. *In* "Viewpoints in Biology" (J. D. Carthy and C. L. Duddington, eds.), Vol. I, pp. 151–200. Butterworths, London.

Ehrlich, H. G., and M. A. Ehrlich. (1963). Electron miscroscopy of the host-parasite relationships in stem rust of wheat. *Am. J. Botany* **50**:123–130.

Fincham, J. R. S., and P. R. Day. (1963). "Fungal Genetics," 300 pp. Blackwell, Oxford.

Fraymouth, J. (1956). Haustoria of the Peronosporales. *Brit. Mycol. Soc. Trans.* **39**:79–107.

Garrett, S. D. (1956). "Biology of Root-Infecting Fungi," 293 pp. Cambridge Univ. Press, London and New York.

Garrett, S. D. (1963). "Soil Fungi and Soil Fertility," 165 pp. Macmillan (Pergamon), New York.

Gäumann, E. A., and C. W. Dodge. (1928). "Comparative Morphology of Fungi," 701 pp. McGraw-Hill, New York.

Girbardt, M. (1958). Über die Substruktur von *Polystictus versicolor* L. *Arch. Mikrobiol.* **28**:255–269.

Girbardt, M. (1961). Licht- und Elektronen mikroskopische Untersuchungen an

olystictus versicolor. II. Die Feinstruktur von Grund Plasma und Mitochondrien. *rch. Mikrobiol.* 39:351–359.
lieb, D. (1950). The physiology of spore germination in fungi. *Botan. Rev.* 3:229–257.
gory, P. H. (1961). "The Microbiology of the Atmosphere," 251 pp. Leonard ill, London; Wiley (Interscience), New York.
ckel, E. (1866). "Generelle Morphologie der Organismen. Allgemeine Grund-ge der organischen Formen-Wissenschaft, mechanisch begründet durch die von harles Darwin reformirte Descendenz-Theorie," 2 vols. Reimer, Berlin.
ckel, E. (1878). "Das Protistenreich. Eine populäre Übersicht über das Formenge-et der niedersten Lebewesen. Mit einem wissenschaftlichen Anhange: System r Protisten." Gunther, Leipzig.
ckel, E. (1894). "Systematische Phylogenie, Entwurf eines natürlichen Systems r Organismen auf Grund ihrer Stammesgeschichte. Erster Theil: Systematische hylogenie der Protisten und Pflanzen." Reimer, Berlin.
/ker, L. E., and P. McV. Abbott. (1963). Fine structure of vegetative hyphae of *hizopus*. *J. Gen. Microbiol.* 30:401–408.
cman, C. J. (1958). Presidential Address. *Phytophthora*—plant destroyer. *Brit. fycol. Soc. Trans.* 41:1–13.
hes, S. J. (1953). Conidiophores, conidia and classification. *Can. J. Botany* 1:577–659.
ld, C. T. (1953). "Dispersal in Fungi," 197 pp. Oxford Univ. Press, (Clarendon), ondon and New York.
s, J. L. (1952). Heterokaryosis: a system of adaptation in wild fungi. *Proc. Roy. oc.* **B140**:83–99.
ison, T. W., Jr. (1956). "The Genus Achlya: Morphology and Taxonomy," 180 p. Univ. of Michigan Press, Ann Arbor, Michigan.
ling, J. S. (1939). Studies on *Rhizophydium*. III. Germination of the resting pores. *Bull. Torrey Botan. Club* 66:281–286.
ge, B. J. (1953). Studies of species of *Phytophthora*. Ph.D. Thesis, Univ. of Birm-igham, England.
tin, G. W. (1960). The systematic position of the Myxomycetes. *Mycologia* 2:119–129
son, E. W. (1933). Annotated account of fungi received at the Imperial Myco-gical Institute. List II (Fascicle 2). *Mycol. Papers No.* 3.
son, E. W. (1937). Annotated account of fungi received at the Imperial Myco-gical Institute. List II (Fascicle 3). General Part. *Mycol. Papers No.* 4.
son, E. W. (1941). Annotated account of fungi received at the Imperial Myco-gical Institute. List II (Fascicle 3). Special Part. *Mycol. Papers No.* 5.
ther, K. (1942). Heterothally as an outbreeding mechanism in fungi. *Nature* 49:54–56.
ore, R. T., and J. H. McAlear. (1961). Fine structure of Mycota. 5. Lomasomes—reviously uncharacterized hyphal structures. *Mycologia* 53:194–200.
ore, R. T., and J. H. McAlear. (1962). Fine structure of Mycota. 7. Observations n septa of Ascomycetes and Basidiomycetes. *Am. J. Botany* 49:86–94.
sse, B. (1963). Vesicular-arbuscular mycorrhiza: an extreme form of fungal daptation. *Symp. Soc. Gen. Microbiol.* 13:146–170.
ller, H. G. (1958). The constricting ring mechanism of two predacious Hyphomy-etes. *Brit. Mycol. Soc. Trans.* 41:341–364.
kerson, W. J. (1959). A report on Symposium VI. *In* "Biochemistry of Morpho-

genesis" (W. J. Nickerson, ed.) *Proc. 4th Intern. Cong. Biochem., Vienna, 1958* VI:242–260. Macmillan (Pergamon), New York.

Olive, L. S (1953). The structure and behaviour of fungus nuclei. *Botan. Rev.* **19**: 439–586.

Olthof, T. H. A., and R. H. Estey. (1963). A nematoxin produced by the nematophagous fungus *Arthrobotrys oligospora* Fresenius. *Nature* **197**:514–515.

Persoon, C. H. (1801). "Synopsis methodica Fungorum." Göttingen.

Peyton, G. A., and C. C. Bowen. (1963). The host-parasite interface of *Peronospora manshurica* on *Glycine max*. *Am. J. Botany* **50**:787–797.

Plomley, N. J. B. (1959). Formation of the colony in the fungus *Chaetomium*. *Australian J. Biol. Sci.* **12**:53–64.

Plunkett, B. E. (1958). Translocation and pileus formation in *Polyporus brumalis*. *Ann. Botany (London)* [N. S.] **22**:237–249.

Pontecorvo, G. (1956). The parasexual cycle in fungi *Ann. Rev. Microbiol.* **10**:393–400.

Prowse, G. A. (1954). *Sommerstoffia spinosa* and *Zoophagus insidians* predacious on rotifers, and *Rozellopsis inflata* the endoparasite of *Zoophagus*. *Brit. Mycol. Soc. Trans.* **37**:134–150.

Raper, J. R. (1954). Life cycles, sexuality and sexual mechanisms in the fungi. *In* "Sex in Microorganisms" (D. H. Wenrich, ed.), pp. 42–81. Am. Assoc. Advance. Sci., Washington, D.C.

Robertson, N. F. (1961). Mycology. *In* "Contemporary Botanical Thought" (A. M. MacLeod and L. S. Cobley, eds.), pp. 133–148. Oliver & Boyd, Edinburgh and London.

Robinow, C. F. (1962). Some observations on the mode of division of somatic nuclei of *Mucor* and *Allomyces*. *Arch. Mikrobiol.* **42**:369–377.

Robinow, C. F. (1963). Observations on cell growth, mitosis and division in the fungus *Basidiobolus ranarum*. *J. Cell Biol.* **17**:123–152.

Ryan, F. J., G. W. Beadle, and E. L. Tatum. (1943). The tube method of measuring the growth rate of *Neurospora*. *Am. J. Botany* **30**:784–799.

Saccardo, P. A. (1880). Conspectus genera fungorum italiae inferiorum nempe ad Sphaeropsideas, Melanconieas et Hyphomycetas pertinentium, systemate sporologico dispositorum. *Michelia* **2**:1–38.

Saccardo, P. A. (1884). "Sylloge fungorum omnium hucusque cognitorum," Vol. 3. Pavia.

Saccardo, P. A. (1886). "Sylloge fungorum omnium hucusque cognitorum," Vol. 4. Pavia.

Sansome, E. R. (1963). Meiosis in *Pythium de Baryanum* and its significance in the life-history of the Biflagellatae. *Brit. Mycol. Soc. Trans.* **46**:63–72.

Schröter, A. (1905). Über Protoplasmaströmung bei Mucorineen. *Flora (Jena)* **95**:1–30.

Schütte. K. H. (1956). Translocation in the fungi. *New Phytologist* **55**:164–182.

Shatkin, A. J., and E. L. Tatum. (1959). Electron microscopy of *Neurospora crassa* mycelia. *J. Biophys. Biochem. Cytol.* **6**:423–426.

Sleigh, M. A. (1962). "The Biology of Cilia and Flagella," 242 pp. Macmillan, New York.

Smith, J. H. (1923). On the apical growth of fungal hyphae. *Ann. Botany (London)* **37**:341–343.

Sparrow, F. K. (1958). Interrelationships and phylogeny of the aquatic Phycomycetes. *Mycologia* **50**:797–813.

Sparrow, F. K. (1960). "Aquatic Phycomycetes," 2nd ed., 1187 pp. Univ. of Mich. Press, Ann Arbor, Michigan.

Stanier, R. Y., and C. B. van Niel. (1962). The concept of a bacterium. *Arch. Mikrobiol.* **42**:17–35.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. (1963). "The Microbial World," 2nd ed., 753 pp. Prentice-Hall, Englewood Cliffs, New Jersey.

Townsend, B. B. (1954). Morphology and development of fungal rhizomorphs. *Brit. Mycol. Soc. Trans.* **37**:222–233.

Townsend, B. B. and H. J. Willetts, (1954). The development of sclerotia of certain fungi. *Brit. Mycol. Soc. Trans.* **37**:213–221.

Tubaki, K. (1958). Studies on the Japanese hyphomycetes. V. Leaf and stem group with a discussion of the classification of hyphomycetes and their perfect stages. *J. Hattori Botan. Lab.* **20**:142–244.

Vuillemin, P. (1910). Matériaux pour une classification rationelle des Fungi Imperfecti. *Compt. Rend.* **150**:882–884.

Vuillemin, P. (1912). "Les champignons. Essai de classification." Doin, Paris.

Wakefield, E. M., and G. R. Bisby. (1941). List of Hyphomycetes recorded for Britain. *Brit. Mycol. Soc. Trans.* **25**:49–126.

Ward, E. W. B., and K. W. Ciurysek, (1962). Somatic mitosis in *Neurospora crassa*. *Am. J. Botany* **49**:393–399.

Waterhouse, G. M. (1962). Presidential Address. The zoospore. *Brit. Mycol. Soc. Trans.* **45**:1–20.

Weisz, P. B. (1961). "Elements of Biology," 491 pp. McGraw-Hill, New York.

Weisz, P. B., and M. S. Fuller. (1962). "The Science of Botany," 562 pp. McGraw-Hill, New York.

Whitehouse, H. L. K. (1949). Heterothallism and sex in the fungi. *Biol. Rev. Cambridge Phil. Soc.* **24**:411–447.

Whittaker, R. H. (1959). On the broad classification of organisms. *Quart. Rev. Biol.* **32**:210–226.

Zalokar, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* **46**:602–610.

Cell Components

CHAPTER 3

The Cell Wall[1]

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I. HISTORICAL RÉSUMÉ

Early in the nineteenth century, Braconnot (1811) published the results of his chemical analysis of several mushroom species. In all he noted a markedly resistant component which remained following protracted treatment of the material with strong alkali. Believing that he had uncovered a unique fungal characteristic, Braconnot named the alkali-resistant substance "fungine." This investigator, displaying considerable ability with the techniques of analytical chemistry that were then available, was able to determine that fungine was a nitrogenous substance and pointed out that its nitrogen content was less than that of protein.

In studies of the nature of the integuments of insects Odier (1823) also discovered a component that resisted the action of alkali. For this substance he proposed the name "chitin." Odier and others failed to recognize Braconnot's elucidation of the properties of fungine, and consequently the term chitin gained general acceptance. The latter term, therefore, applies to the resistant nitrogenous material that is found in the walls of most (but not all) fungi and which is of widespread occurrence in the cuticular structures of invertebrates. The term chitin (Gr. *chitōn*) signifies a mantle or tunic and is, therefore, applied appropriately to fungi and invertebrates alike.

The widespread occurrence of chitin in the cell walls of fungi came to light in the classical investigations of van Wisselingh (1898). This worker devised a cytochemical test (see Section IV, B, 1, b) involving the alka-

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line conversion of chitin to chitosan (see Section IV, B, 2). The test was applied to more than one hundred genera of fungi with generally positive results, but the reliability of this cytochemical method has been challenged in numerous instances. This fundamental difference between the cell walls of many fungi and the cellulosic walls of green plants has been of some concern to many mycologists. Consequently, over the years the van Wisselingh test has been utilized repeatedly as an adjunct to morphological studies of the fungi.

II. MICROSCOPIC STRUCTURE OF CELL WALLS

It is noteworthy that the gross morphology of fungi is, to a great degree, dependent upon the conformation of their cell walls. The walls of fungi, possessing considerable rigidity, lend stability to the shapes of vegetative hyphae and various reproductive structures. The foregoing statement is substantiated in numerous textbooks of mycology (e.g., Alexopoulos, 1962), where illustrations show discharged sporangia of phycomycetes or spent asci, to cite but two examples. Emerson (1958) has provided illustrations demonstrating this principle with the sporangia of aquatic phycomycetes. Also, the characteristic pseudoseptations of phycomycetes are partial intrusions of the hyphal walls into the hyphal interior and may be seen to persist after chemical removal of the cytoplasm (Aronson and Machlis, 1959). Finally we may note a multitude of cell wall ornamentations which characterize resistant sporangia, zygospores, and ascospores.

The preceding remarks indicate that striking features of cell wall form in the fungi are to be seen in the walls of structures that are markedly differentiated and have distinct functional attributes. However, most of the total wall substance of filamentous fungi is part of an extensive hyphal wall continuum, which, when viewed by conventional light microscopy, shows little, if any, differentiation from one wall segment to the next. Little is known of ontogenetic changes within the vegetative hyphal walls of fungi, or whether they occur generally. However, functional differentiation in the cytoplasm of *Neurospora* hyphae has been reported by Zalokar (1959), and it is conceivable that this may be accompanied by ontogenetic changes in the fine structure of the cell wall. This matter is carried further in Section III.

III. PHYSICAL CONFORMATION OF CELL WALLS

A. General Remarks

By means of polarization optics, X-ray diffraction, electron microscopy, and biochemical analysis, it is possible to gain some insight into the organi-

ation of cell walls at a level not revealed by light microscopy (Frey-Wyssling, 1953; Preston, 1952). In the past, these techniques have been employed infrequently in studies of fungal walls. A notable exception is the cell wall of the *Phycomyces* sporangiophore, which has attracted several investigators and which, at times, has been offered as a prototype of fungal wall structure. However, it is open to question whether or not the *Phycomyces* sporangiophore wall is representative of the walls of fungi in general, and, for the present, it should be regarded as a special case.

B. Fibrous Structure

Recent studies of the structure of cell walls of plants have brought about the evolution of a concept in which cell walls are regarded as a two-phase system—one phase consisting of the microfibrils which are embedded within the other phase, referred to as the amorphous matrix (Figs. 1 and

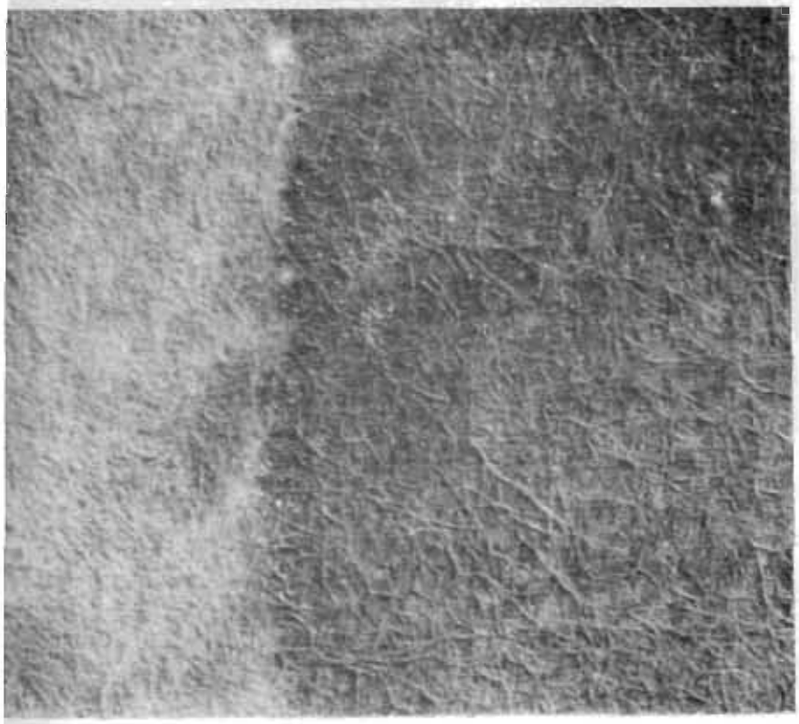


FIG. 1. Electron micrograph of a portion of a hyphal wall of *Allomyces macrogynus*, showing fibrous texture and masking of fibrils by matrix substances. Magnification: × 21,000. Reproduced from Aronson and Preston (1960a).

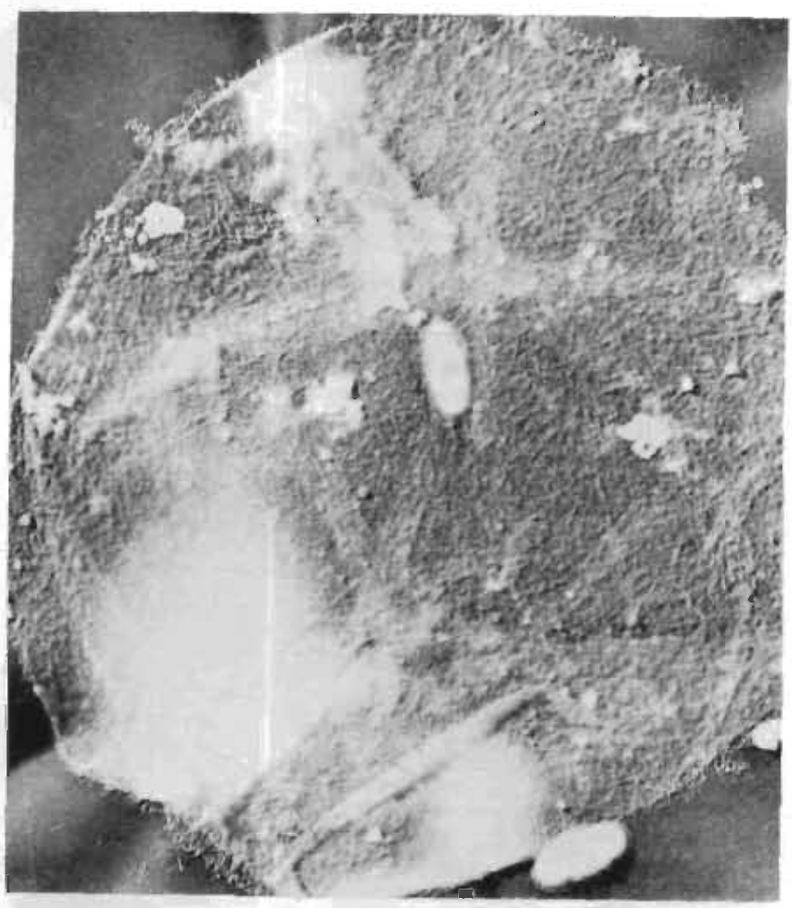


FIG. 2. Electron micrograph of the cell wall of *Rhizophydium sphaerotheca*. Magnification: × 11,520. (Aronson and Preston, previously unpublished photograph.)

2). The literature on this topic is vast and has been the subject of several reviews (e.g., Setterfield and Bayley, 1961). The fibrous texture of fungal walls was observed for the first time by Frey-Wyssling and Mühlethaler (1950) in the sporangiophore of *Phycomyces*. The existence of such fibrous structures, however, had been deduced much earlier through the application of polarization optics (Oort and Roelofsen, 1932) and X-ray diffraction (van Iterson *et al.*, 1936). Indeed prior to pioneering electron microscopy of plant cell walls (e.g., Frey-Wyssling *et al.*, 1948; Preston *et al.*, 1948), it was commonplace for investigators to make reference to

chains and their orientation in the cell walls of fungi (e.g., Castle,
. The principles involved in the application of the techniques referred
ve have been treated in sufficient detail by Preston (1952).
owing the initial observations of the fibrillar structure in the *Phy-*
es wall, the presence of a fibrous structure received confirmation
h the work of Roelofsen (1951) and Middlebrook and Preston
). The scope of observations in this area has since been widened to
: many other fungi. Houwink and Kreger (1953) demonstrated a
texture in the chemically treated walls of yeasts, and recently,
of the aquatic phycomycetes have been investigated by Aronson
eston (1960a), leading to the demonstration of a fibrous wall struc-
all cases.

C. Lamellae

:ral electron microscopic analyses have shown that the cell wall is
ıminate, as in the *Phycomyces* sporangiophore (*e.g.*, Frey-Wyssling
ühlethaler, 1950), where the primary wall of the growth zone has
esolved into two lamellae that differ in their microfibrillar orienta-
As the growth potential diminishes below the apical 2 mm of the
giophore, an inner lamella, designated as the secondary wall, is
:d to these outer layers of fibrils. A considerable amount of discus-
the literature has attempted to reconcile fibril textures with the oc-
ce of spiral growth in the sporangiophore (Castle, 1942; Roelofsen,
Middlebrook and Preston, 1952). That the cell wall is, in some
ı, implicated in spiral growth in *Phycomyces* most workers would
but the mechanism involved remains to be elucidated. A recent series
estigations (e.g., Probine, 1963) has attempted to explain spiral
ı and reversal of spiraling in *Nitella*. The basis for the proposed
growth theory lies in the observed mechanical anisotropy of the cell
and it was pointed out that the same explanation might be applied to
)wth of the *Phycomyces* sporangiophore.
:inct lamellae have been observed also in the hyphal walls of *Al-*
es (Aronson and Preston, 1960b). In Fig. 3 a bilaminate condition
ıtrated. The oriented fibrils of the inner layer are aligned parallel to
ıjor axis of the hypha. In this respect the wall texture of *Allomyces*
similarity to the secondary wall of *Phycomyces*. Evidence of lamina-
ıas not been limited to these observations of fibrous texture. Agar and
as (1955) prepared ultrathin sections of cells of *Saccharomyces*
revealed a multilaminate wall structure when viewed in the electron
;cope. The laminations appeared especially prominent in the bud scar
s. Studies by Mundkur (1960) and Vitols *et al.* (1961), both deal-

FIG. 3. Electron micrograph of a portion of a hyphal wall of *Allomyces macrogynus*, showing contrasting fibrous textures in inner and outer lamellae. Magnification: × 18,135. Reproduced from Aronson and Preston (1960b).

ing with *Saccharomyces*, have demonstrated in the walls a stratification that indicates differences in chemical constitution. Mundkur's work provides evidence for a thin outer layer of mannan which merges with a relatively thick inner layer of glucan (see Section IV, C, 2). It is noteworthy that the stratification of yeast cell wall components observed in the electron microscope has been deduced from biochemical analyses by Eddy (1958). This investigator indicated that a phosphomannan, in part, constitutes the outer and inner stratum of the yeast cell wall. Evidence in support of this view was derived from studies of the action of papain which brings about a simultaneous release of mannan associated with nondialyzable phosphorus and a loss of electrophoretic mobility (attributable to negative charges).

D. Septations

Virtually nothing is known of the fine structure of the pseudoseptations of phycomycetes. However, using ordinary light microscopy, it has been

observed (Coker, 1930) that the pseudosepta of *Allomyces* consist of strands of wall material resembling the spokes of a wheel. These spokes converge forming a hublike structure. Electron microscopy should be most revealing if applied to a study of these structures. The septations of selected ascomycetes and basidiomycetes have been investigated by Moore and McAlear (1962), who, using electron microscopy of ultrathin sections, noted rather simple septa, with single perforations in the ascomycetous forms. In basidiomycetes, on the other hand, they have demonstrated cross walls of complex fabrication. The septa are traversed by pores, the pore diameters tapering toward the apertures on both ends; the pore apertures are further associated with hemispherical, membranous structures ("parenthesomes"), the function of which is unknown at the present time.

E. Crystalline Components

In the fungi, as in green plants, the cell walls are composed of polysaccharides to a great extent. In this multitude of polysaccharides attention must be focused on the most ubiquitous of all biologically elaborated substances—cellulose—which has been the most thoroughly studied of all the cell wall carbohydrates (see Section IV, C, 1). Through the use of X-ray diffraction and polarization optical analyses it has been shown that in cellulose there is a tendency for the long chainlike molecules to assume a parallel arrangement (at least over portions of their total lengths) with regular spacing between the adjacent chains. These well-ordered aggregations (crystallites) of cellulose molecules constitute what have been generally referred to as *micelles*. Several workers have written at length on the crystalline properties of cellulose (e.g., Preston, 1952), with reference primarily to green plants. Nevertheless, these remarks serve to describe fungal cellulose which occurs, for the most part, in the biflagellate phycomycetes (see Section IV, C, 1). In most fungi, however, the role of cellulose (*i.e.*, to provide a skeletal framework for the cell wall) is assumed by chitin (see Section IV, B, 1). For purposes of the present discussion it will suffice to state that, in a general way, chitin may be regarded as being similar to cellulose insofar as chitin consists of long molecules that aggregate into crystallites.

The crystalline properties of chitin and cellulose can be exploited for the purpose of determining molecular orientations, but, in addition, X-ray diffraction, employing the so-called powder method, can be utilized as a means for their detection (Fig. 4). The use of the X-ray powder method affords simplicity and relative ease of interpretation and has been used repeatedly for the detection of chitin, cellulose, and other crystalline substances (e.g., Frey, 1950; Blank, 1954; Kreger, 1954; Aronson and Preston, 1960a).

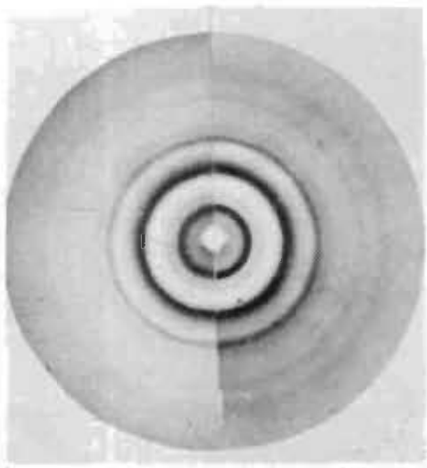


FIG. 4. X-ray powder diagrams of *Allomyces* chitin (left) and crustacean chitin (right).

IV. CHEMICAL COMPOSITION OF CELL WALLS

A. General Considerations

Since the early investigations of van Wisselingh (1898) a number of workers have taken up the study of the chemical constitution of fungal walls. Up to the most recent times, however, progress has been slow; this has been due in part to a lack of analytical methods that fall within the sphere of mycology, and also to a concentration of interest on the occurrence of chitin and cellulose in various taxa. Indeed, the distribution of chitin and cellulose has led to several attempts to deduce taxonomic and phylogenetic relationships among the fungi and between the fungi and the algae. Wettstein (1921) was the first investigator to utilize cell wall composition as a criterion for assessing relationships among major taxa. Wettstein's views were most significant with respect to the phycomycetes, for which he supported a concept of polyphyletic origin. In his studies, Wettstein employed cytochemical methods of analysis some of which now appear to have been in error, particularly with regard to the Monoblepharidales. This taxon, which Wettstein reported as having cellulosic walls, is clearly a chitinous wall assemblage (Aronson and Preston, 1960a; Aronson, 1962). Interest in cell wall constitution as a means of determining relationships was revived by Nabel (1939), who reported positive cytochemical tests for both chitin and cellulose in the cell walls of *Rhizidiomyces bivellatus*, which was described as a new species of the Chytridiales.

Nabel proposed that, in the possession of both polysaccharides, *Rhizidiomyces* established phylogenetic continuity between the chitinous Chytridiales and Blastocladiales on the one hand, and the cellulosic Oomycetes on the other. Fuller and Barshad (1960) and Fuller (1960) have carried out X-ray and cytochemical analyses that confirm the simultaneous presence of chitin and cellulose in the walls of *Rhizidiomyces*. These investigators reaffirmed the basic ideas of Wettstein, but pointed out that, whereas Wettstein recognized but two groups of aquatic phycomycetes (cellulosic or chitinous), these organisms may now be separated into three categories, each of which is distinguished by wall composition (cellulose, chitin, or both) and mode of flagellation. This latter work does not attempt to establish cell wall composition as a criterion for judging phylogenetic relationships. Rather, it is merely pointed out that cell wall composition in the aquatic phycomycetes correlates well with accepted taxonomic groups. To limit the significance of cell wall composition in this way seems entirely justifiable.

Another traditional point of convergence between wall composition and systematics is seen in the use of the amyloid reaction. The reaction of I_2-KI with cell wall and cytoplasmic constituents has been employed as a technique for the classification of higher fungi (e.g., Singer, 1962). A positive reaction with I_2-KI [or Melzer's reagent: I_2-KI + chloral hydrate (Melzer, 1924)] would generally be considered to be indicative of starch, glycogen, or related substances (e.g., dextrins). Other polysaccharides from lower plants are known to react with I_2-KI (e.g., A. B. Foster and Stacey, 1958) and some inorganic complexes are also known (Percival, 1950). As Singer (1962) indicates, a spectrum of hues and intensities is encountered in the application of this technique. The identity of substances (some of which appear to reside in cell walls) reactive to the iodine reagent can be known only when the fungi in question are subjected to satisfactory techniques of biochemical analysis. It should be noted that the glycogen of yeast cells, at one time thought to be a cell wall constituent, was not found to be a part of the cell walls when methods for wall isolation and analysis were employed (Northcote and Horne, 1952).

In addition to considerations of the cell wall as a criterion for establishing systematic relationships, the question of the simultaneous occurrence of chitin and cellulose has been raised repeatedly. The contending views have recently been summarized by Fuller and Barshad (1960), who have, as indicated above, solved this problem with one fungus, *Rhizidiomyces*. Other claims of the occurrence of both substances include those of Thomas (1928, 1942, 1943) for *Fusarium, Pythium,* and *Phytophthora* species and of Hopkins (1929) for *Mucor rouxii*. Thomas's conclusions, based primarily upon cytochemical and solubility tests, do not appear to be well

founded and are not in accord with the X-ray investigations of Frey (1950) and the biochemical studies of Crook and Johnston (1962). Similarly, the conclusions of Hopkins (1929) must be discarded in favor of those of Frey (1950) and Bartnicki-Garcia and Nickerson (1962). In the light of present information, therefore, *Rhizidiomyces* appears to be unique.

The occurrence of chitin and cellulose and the relation between these substances and systematics has been for most mycologists the most intriguing aspect of fungal wall composition. These considerations, however, tended to obscure the complexity of fungal walls. Until comparatively recent times, the few investigations purporting to deal with wall constituents other than chitin and cellulose suffered from serious technical defects. Taking the investigations of Thomas (1928, 1930, 1942, 1943) as examples, surface mycelial mats were extracted with various reagents and the extracts were analyzed. Many of the extracts gave indications of being known cell wall constituents, but it is impossible to rule out the possibility that the extracted material originated from the cytoplasm. On the other hand, many investigations have employed chemically prepared walls using procedures that generally involved alkaline digestion of the cytoplasm (e.g., Norman and Peterson, 1932; Aronson and Machlis, 1959; Blank, 1953). It is clear that chemical methods of cell wall preparation may remove certain wall constituents (Kreger, 1954). Recent years have seen the development of mechanical methods for isolating cell walls from microorganisms

TABLE I

THE CHEMICAL COMPOSITION OF THE WALLS OF SELECTED FUNGI[a]

Constituent	*Saccharomyces cerevisiae* (bakers' yeast)	*Allomyces macrogynus*	*Mucor rouxii* (filamentous form
Nitrogen	2.1	5.5	—
Phosphate	0.31	—	23.3
Lipid	8.5	—	7.8
Protein	13.0	10	6.3
Chitin	ca. 1.0	58	9.4
Chitosan	—	—	32.7
Glucan	28.8	16	—
Mannan	31.0	—	3.8
Other carbohydrates	—	—	9.5
Ash	—	8	ca. 2.0[b]
References	Northcote and Horne (1952)	Aronson and Machlis (1959)	After Bartnicki-Garcia and Nickerson (1962

[a] Values stated as percentage, dry weight, of walls.

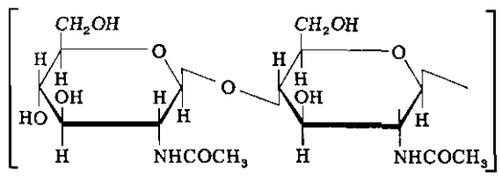
[b] Total ash excluding phosphate.

These techniques have met with marked success in studies of the cell walls of bacteria (Salton, 1961), and are also applicable to cell wall studies in fungi (Kreger, 1954; Aronson and Machlis, 1959; Crook and Johnston, 1962). Largely through the use of these newer methods, a marked degree of complexity and diversity in fungal wall structure has become evident, even though but a few species have been analyzed in detail. Table I brings together some of the analytical data obtained in studies of selected fungi. A systematic survey of wall constituents appears in the sections immediately following.

B. Aminopolysaccharides

1. Chitin

a. Structure and Properties. A most satisfactory compilation of the chemical and physical properties of chitin has been set forth by A. B. Foster and Webber (1960), and but a few salient features are mentioned here. Chitin is a linear molecule, constituted entirely of β-1,4 linked *N*-acetylglucosamine residues (I).



(I)

The long chains of *N*-acetylglucosamine units may achieve a molecular weight of the same order of magnitude as the molecular weight of cellulose (see Section IV, C, 1). Crystalline chitin forms a unit cell that is orthorhombic, having the dimensions, $a = 4.76$ Å, $b = 10.28$ Å, and $c = 18.85$ Å (Carlström, 1957) and containing four acetylglucosamine residues. The resistance of chitin to acidic hydrolysis is explained by A. B. Foster and Stacey (1952) as being due to the hydrolysis of the amide bonds in the acetylamino side chains, which leaves exposed cationic amino groups. These groups electrostatically repel hydronium ions.

b. Methods of Detection. A number of procedures have been used in the detection of chitin. The microchemical test of van Wisselingh (1898) has been used most extensively. This test involves the partial alkaline deacetylation of chitin, giving chitosan, which is actually detected. The reliability of the van Wisselingh test has been questioned by several investigators (e.g., Frey, 1950; A. B. Foster and Webber, 1960), and the work of Hopkins (1929) indicates that the test may lead to erratic results, differing from one day to the next on material from the same culture.

The use of X-ray powder diagrams to detect chitin has become a popular technique and, in most instances, can be regarded as the most reliable of methods. Such studies have been carried out on the walls of filamentous fungi (e.g., Frey, 1950; Aronson and Preston, 1960a), yeasts (Kreger, 1954; Houwink and Kreger, 1953), and a number of pathogenic fungi (Blank, 1953, 1954; Blank and Burke, 1954). Figure 4 shows a typical X-ray powder diagram obtained from fungi as compared with a diagram of crustacean chitin. The interplanar spacings corresponding to the more intense chitin reflections are listed in Table II along with characteristic

TABLE II

INTERPLANAR SPACINGS (A) COMMONLY ENCOUNTERED IN X-RAY POWDER ANALYSES

Chitin (Carlström, 1957)	Chitosan (Bartnicki-Garcia and Nickerson, 1962)	Cellulose I (Gezelius, 1959)	Cellulose II[a] (Fuller and Barshad, 1960)
9.54	4.41	6.0	7.48
6.96	—	5.2	4.57
4.65	—	4.3	4.13
3.38	—	3.9	—
—	—	2.5	—

[a] Mercerized cellulose.

spacings of other substances that have been detected in fungal walls by the X-ray method.

More comprehensive treatments of the techniques of chitin detection may be found by consulting Tracey (1955) and A. B. Foster and Webber (1960).

c. Occurrence. The occurrence of chitin in the cell walls of fungi has intrigued mycologists for a great many years, and in its detection all methods, at one time or another, have been employed. It would be presumptuous to state that our knowledge of the occurrence of chitin in fungi is complete, but there is sufficient information available so that we may hold to the belief that chitin occurs in most taxa. It is of interest to note that chitin is a prominent constituent of the cuticular structures of invertebrates and is believed to occur also in certain green algae (Tracey, 1955).

Table III lists those fungi for which the presence of chitin is fairly well established. This tabulation, admittedly, reflects the author's bias which for the most part favors the X-ray method of chitin detection. However, other methods have been accepted wherever significant methodological refinements have been noted or where the results compare favorably with those for closely allied fungi.

TABLE III

THE OCCURRENCE OF CHITIN IN FUNGAL WALLS

Classification[a]	Method	References
Chytridiales		
Chytridium	X-ray	Aronson and Preston (1960a)
Rhizophydium	X-ray	Aronson and Preston (1960a)
Blastocladiales		
Allomyces	X-ray	Frey (1950)
Blastocladiella	X-ray	Frey (1950)
Monoblepharidales		
Monoblepharis	X-ray	Aronson (1962)
Monoblepharella	X-ray	Aronson and Preston (1960a)
Hyphochytriales		
Rhizidiomyces	X-ray	Fuller and Barshad (1960)
Mucorales		
Mucor	X-ray	Frey (1950)
Rhizopus	X-ray	Frey (1950)
Phycomyces	X-ray	van Iterson *et al.* (1936)
Absidia	Macrochemical	Schmidt (1936)
Cunninghamella	Macrochemical	Schmidt (1936)
Mortierella	Macrochemical	Schmidt (1936)
Entomophthorales		
Entomophthora	X-ray	Frey (1950)
Basidiobolus	X-ray	Frey (1950)
Protomycetales		
Coccidioides	X-ray	Blank and Burke (1954)
Endomycetales		
Endomyces	X-ray	Frey (1950)
Eremascus	Macrochemical	Schmidt (1936)
Endomycopsis	Macrochemical	Schmidt (1936)
Debaryomyces	Microchemical	Roelofsen and Hoette (1951)
Hansenula	Microchemical	Roelofsen and Hoette (1951)
Pichia	Microchemical	Roelofsen and Hoette (1951)
Zygosaccharomyces	Microchemical	Roelofsen and Hoette (1951)
Saccharomyces	Microchemical	Roelofsen and Hoette (1951)
Candida	Microchemical	Roelofsen and Hoette (1951)
Nadsonia	X-ray	Kreger (1954)
Eurotiales		
Aspergillus	Macrochemical	Behr (1930)
Ctenomyces	X-ray	Blank (1953)
Epidermophyton	X-ray	Blank (1953)
Penicillium	X-ray	Kreger (1954)
Microsporum	X-ray	Blank (1953)
Trichophyton	X-ray	Blank (1953)
Hypocreales		
Fusarium	X-ray	Frey (1950)
Nectria	X-ray	Frey (1950)
Trichoderma	X-ray	Frey (1950)

TABLE III (Continued)

Classification[a]	Method	References
Helotiales		
Sclerotinia	X-ray	Frey (1950)
Moniliales		
Rhodotorula	Microchemical	Roelofsen and Hoette (1951)
Schizoblastosporon	Microchemical	Roelofsen and Hoette (1951)
Torulopsis	Microchemical	Roelofsen and Hoette (1951)
Trichosporon	Microchemical	Roelofsen and Hoette (1951)
Trigonopsis	Microchemical	Roelofsen and Hoette (1951)
Histoplasma	X-ray	Blank (1954)
Blastomyces	X-ray	Blank (1954)
Paracoccidioides	X-ray	Blank (1954)
Sporotrichum	X-ray	Blank (1954)
Tremellales		
Sporobolomyces	X-ray	Frey (1950)
Ustilaginales		
Ustilago (basidia)	Microchemical	Nabel (1939)
Ustilago (sporidia)	X-ray	Aronson (1961)
Polyporales		
Cantharellus	X-ray	Gonell (1926)
Fomes	Macrochemical	Schmidt (1936)
Polystictus	Macrochemical	Schmidt (1936)
Polyporus	Macrochemical	Proskuriakow (1926)
Agaricales		
Boletus	Macrochemical	Scholl (1908)
Lactarius	Macrochemical	Proskuriakow (1926)
Agaricus	Macrochemical	Proskuriakow (1926)
Armillaria	Macrochemical	Proskuriakow (1926)
Coprinus	X-ray	Frey (1950)
Hypholoma	X-ray	Frey (1950)

[a] After Alexopoulos (1962); imperfect genera associated with ascomycetes are included under the appropriate ascomycetous orders.

2. *Chitosan*

Chitosan may be regarded as a deacetylated form of chitin. Its properties have been studied in investigations of the alkaline degradation of chitin, and, as pointed out by A. B. Foster and Webber (1960), its acetyl content varies from relatively high values to almost nil. Chitosan can be detected by dilute acid extraction, followed by the chitosan sulfate test (Roelofsen and Hoette, 1951) or by X-ray diffraction (Kreger, 1954; Bartnicki-Garcia and Nickerson, 1962). Its powder diagram yields an X-ray reflection corresponding to an interplanar spacing of 4.41 Å. Thus far, this substance has been found in the walls of *Phycomyces blakesleeanus* (Kreger, 1954) and is the major component of the walls of *Mucor rouxii* (Bartnicki-Garcia and Nickerson, 1962). In the investigation of *Mucor* cell walls, it

s found that the chitosan contained little, if any, *N*-acetylation. Whether not this holds for *Phycomyces* chitosan is unknown. Since the natural :urrence of chitosan is known only in the Mucorales, it would be of erest to determine whether it occurs throughout this order. Outside of s group, its occurrence is purely a matter of conjecture, but as its recog-ion is a recent development it could easily have gone unnoticed in lier investigations.

Other Aminopolysaccharides

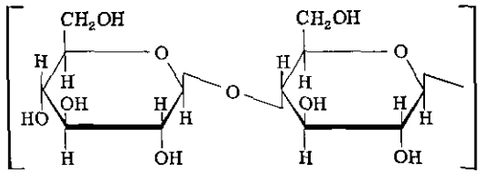
Evidence for other types of nitrogenous polysaccharides has come to ht in recent investigations. Harold (1962) has isolated from walls of *urospora crassa* a polymer of galactosamine which is capable of binding lyphosphate. Since the binding of polyphosphate anions would depend on the cationic nature of a polygalactosamine, the substance must have ntained free amino groups. It is unlikely that this type of polysaccharide unique since Crook and Johnston (1962) have found galactosamine in id hydrolyzates of mechanically isolated cell walls from *N. sitophila, spergillus niger,* and *Botrytis cinerea.*

The occurrence of glucosamine in forms distinct from chitin and chitosan is been demonstrated in isolated cell walls of bakers' yeast (Korn and orthcote, 1960). The amino sugar occurs in association with mannan and otein or mannan, glucan, and protein. The structures of these glycopro-ins are unknown, but it has been suggested that glucosamine provides a eans for linking protein and polysaccharide (Korn and Northcote, 1960). he possibility of ester linkages between protein and polysaccharide has so been suggested. This view is based upon the high percentage of acidic nino acids in cell wall protein fractions and evidence of ester bond cleav-ge during chemical and enzymatic fractionation of wall components Nickerson, 1963).

C. Nonaminopolysaccharides

. *Cellulose*

a. Structure and Properties. Cellulose is a linear polysaccharide, com-osed of β-1,4 linked glucose residues (II). The acid hydrolysis of the



(II)

glycosidic linkages (in contrast to chitin) proceeds with comparative ease. According to Hirst (1962), the cellulose molecule may achieve chain lengths of up to 4000 glucose residues, corresponding to a molecular weight of about 650,000. Others have placed much higher values on the molecular weight (e.g., Northcote, 1958).

The unit cell of crystalline cellulose is monoclinic, composed of four glucose residues, and has the dimensions $a = 8.34$ Å, $b = 10.3$ Å, and $c = 7.9$ Å (Preston, 1952). These are the unit cell dimensions for cellulose I which can be converted to cellulose II (also termed mercerized cellulose or cellulose hydrate), by swelling in alkali. Cellulose II differs somewhat from cellulose I in its crystalline properties. A voluminous literature on cellulose exists and many aspects of the properties of this substance lie outside the scope of this discussion. A start in that direction, however, may be made by consulting Preston (1952).

b. Methods of Detection. As is the case with chitin, there are numerous methods of cellulose detection. Many of these are inconclusive when applied singly but, when employed in various combinations, can yield satisfactory results.

The cytochemical method employing I_2-KI-70% H_2SO_4 has been used widely and usually (but not always) leads to a blue color in cellulosic walls. In certain instances other wall constituents may mask the cellulose that is present (Preston, 1952). Another test considered to be indicative of cellulose is solubility in cuprammonium hydroxide (Schweitzer's reagent). The dissolved material may be regenerated as cellulose II, upon addition of dilute acid.

The cytochemical and solubility tests have been employed in numerous instances, but these procedures suffer from a lack of specificity, which is indicated, for example, in the work of Thomas (1943) on *Phytophthora*.

The detection of cellulose by X-ray diffraction has been accomplished successfully in several instances employing the powder method. Both cellulose I and cellulose II have been detected in the Acrasiales (Gezelius and Rånby, 1957; Gezelius, 1959), although, taking the cellulosic fungi as a whole, cellulose I is the most commonly occurring form. Interplanar spacings indicative of these two forms of cellulose appear in Table II.

c. Occurrence. The biflagellate phycomycetes appear to be an assemblage characterized by cellulosic cell walls. In other groups (Acrasiales, Hyphochytriales) the number of genera that are known to produce cellulose are too few to allow any far-ranging conclusions to be drawn (Table IV). In addition to its occurrence in green plants, cellulose is known to occur in the bacterium *Acetobacter xylinum* (e.g., Hassid, 1962), in amoebae (Tomlinson and Jones, 1962), in tunicates (Whistler and Smart, 1953), and in mammals, including man (Hall and Saxl, 1961).

TABLE IV

THE OCCURRENCE OF CELLULOSE IN FUNGAL WALLS

Classification[a]	Method	References
ıcrasiales		
Acytostelium[b]	X-ray	Gezelius (1959)
Dictyostelium[b]	X-ray	Gezelius and Rånby (1957)
Hyphochytriales		
Rhizidiomyces	X-ray	Fuller and Barshad (1960)
.agenidiales		
Lagenidium	X-ray	Frey (1950)
aprolegniales		
Saprolegnia	X-ray	Frey (1950)
Achyla	X-ray	Frey (1950)
.eptomitales		
Apodachlya	Microchemical	Nabel (1939)
Sapromyces	Microchemical	Nabel (1939)
Peronosporales		
Pythium	Microchemical	Nabel (1939)
Phytophthora	X-ray	Frey (1950)

[a] After Alexopoulos (1962).
[b] Cellulose detected in exocellular sheaths.

'. *Glucans*

Polysaccharides distinct from cellulose but composed of glucose residues ıre of common occurrence in fungal walls. The most thoroughly studied is 'east glucan, some properties of which have been reviewed by Nickerson *'t al.* (1961). Yeast glucan is weakly crystalline, but its crystallinity is nhanced by treatment with dilute acid (Houwink and Kreger, 1953). Ac-ording to Houwink and Kreger (1953), the dilute acid conversion of lucan to what they term "hydroglucan" is accompanied by the formation)f aggregates of microfibrils. It is significant that yeast glucan may be com-)lexed with protein (see Section IV, D) in the native cell walls (e.g., Nickerson, *et al.,* 1961). This polysaccharide is known to occur in a variety)f yeasts (Houwink and Kreger, 1953; Kreger, 1954) and has also been letected in the walls of *Penicillium notatum* (Kreger, 1954). X-ray data ndicate that the derivative, hydroglucan, is similar to, if not identical with,)aramylon, the storage glucan found in *Euglena* (Kreger and Mceuse, 1952).

Another glucan that has received considerable attention as a fungal wall :onstituent is callose, which occurs widely throughout the plant kingdom,)ut is especially prominent in the phloem of vascular plants. In a number)f studies, Mangin drew attention to this polysaccharide which is soluble in dilute alkali and has an affinity for basic dyes (e.g., Mangin, 1890; see

also references in J. W. Foster, 1949, and Cochrane, 1958). In addition to the aforementioned properties, it is known that the glucose residues in callose are β-1,3 linked. The presence of glucose moieties alone does not explain the basis for the acidic character of this polysaccharide, and, therefore, it is of interest to consider the findings of Thomas (1930). This investigator isolated an alkali-soluble glucan from *Sclerotinia*. The properties of callose were exhibited by the polysaccharide, which yielded glucose and no other sugar upon acid hydrolysis. In addition, the substance contained an appreciable quantity of nondialyzable phosphate, which led Thomas to propose that the bound phosphate was responsible for the affinity of callose for basic dyes. This explanation has considerable merit, but at the same time it raises a question of the specificity of basic dye staining. It appears that any polysaccharide–phosphate complex would have the potential for fixing basic dyes.

Other glucans have been detected in some fungi, but they have not been characterized to any extent. Such is the case, for example, with *Aspergillus fischeri* (Norman and Peterson, 1932), *Allomyces macrogynus* (Aronson and Machlis, 1959), and *Neurospora crassa* (de Terra and Tatum, 1961).

With respect to the uncharacterized polysaccharides just mentioned, it should not be inferred that these are homopolymeric substances necessarily. Since information has been derived largely from analysis of the products of cell wall hydrolysis, it must be recognized that in the intact wall two or more types of monosaccharide constituents may participate in the formation of a heteropolysaccharide. It must be further noted that this qualification may apply also in other instances (see Sections IV, C, 3 and 4).

3. Mannans

The occurrence of polymers of mannose has been noted primarily in yeast cell walls (e.g., Kreger, 1954) although there is little doubt that they are of widespread distribution in the walls of fungi. While mannans (as intact polysaccharides) have not been isolated from the walls of filamentous fungi, mannose has been found in acid hydrolyzates of isolated walls (Bartnicki-Garcia and Nickerson, 1962; Hamilton and Knight, 1962; Crook and Johnston, 1962).

4. Other Neutral Polysaccharides

Based upon the detection of certain monosaccharides in acid hydrolyzates of isolated cell walls, the presence of other types of wall polysaccharides may be assumed. Galactose has been found to be a cell wall constituent in all major classes of fungi (e.g., Crook and Johnston, 1962). Similarly, the occurrence of 6-deoxyhexoses (methylpentoses) has come to light quite recently. Of all the 6-deoxyhexoses, fucose appears to be the

ost common. It is especially prominent in the Mucorales (Bartnicki-arcia and Nickerson, 1962; Crook and Johnston, 1962). Hamilton and night (1962) have found another 6-deoxyhexose, rhamnose, in the walls *Penicillium chrysogenum*. Pentoses are of unusual occurrence in cell all hydrolyzates, but xylose has been found in the course of wall studies *Penicillium chrysogenum* (Hamilton and Knight, 1962) and *Polystictus nguineus* (Crook and Johnston, 1962).

The occurrence of these constituents and those previously mentioned test to a complexity of structure in fungal walls that heretofore has been ıecognized.

Polyuronides

An unexpected fact of fungal wall chemistry is that uronic acids have ·ver been conclusively demonstrated. For many years it has been assumed at fungal walls contain pectic substances. The basis for this assumption e results obtained primarily with ruthenium red staining, solubility tests, ıd qualitative tests on extracts (e.g., Graham, 1960; Thomas, 1928, 1942,)43). Once again the evidence indicates that there is danger in plaeing o much reliance upon this kind of analysis. While the demonstration of onic acids is attended by technical difficulties, it should be possible to :tect them chromatographically in cell wall hydrolyzates; but this has not :en accomplished. In species of *Phytophthora,* for example, Thomas 943) reported the presence of pectic substances detected by cytochemical ıd solubility tests whereas Crook and Johnston (1962), working with rmic acid hydrolyzates of isolated walls, found no trace of any uronic ·id.

D. Proteins

The presence of proteins as an integral part of the cell wall has rarely :en established. However, some earlier studies (e.g., Thomas, 1928), nploying cytochemical and solubility tests, led to the conclusion that ·oteins were present. Again the results of early investigations are quite nbiguous since workers utilized intact mycelia and it is difficult to see how latively small quantities of cell wall protein (if present) could be dis-ıguished from the bulk of the cellular protein located in the cytoplasm. ı several recent investigations, in which cell walls have been isolated echanically, protein has been detected (e.g., Aronson and Machlis, 1959; artnicki-Garcia and Nickerson, 1962; Crook and Johnston, 1962). How-·er, even when working with mechanically isolated walls, there is always e possibility that contaminating cytoplasm is present.

In studies with yeasts, on the other hand, the results have been much ıore definitive. Nickerson and his co-workers (e.g., Nickerson *et al.,* 1961)

have demonstrated that it is possible to extract polysaccharide–protein complexes from isolated cell walls of *Candida albicans*. Working with several species of *Saccharomyces*, Eddy (1958) has determined that a mannan–protein complex is released from isolated walls by the action of papain. Similarly, Korn and Northcote (1960), employing anhydrous ethylene diamine have isolated three polysaccharide–protein complexes from the walls of bakers' yeast. In all these yeasts, the polysaccharide moieties are known to be integral parts of the walls. Furthermore, it is likely that the polysaccharide and protein components of the complexes are covalently linked. The conclusion, therefore, that protein is indeed a cell wall component in yeasts is inescapable. It is unlikely that yeasts are unique in this respect, but the conclusive demonstration of protein, if present, in the walls of filamentous fungi will require considerable technological improvements.

E. Other Constituents

1. Lignin

The literature contains contesting views on whether or not lignin occurs in fungal walls. J. W. Foster (1949) has summarized much of the literature dealing with fungal substances of an aromatic nature which are resistant to acid hydrolysis. Since there can be no single definition for the term lignin (Northcote, 1958), certain fungal constituents may be described as "lignin-like" substances (Bu'Lock and Smith, 1961; Siegel, 1962).

2. Lipids

The presence of lipoidal material in isolated walls is variable depending upon the fungus investigated. Aronson and Machlis (1959) could not detect lipids in isolated walls of *Allomyces macrogynus*. However, Northcote and Horne (1952) reported over 3% lipid in yeast cell walls, and Bartnicki-Garcia and Nickerson (1962) have discriminated between easily extractable and bound lipids in walls isolated from *Mucor rouxii*. It is not known to what extent, if at all, these lipoidal substances are integrated into the cell wall fabric. In the sporangiophore wall of *Phycomyces*, lipoidal material accounts for better than 25% of the dry weight (Kreger, 1954). In this instance the material is mostly cuticular in nature. Nickerson (1963) in calling attention to the general tendency to overlook the importance of lipid materials, has cited evidence for a possible role for lipids in the architecture of the yeast cell wall.

3. Inorganic Constituents

Very little is known of the nature and amounts of inorganic wall components, but it is likely that some are present in the walls of all fungi. In

certain instances the presence of inorganic substances may be fortuitous, due to sparingly soluble salt depositions from the culture media. In some cases, on the other hand, the presence of inorganic components is clearly related to the chemical nature of the wall, which exhibits a binding capacity for ions. This is the case with *Neurospora* ascospore walls (e.g., Sussman *et al.*, 1957), where as much as 11% of the spores' total cations may be bound to the wall. A similar example is found in the binding of polyphosphate ions to polygalactosamine and protein in the walls of *Neurospora* (Harold, 1962). Chitosan may be involved in the binding of phosphate in the walls of *Mucor rouxii* (Bartnicki-Garcia and Nickerson, 1962). Indeed, in this latter case, the cell walls may be composed of rather large amounts of the salt chitosan phosphate. In addition, it has been noted that phosphate is associated with several glycoprotein fractions from isolated yeast cell walls (Korn and Northcote, 1960).

4. Nucleic Acid Derivatives

Most investigations of mechanically isolated walls have not looked into nucleic acid components. Since ribose is rarely present in wall preparations, RNA could not be present in any great amount, if at all. However, UV absorption of extracts of walls of *Allomyces macrogynus* indicated that RNA possibly was present (Aronson and Machlis, 1959), but definitive studies were not made. Bartnicki-Garcia and Nickerson (1962) detected significant quantities of adenine, guanine, cytosine, and uracil in both yeast phase and filamentous phase walls of *Mucor rouxii*. As ribose was detected only in cell wall hydrolyzates of the yeast phase, the presence of the purine and pyrimidine bases is not easily interpreted, although the lability of ribose to acid could possibly explain its absence in the filamentous phase walls, assuming it was present initially in relatively small amounts. These workers expressed the view that the nucleic acid derivatives in *Mucor* walls were not derived from cytoplasmic contamination.

5. Melanins

The presence of melanin-type pigments in cell walls is probable although definitive diagnostic studies have not been made. Bessey (1950) has referred to certain pigments as melanin and stresses that in many instances the pigment resides in the cell wall. Emerson and Fox (1940) have indicated that the brown pigment of the outer walls of resistant sporangia of *Allomyces* is probably a melanin-like substance. Also, Lowry and Sussman (1958) referred to a melanized layer, the epispore, in ascospore walls of *Neurospora tetrasperma*.

F. Biosynthesis of Polysaccharides

Recent years have seen the development of a concept that assigns significant role to nucleoside diphosphate sugars in polysaccharide synthe sis (e.g., Hassid, 1962). Sugar nucleotides are considered to be precurso of several types of polysaccharides. Of particular interest is the work c Glaser and Brown (1957), who found that an enzyme preparation fro *Neurospora crassa* would catalyze the incorporation of uridine diphosphat *N*-acetyl-D-glucosamine into an insoluble aminopolysaccharide. The produ was undoubtedly a β-1,4 linked polymer of *N*-acetylglucosamine and w sensitive to chitinase. In this case, the position and stereochemistry of tl glycosidic linkages are the principal criteria for assessing the nature of tl synthesized product. At the very least, however, chitin synthesis probab occurs in two stages. The first of these, involving the formation of polysa charide chains, would seem to be similar to, if not identical with, the *vitro* synthesis just referred to. The second stage, involving the fabricati of chitin fibrils with their concomitant deposition, remains to be elucidate

Until very recently the synthesis of cellulose from uridine diphosph D-glucose (UDPG) had been suspected, but a definitive demonstration this synthesis had not been accomplished (Hassid, 1962). The inabil to demonstrate UDPG participation in cellulose synthesis in green pla now seems to have been explained by the work of Elbein *et al.* (196- In this work, employing extracts of mung bean seedlings, it was shown tl guanosine diphosphate D-glucose serves as the glucosyl donor in the *vitro* synthesis of a β-1,4 glucan. The product was indistinguishable fr cellulose using various chemical criteria. UDPG is inactive in this syste but the incorporation of D-glucose into polysaccharide is markedly hanced by the presence of guanosine diphosphate mannose (Hassid, 196

Work carried out on the cellulose-forming bacterium *Acetoba xylinum* does not indicate that the above mechanism of glucose activat is operative in this organism (e.g., Colvin, 1964). Obviously, the ini phase of cellulose formation in the cellulosic fungi can be known v certainty only when enzymological studies are carried out with this gr Whatever the course of glucose activation, the mechanism of fabricatio the physical entities (*viz.*, microfibrils) must also be illuminated, as indicated also in connection with chitin formation. Colvin (1964) has c sidered the latter problem in more detail than can be presented here.

V. FUNCTIONAL ATTRIBUTES OF FUNGAL WALLS

The ultimate goal in elucidating the properties of cell walls is to determine to what extent the cell wall is active in the physiological and biochemical activities of the cell. Sussman (1957) has drawn attention to the cell surface in fungi, pointing out that it is not an inert secretion, but rather a region of manifold activities. Such activity is manifested in the surface localization of certain enzymes (see Sussman, 1957, for specific references). It is not known precisely to what extent surface enzymes are physically integrated into the cell wall, but such a locus would apparently be appropriate for enzymes involved in the synthesis of wall components and extracellular polysaccharides. In addition, the cell wall may function as a reservoir for various ions (e.g., Harold, 1962; Sussman *et al.*, 1957; Lowry and Sussman, 1958). Work by Lingappa and Sussman (1959) indicates that heat resistance of ascospores of *Neurospora* is related to the presence of the exospore, one of the spore wall layers.

The relationship between cell wall properties and growth of cells has been the subject of considerable research and discussion. In higher plants the cell wall occupies a central position in the growth process (e.g., Setterfield and Bayley, 1961). The general view regarding fungi is that growth occurs as a prolongation of the hyphal tips (e.g., Smith, 1923; Zalokar, 1959), and it implies that hyphal wall extension occurs through the apical deposition of newly synthesized wall material without the occurrence of any plastic extension of preexisting wall material. This may well be the case, although strictly apical extension of hyphal walls has not been clearly demonstrated. However, processes occur during growth of fungi that indicate that the tip growth concept requires qualification. The subapical growth of the *Phycomyces* sporangiophore is a well-known example and one in which the properties of the cell wall have been implicated (see Section III, C). In addition we should note the formation of trophocysts and subsporangial swellings in *Pilobolus* and the phototropic curvatures of asci in fungi such as *Ascobolus* (e.g., Ingold, 1961). The latter examples indicate that plastic extension of cell wall material may be occurring. Clearly, the relationship between wall structure and growth in fungi requires careful scrutiny and, hopefully, further investigation.

Nickerson and his collaborators (e.g., Nickerson *et al.*, 1961) have suggested that glycoproteins in the walls of *Candida albicans* are functional in cell division. It has been suggested that a "protein disulfide reductase" brings about the reductive cleavage of disulfide bonds in glucomannan protein, which in turn brings about a localized weakness in the cell wall. The action

of turgor pressure at this site would cause the wall to "blow out," giving rise to a bud initial.

In addition there is evidence to suggest that the cell wall is involved in morphogenetic processes. Working with *Neurospora crassa,* de Terra and Tatum (1961) found that morphological alterations induced by sorbose are accompanied by an alteration of the ratio of glucosamine to glucose in the cell walls. Also, in *Mucor rouxii,* the yeast phase cell walls contain five times more mannan than the walls of the filamentous phase (Bartnicki-Garcia and Nickerson, 1962).

Finally, there is evidence to suggest that cell wall composition is involved in the mating of heterothallic yeasts. Work on *Hansenula wingei* indicates that cell wall protein from one strain interacts with cell wall polysaccharide of the opposite mating type (e.g., Brock, 1959). It is believed that this type of molecular interaction at the cell surfaces is responsible for the agglutination properties of these yeasts. Obviously, this could also provide a means for the initial phase of sexual union in this organism.

Thus it can be stated with assurance that there are ample grounds for viewing the cell walls of fungi as functional entities. The continued investigation of their chemical and physical properties holds inherent interest, and it is likely that further attempts to gain knowledge of cell walls will contribute markedly to the elucidation of many activities of the fungi.

REFERENCES

Agar, H. D., and H. C. Douglas. (1955). Studies of budding and cell wall structure of yeast. *J. Bacteriol.* 70:427–434.

Alexopoulos, C. J. (1962). "Introductory Mycology," 2nd ed., 613 pp. Wiley, New York.

Aronson, J. M. (1961). Wall structure of the sprout cells of *Ustilago. Am. J. Botany* 48:538.

Aronson, J. M. (1962). Hyphal wall structure in *Monoblepharis. Am. J. Botany* 49:665.

Aronson, J. M., and L. Machlis. (1959). The chemical composition of the hyphal walls of the fungus *Allomyces. Am. J. Botany* 46:292–300.

Aronson, J. M., and R. D. Preston. (1960a). An electron microscopic and X-ray analysis of the walls of selected lower Phycomycetes. *Proc. Roy. Soc.* B152:346–352.

Aronson, J. M., and R. D. Preston. (1960b). The microfibrillar structure of the cell walls of the filamentous fungus, *Allomyces. J. Biophys. Biochem. Cytol.* 8:247–256.

Bartnicki-Garcia, S., and W. J. Nickerson. (1962). Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii. Biochim. Biophys. Acta* 58:102–119.

Behr, G. (1930). Über Autolyse bei *Aspergillus niger. Arch. Mikrobiol.* 1:418–444.

Bessey, E. A. (1950). "Morphology and Taxonomy of Fungi," 791 pp. McGraw-Hill (Blakiston), New York.

Blank, F. (1953). The chemical composition of the cell walls of dermatophytes. *Biochim. Biophys. Acta* **10**:110–113.

Blank, F. (1954). On the cell walls of dimorphic fungi causing systemic infections. *Can. J. Microbiol.* **1**:1–5.

Blank, F., and R. C. Burke. (1954). Chemical composition of the cell wall of *Coccidioides immitis*. *Nature* **173**:829.

Braconnot, H. (1811). Recherches analytiques sur la nature des champignons. *Ann. Chim.* **79**:265–304.

Brock, T. D. (1959). Biochemical basis of mating in yeast. *Science* **129**:960–961.

Bu'Lock, J. D., and H. G. Smith. (1961). A fungus pigment of novel type, and the nature of fungus "Lignin." *Experientia* **17**:553.

Carlström, D. (1957). The crystal structure of α-chitin. *J. Biophys. Biochem. Cytol.* **3**:669–683.

Castle, E. S. (1942). Spiral growth and reversal of spiraling in *Phycomyces*, and their bearing on primary wall structure. *Am. J. Botany* **29**:664–672.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Coker, W. C. (1930). Notes on fungi, with a description of a new species of *Ditiola*. *J. Elisha Mitchell Sci. Soc.* **46**:117–120.

Colvin, J. R. (1964). *In* "The Formation of Wood in Forest Trees" (M. H. Zimmermann, ed.), pp. 189–201. Academic Press, New York.

Crook, E. M., and I. R. Johnston. (1962). The qualitative analysis of the cell walls of selected species of fungi. *Biochem. J.* **83**:325–331.

de Terra, N., and E. L. Tatum. (1961). Colonial growth of *Neurospora*. *Science* **134**:1066–1068.

Eddy, A. A. (1958). The structure of the yeast cell wall II. Degradative studies with enzymes. *Proc. Roy. Soc.* **B149**:425–440.

Elbein, A. D., G. A. Barber, and W. Z. Hassid. (1964). The synthesis of cellulose by an enzyme system from a higher plant. *J. Am. Chem. Soc.* **86**:309–310.

Emerson, R. (1958). Mycological organization. *Mycologia* **50**:589–621.

Emerson, R., and D. L. Fox. (1940). γ-Carotene in the sexual phase of the aquatic fungus *Allomyces*. *Proc. Roy. Soc.* **B128**:275–293.

Foster, A. B., and M. Stacey. (1952). *In* "Advances in Carbohydrate Chemistry" (C. S. Hudson *et al.*, eds.), Vol. 7, pp. 247–288. Academic Press, New York.

Foster, A. B., and M. Stacey. (1958). *In* "Handbuch der Pflanzenphysiologie" (W. Ruhland, ed.), Vol. VI, pp. 331–344. Springer, Berlin.

Foster, A. B., and J. M. Webber. (1960). *In* "Advances in Carbohydrate Chemistry" (M. L. Wolfrom and R. S. Tipson, eds.), Vol. 15, pp. 371–393. Academic Press, New York.

Foster, J. W. (1949). "Chemical Activities of Fungi," 648 pp. Academic Press, New York.

Frey, R. (1950). Chitin und zellulose in pilzzellwanden. *Ber. Schweiz. Botan. Ges.* **60**:199–230.

Frey-Wyssling, A. (1953). "Submicroscopic Morphology of Protoplasm," 2nd. English ed., 411 pp. Elsevier, Amsterdam.

Frey-Wyssling, A., and K. Mühlethaler. (1950). Der submikroskopische feinbau von chitinzellwanden. *Vierteljahresschr. Naturforsch. Ges. Zurich* **95**:45–52.

Frey-Wyssling, A., K. Mühlethaler, and R. W. G. Wyckoff (1948). Mikrofibrillenbau der pflanzlichen Zellwände. *Experientia* **4**:475–476.

Fuller, M. S. (1960). Biochemical and microchemical study of the cell walls of *Rhizidiomyces sp. Am. J. Botany* **47**:838–842.

Fuller, M. S., and I. Barshad. (1960). Chitin and cellulose in the cell walls of *Rhizidiomyces sp. Am. J. Botany* **47**:105–109.

Gezelius, K. (1959). The ultrastructure of cells and cellulose membranes in Acrasiae. *Exptl. Cell Res.* **18**:425–453.

Gezelius, K., and B. Ranby. (1957). Morphology and fine structure of the slime mold, *Dictyostelium discoideum. Exptl. Cell Res.* **12**:265–289.

Glaser, L., and D. H. Brown. (1957). The synthesis of chitin in cell-free extracts of *Neurospora crassa. J. Biol. Chem.* **228**:729–742.

Gonell, H. W. (1926). Röntgenographische Studien an Chitin. *Z. Physiol. Chem.* **152**:18–30.

Graham, S. O. (1960). The morphology and a chemical analysis of the teliospore of the dwarf bunt fungus, *Tilletia contraversa. Mycologia* **52**:97–118.

Hall, D. A., and H. Saxl. (1961). Studies of human and tunicate cellulose and of their relation to reticulin. *Proc. Roy. Soc.* **B155**:202–217.

Hamilton, P. B., and S. G. Knight. (1962). An analysis of the cell walls of *Penicillium chrysogenum. Arch. Biochem. Biophys.* **99**:282–287.

Harold, F. M. (1962). Binding of inorganic polyphosphate to the cell wall of *Neurospora crassa. Biochim. Biophys. Acta* **57**:59–66.

Hassid, W. Z. (1962). *In* "The Structure and Biosynthesis of Macromolecules" (D. J. Bell and J. K. Grant, eds.), pp. 63–79. Cambridge Univ. Press, London and New York.

Hassid, W. Z. (1964). Personal communication.

Hirst, E. L. (1962). *In* "The Structure and Biosynthesis of Macromolecules" (D. J. Bell and J. K. Grant, eds.), pp. 45–62. Cambridge Univ. Press, London and New York.

Hopkins, E. W. (1929). Microchemical tests on the cell walls of certain fungi. Cellulose and chitin. *Trans. Wisconsin Acad. Sci.* **24**:187–196.

Houwink, A. L., and D. R. Kreger. (1953). Observations on the cell wall of yeasts. An electron microscope and X-ray diffraction study. *Antonie van Leeuwenhoek, J. Microbiol. Serol.* **19**:1–24.

Ingold, C. T. (1961). "The Biology of Fungi," 124 pp. Hutchinson Educational, London.

Korn, E. D., and D. H. Northcote. (1960). Physical and chemical properties of polysaccharides and glycoproteins of the yeast-cell wall. *Biochem. J.* **75**:12–17.

Kreger, D. R. (1954). Observations on cell walls of yeasts and some other fungi by X-ray diffraction and solubility tests. *Biochim. Biophys. Acta* **13**:1–9.

Kreger, D. R., and B. J. D. Meeuse. (1952). X-ray diagrams of *Euglena*-Paramylon, of the acid-insoluble glucan of yeast cell walls and of Laminarin. *Biochim. Biophys. Acta* **9**:699–700.

Lingappa, Y., and A. S. Sussman. (1959). Changes in the heat-resistance of ascospores of *Neurospora* upon germination. *Am. J. Botany* **46**:671–678.

Lowry, R. J., and A. S. Sussman. (1958). Wall structure of ascospores of *Neurospora tetrasperma. Am. J. Botany* **45**:397–403.

Mangin, L. (1890). Sur la callose, nouvelle substance fondamentale existant dans la membrane. *Compt. Rend.* **110**:644–647.

Melzer, M. V. (1924). L'ornementation des spores de Russules. *Bull. Soc. Mycol. France* **40**:78–81.

Middlebrook, M. J., and R. D. Preston. (1952). Spiral growth and spiral structure III. Wall structure in the growth zone of *Phycomyces. Biochim. Biophys. Acta* **9**: 32–48.

Moore, R. T., and J. H. McAlear. (1962). Fine structure of Mycota. 7. Observations on septa of Ascomycetes and Basidiomycetes. *Am. J. Botany* 49:86–94.

Mundkur, B. (1960). Electron microscopical studies of frozen-dried yeast. *Exptl. Cell Res.* 20:28–42.

Nabel, K. (1939). Über die Membran niederer Pilze, besonders von *Rhizidiomyces bivellatus* nov. spez. *Arch. Mikrobiol.* 10:515–541.

Nickerson, W. J. (1963). Symposium on biochemical bases of morphogenesis in fungi. IV. Molecular bases of form in yeasts. *Bacteriol. Rev.* 27:305–324.

Nickerson, W. J., G. Falcone, and G. Kessler. (1961). *In* "Macromolecular Complexes" (M. V. Edds, Jr., ed.), pp. 205–228. Ronald Press, New York.

Norman, A. G., and W. H. Peterson. (1932). The chemistry of mould tissue. II. The resistant cell-wall material. *Biochem. J.* 26:1946–1953.

Northcote, D. H. (1958). The cell walls of higher plants: their composition, structure and growth. *Biol. Rev. Cambridge Phil. Soc.* 33:53–102.

Northcote, D. H., and R. W. Horne. (1952). The chemical composition and structure of the yeast cell wall. *Biochem. J.* 51:232–236.

Odier, A. (1823). Mémoires sur la composition chimique des parties cornées des insectes. *Mem. Soc. Hist. Nat. Paris* 1:29–42.

Oort, A. J. P., and P. A. Roelofsen. (1932). Spiralwachstum, Wandbau und Plasmaströmung bei *Phycomyces. Koninkl. Ned. Akad. Wetenschap., Proc.* C35:898–908.

Percival, E. G. V. (1950). "Structural Carbohydrate Chemistry," 246 pp. Prentice-Hall, Englewood Cliffs, New Jersey.

Preston, R. D. (1952). "The Molecular Architecture of Plant Cell Walls," 211 pp. Chapman & Hall, London.

Preston, R. D., E. Nicolai, R. Reed, and A. Millard. (1948). An electron microscope study of cellulose in the wall of *Valonia ventricosa. Nature* 162:665.

Probine, M. C. (1963). Cell growth and the structure and mechanical properties of the wall in internodal cells of *Nitella opaca*. III. Spiral growth and cell wall structure. *J. Exptl. Botany* 14:101–113.

Proskuriakow, N. J. (1926). Über die Beteiligung des Chitins am Aufbau der Pilzzellwand. *Biochem. Z.* 167:68–76.

Roelofsen, P. A. (1951). Cell wall structure in the growth-zone of *Phycomyces* sporangiophores II. Double refraction and electron microscopy. *Biochim. Biophys. Acta* 6:357–373.

Roelofsen, P. A., and I. Hoette. (1951). Chitin in the cell wall of yeasts. *Antonie van Leeuwenhoek, J. Microbiol. Serol.* 17:27–43.

Salton, M. R. J. (1961). "Microbial Cell Walls," 94 pp. Wiley, New York.

Schmidt, M. (1936). Makrochemische Untersuchungen über das Vorkommen von Chitin bei Mikroorganismen. *Arch. Mikrobiol.* 7:241–260.

Scholl, E. (1908). Die Reindarstellung des Chitins aus *Boletus edulis. Monatsh. Chem.* 29:1023–1036.

Setterfield, G., and S. T. Bayley. (1961). Structure and physiology of cell walls. *Ann. Rev. Plant Physiol.* 12:35–62.

Siegel, S. M. (1962). "The Plant Cell Wall," 123 pp. Pergamon Press, New York.

Singer, R. (1962). "The Agaricales in Modern Taxonomy," 915 pp. Cramer, Weinheim.

Smith, J. H. (1923). On the apical growth of fungal hyphae. *Ann. Botany* 37:341–343.

Sussman, A. S. (1957). Physiological and genetic adaptability in the fungi. *Mycologia* 49:29–43.

Sussman, A. S., B. von Böventer-Heidenhain, and R. J. Lowry. (1957). Physiology of the cell surface of *Neurospora* ascospores. IV. The functions of surface binding sites. *Plant Physiol.* **32**:586–590.

Thomas, R. C. (1928). Composition of fungus hyphae. I. The Fusaria. *Am. J. Botany* **15**:537–547.

Thomas, R. C. (1930). Composition of fungus hyphae. II. *Sclerotinia. Am. J. Botany* **17**:779–787.

Thomas, R. C. (1942). Composition of fungus hyphae. III. The Pythiaceae. *Ohio J. Sci.* **42**:60–62.

Thomas, R. C. (1943). Composition of fungus hyphae. IV. *Phytophthora. Ohio J. Sci.* **43**:135–138.

Tomlinson, G., and E. A. Jones. (1962). Isolation of cellulose from the cyst wall of a soil Amoeba. *Biochim. Biophys. Acta* **63**:194–200.

Tracey, M. V. (1955). *In* "Moderne Methoden der Pflanzenanalyse" (K. Paech and M. V. Tracey, eds.), Vol. II, pp. 264–274. Springer, Berlin.

van Iterson, G., Jr., K. H. Meyer, and W. Lotmar. (1936). Ueber den Feinbau des pflanzlichen Chitins. *Rec. Trav. Chim.* **55**:61–63.

van Wisselingh, C. (1898). Mikrochemische Untersuchungen über die Zellwände der Fungi. *Jahrb. Wiss. Botan.* **31**:619–687.

Vitols, E., R. J. North, and A. W. Linnane. (1961). Studies on the oxidative metabolism of *Saccharomyces cerevisiae* I. Observations on the fine structure of the yeast cell. *J. Biophys. Biochem. Cytol.* **9**:689–699.

Wettstein, F. (1921). Das Vorkommen von Chitin und seine Verwertung als systematisch-phylogenetisches Merkmal im Pflanzenreich. *Sitzber. Akad. Wiss. Wien. Math.-Naturw. Kl., Abt. I* **130**:3–20.

Whistler, R. L., and C. L. Smart. (1953). "Polysaccharide Chemistry," 493 pp. Academic Press, New York.

Zalokar, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* **46**:602–610.

NOTE ADDED IN PROOF

Recently new investigations have been reported that have considerable significance with respect to certain subjects considered in this chapter. M. A. Rosinski and R. J. Campana [Chemical analysis of the cell wall of *Ceratocystis ulmi*. *Mycologia* **56**:738–744 (1964)], have provided good evidence for the simultaneous presence of chitin and cellulose in *Ceratocystis ulmi* which now shares this unusual feature with *Rhizidiomyces* (see Section IV, A). Also, the presence of melanin (see Section IV, E, 5) has been demonstrated clearly in spore walls of *Mucor rouxii* [S. Bartnicki-Garcia and E. Reyes, Chemistry of spore wall differentiation in *Mucor rouxii*. *Arch. Biochem. Biophys.* **108**:125–133 (1964)].

CHAPTER 4

Flagella

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I. INTRODUCTION

In fungi the flagellum is the organ of locomotion for zoospores and motile gametes (planogametes) which are found in certain lower fungi (Myxomycetes and Phycomycetes except the Zygomycetes and the more advanced members of the Peronosporaceae). Two types of planogametes occur—isogamous planogametes, which are morphologically similar and alike in size, and anisogamous planogametes (as in *Allomyces*), which are of two sizes. In the Monoblepharidales the male gamete only is motile while the nonmotile female gamete is enclosed in a special organ, the oogonium. The distinction between zoospores and motile gametes is not always clear because in some fungi the motile cells may act either as asexual spores or as gametes.

The term "cilium" has been used frequently for flagellum, and both terms are still used interchangeably. A clear distinction cannot always be made between the two types of appendages, but in fungi it is preferable to use "flagellum" on account of the relative length, the occurrence as a single unit, and the independent beat of the organ.

II. GENERAL CHARACTERISTICS

Although a staining method for the microscopical examination of flagella was described by Loeffler (1889), as compared with other fields of research it has taken a long time to elucidate the structure of flagella in fungi. Loeffler described two types of flagella, viz. in a flagellate the presence of lateral hairs on the flagellum and in a ciliate the presence of a thin endpiece to the flagellum. Fischer (1894) introduced the name "Flimmer-

geissel" for the first type and "Peitschengeissel" for the second ("tinsel-type" and "whiplash-type" flagellum, respectively). Vlk (1938) demonstrated the presence of a whiplash-type flagellum in a myxomycete, and later it was shown that under certain conditions many species of Myxomycetes produce two flagella, which are usually designated as being of this type (cf. Alexopoulos, 1963). The next year Vlk (1939) published data on some of the Saprolegniaceae, from which it appeared that both the primary and the secondary zoospores have one flagellum of each type; the structure and action of the flagella in some aquatic Phycomycetes was described by Couch (1938, 1941). Couch demonstrated that the single, posterior flagellum of the Chytridiales, Blastocladiales, and Monoblepharidales is of the whiplash type. In the Hyphochytridiomycetes a single, anterior flagellum of the tinsel type was found. In members of the Oomycetes Couch found a posterior whiplash-type flagellum, as occurs in the chytrids, and an anterior flagellum of the tinsel type. After the work by Vlk and Couch, data on the structure of flagella were still lacking for the Plasmodiophoromycetes. Although from Ledingham (1934, 1935) and from several later publications (cf. Miller, 1958) it appears that the motile cells of the Plasmodiophoromycetes possess two apically attached flagella of different length, these flagella were first described by Miller as whiplash-type flagella.

On the basis of these data two types of flagella can be distinguished in fungi, viz. the whiplash and the tinsel type, and four types of motile cells (Fig. 1): (1) uniflagellate cells with a posterior whiplash-type flagellum (Chytridiales, Blastocladiales, Monoblepharidales); (2) uniflagellate cells

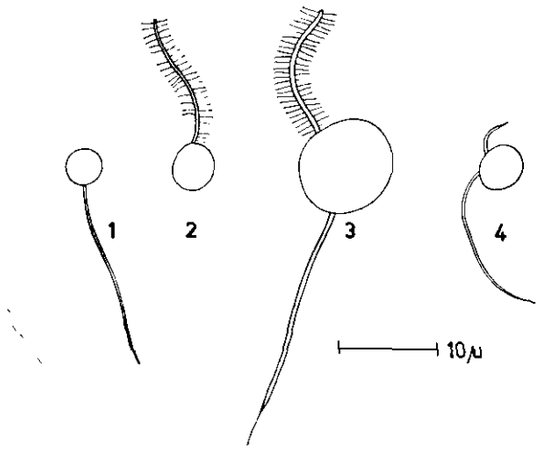

FIG. 1. Zoospore types according to flagellation (for explanation see text). Number 2 was redrawn from Couch (1941); the others are original freehand drawings.

with an anterior tinsel-type flagellum (Hyphochytridiomycetes); (3) biflagellate cells with one flagellum of each type (Oomycetes); (4) biflagellate cells with two whiplash-type flagella (Myxomycetes, Plasmodiophoromycetes).

Directly related to the flagellum is the way in which it is inserted in the body of the motile cell. Cytological examinations of motile cells reveal that there is a kernel-like body at the point of insertion. In line with the name used for such a body in flagellates, the designation "blepharoplast" is used here. The blepharoplast is often connected to the nucleus by means of a strand, the rhizoplast.

III. FINE STRUCTURE OF FLAGELLA

The application of the electron microscope and the phase contrast microscope has provided new possibilities for the investigation of flagella. The shortcoming of the electron microscope that no living motile cells can be examined with it, is partly compensated by the phase contrast microscope, which is an excellent instrument for obtaining contrasting images of living motile cells. In recent years many new data have been obtained on flagella and cilia. The main result is the great uniformity in the internal structure of these appendages, whether of plants or animals. Through this uniformity the difference between flagella and cilia also appears to be smaller than was expected. An excellent survey of what is known about flagella and cilia in general is given by Fawcett (1961).

A. Internal Organization of the Shaft

In the nineteenth century indications were obtained that sperm tails are composed of fine threads. Although this conclusion was at first unacceptable, it was supported by several investigations, including some on plant material, in the first part of this century. The results obtained with the electron microscope have confirmed the correctness of the concept of a fibrillar structure of the flagella (Fig. 2).

In addition, great uniformity was found in the number of fibers composing flagella and cilia. This number appeared to be always eleven, whereby two fibers are distinguished from the remaining nine in that they are thinner, different in length, and have a tendency to remain attached to each other. At first the investigations were restricted to disintegrated flagella so that no exact data could be obtained about the spatial arrangement of the fibers in the intact flagellum. In spite of this Manton and Clarke (1952) succeeded in designing a diagrammatic reconstruction of the position of the fibers within the flagellum. This concept was later confirmed

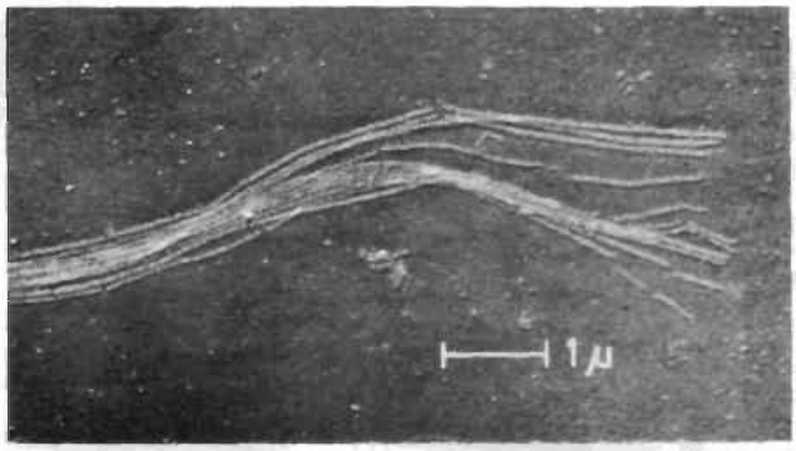

FIG. 2. Flagellum of *Pythium aphanidermatum*, showing disintegration into eleven fibers.

by examination of cross sections. According to this theory the two different fibers would take a central position, surrounded by the nine others. Because they found a groove over the length of the fibers Manton and Clarke came to the conclusion that the separate fibers would consist of two components. Koch (1956) was the first to make observations on this feature in disintegrated fungal flagella, he found in chytrids that there were probably two subfibers in each of the central pair of fibers and more than two subfibers, perhaps three, in each of the nine peripheral fibers. Kole (1957) working with *Synchytrium endobioticum,* found two subfibers in the peripheral fibers, thus confirming Manton and Clarke's opinion. The first electron microscope examination by Manton *et al.* (1951) of motile cells of fungi did not provide data on disintegration of flagella in fibers; it did give indication that in *Saprolegnia ferax,* the posterior flagellum of the secondary zoospores is flattened into a fin. When the investigations were extended to include *Allomyces* and *Olpidium* (Manton *et al.,* 1952), all flagella showed eleven fibers, generally differentiated into nine separate fibers and a central pair. As in *S. ferax* a wide translucent sheath was found in the zoospore of *A. arbuscula.* Furthermore, data on the disintegration of flagella into eleven fibers have become known for *Phytophthora infestans* (Kole and Horstra, 1959) and *Spongospora subterranea* (Kole and Gielink, 1961b). Also in *Pythium aphanidermatum* (Kole and Gielink, 1962a) eleven components were found.

To confirm Manton and Clarke's concept of the spatial arrangement of the fibers it would be necessary to make electron microscope observations

of cross sections. Little is known on this aspect of the motile cells of fungi, but Blondel and Turian (1960) published illustrations of cross sections of flagella of *Allomyces macrogynus* showing that these flagella have nine peripheral fibers and two axial fibers and that each of the peripheral fibers is double.

However, in the meantime many data have become available on cross sections of cilia, ciliated epithelia, and flagella in zoological material (cf. Fawcett, 1961). The uniformity in internal structure appears to be so great that it seems justified to assume a similar structure in motile cells of fungi. A diagrammatic reconstruction of a cross section of a flagellum has been given by Gibbons and Grimstone (1960) on the basis of their research on the flagellar structure of some flagellates (Fig. 3).

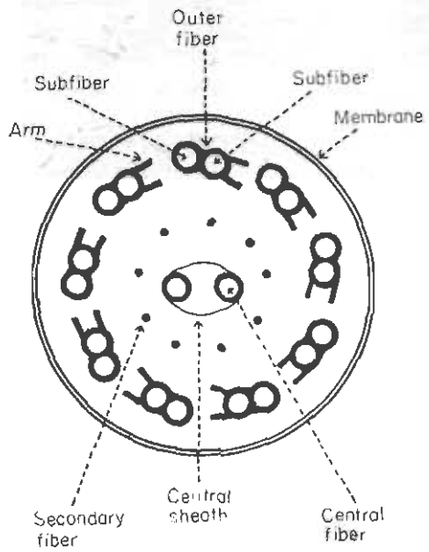

FIG. 3. Diagrammatic transverse section of a protozoan flagellum. From Gibbons and Grimstone (1960).

In this diagram the flagellum is cylindrical. The nine outer and two central fibers are clearly indicated. There are indications that between the outer and inner fibers thinner secondary fibers are present. The two central fibers are approximately circular in cross section and are situated separately. They have a dense annular region and a less dense core. Longitudinal sections suggested that the central fibers consist, at least in part, of coiled filaments forming a helix. Gibbons and Grimstone obtained indications that some form of central sheath envelops the two central fibers. In transverse

section this appeared as a moderately dense line running out from one of the fibers and curving round to join the other. The central sheath may consist of one or more filaments coiled around the pair of central fibers. Each of the nine outer fibers is composed of two subfibers. The longitudinal axis of the two subfibers is inclined slightly inward, positioning one subfiber slightly closer to the center of the flagellum than the other. Like the central fibers, the two subfibers of the doublet structure have a dense periphery and a less dense core. Indications have been found of some form of helical substructure in the outer fibers. One of the subfibers of each outer fiber bears short projections, designated "arms." In transverse sections two arms are usually visible on each outer fiber. Looking along the flagellum from base to tip, the arms always point in a clockwise direction. In longitudinal sections the arms appear not as continuous flanges, but as approximately rectangular structures. There is no certainty about the structures indicated as secondary fibers in the diagrammatic transverse section of Gibbons and Grimstone; they may be a set of radial connections between the central and the outer fibers.

It is amazing how accurately Manton and Clarke's diagrammatic reconstruction of the flagellum, based exclusively on the examination of disintegrated flagella, resembles that which has been found with transverse sections. Contrary to what is indicated by Manton and Clarke, the central fibers appear to be not double, but single. There is no certainty so far about the exact structure of the central sheath. The "arms," which are lacking in Manton and Clarke's diagram, are described by them as "battlements," but they are wrongly interpreted as being spiral or circular bands forming an inner tubular lining.

B. The Whiplash-Type Flagellum

Characteristic of the whiplash-type flagellum are the thin endpiece and the absence of lateral hairs (Fig. 4).

Examination by the electron microscope has shown that there is a fair amount of variation in the form of the endpiece. Manton *et al.* (1951) established that in the primary zoospores of *Saprolegnia ferax* the thickness of the sheath is gradually reduced toward the apex, but the axis remains of almost constant width until it narrows abruptly to form a short endpiece; this narrow endpiece is considerably longer in the posterior flagellum of the secondary zoospores. The whiplash-type flagellum of *Allomyces arbuscula,* according to the electron micrographs of Manton *et al.* (1952), has a gradually tapering endpiece, but in *Olpidium brassicae,* in which the motile cells are smaller and the flagella shorter, the transition to the thin endpiece is more abrupt. In *O. brassicae* a slight terminal swelling on the

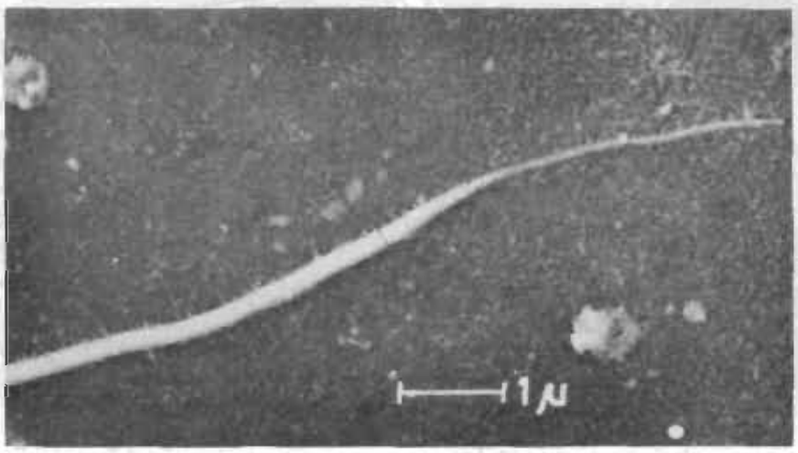

FIG. 4. Whiplash endpiece of the flagellum of *Synchytrium endobioticum*.

end of the whiplash is generally encountered; it is considered to be characteristic. Koch (1956) also found this swelling occasionally in chytrids; according to Kole (1957), a slight terminal swelling on the end of the whiplash flagellum of *Synchytrium endobioticum* sporangial zoospores is sometimes found. In better micrographs of later work this bulbous tip is lacking, however, so that the first observation may be an artifact or abnormality. These *Synchytrium* zoospores have a gradually tapering endpiece. According to Kole and Horstra (1959) the whiplash flagellum of *Phytophthora infestans* is rather abruptly tapering. Electron micrographs of zoosporangial zoospores of *Plasmodiophora brassicae* and *Spongospora subterranea* have been published by Kole and Gielink (1961b). In *S. subterranea* both flagella are of the whiplash type, the long flagellum having a gradually tapering endpiece while the endpiece of the short flagellum narrows abruptly and is very short. However, in *P. brassicae* the long flagellum is of the typical whiplash type and the short flagellum has an obtuse endpiece. The thin endpiece of the short flagellum may have been lost in making the preparations because in some of the *S. subterranea* micrographs it was missing as well. In apparently well-preserved zoospores from the resting spores of *P. brassicae* (Kole and Gielink, 1962b), the long flagellum showed a very distinct whiplash, while the short flagellum again had an obtuse point. In the various micrographs made by Kole (1963) of both uniflagellate and biflagellate motile cells of myxomycetes a whiplash flagellum was seldom found; most of the flagella had an obtuse point and when a whiplash was encountered it was not typical and suggested the beginning of disintegration.

Opinions of the internal structure of the whiplash differ considerably. According to Manton *et al.* (1952) the whiplash in *O. brassicae* is composed of the same eleven strands as the rest of the flagellum, but in a much more destructible condition. However, both Koch (1956) and Kole (1957), on examining motile chytrid cells came to the conclusion that the central pair of fibers extend to the tip while the nine peripheral fibers are shorter and are of different lengths.

Certainty about the internal structure of the whiplash can be reached only on the basis of serial cross sections. Because there are no data as yet for flagella of fungi, we must refer again to Gibbons and Grimstone (1960). From their micrographs of serial cross sections toward the tip it appears that the arms and the secondary fibers both disappear and the outer fibers converge toward the tip. At about the same level the central fibers tend to lose their central position and the symmetrical arrangement, and the even spacing of the outer fibers is partly lost. The double fibers became single as a result of the abrupt termination of one subfiber. The flagella continue to decrease in diameter and the outer fibers, having become single, eventually end. The change from doublet to singlet and the ending of the singlets occurs at different levels in different fibers, so that transverse sections show variable numbers of mixed doublets and singlets. The central fibers are not easily identified after they lose their central position, but they seem to end in the same manner as the outer fibers.

C. The Tinsel-Type Flagellum

The tinsel flagellum, the lateral hairs of which can be observed with the conventional microscope only after staining, and even then not always clearly, can be better studied with an electron microscope (Fig. 5).

In *Saprolegnia ferax* Manton *et al.* (1951, 1952) found that the hairs, extending almost to the tip of the flagellum, are borne singly in two rows down the sides of the axis. Each hair ends in a very delicate apical hair. From partly decomposed flagella it was apparent that the hairs originate from the axis. However, to ascertain in exactly which way this happens it will be necessary to prepare cross sections. The observations by Manton *et al.* are confirmed by Kole and Horstra (1959) for *Phytophthora infestans* and by Nagai and Takahashi (1962) for *S. diclina*. In *Phytophthora* there is an abrupt transition from the basal three-fourths of the hair to the thin hair-like apex. In *Saprolegnia* and *Phytophthora* the lateral hairs are readily found, even in micrographs of decomposed flagella. In *Pythium* (Kole and Gielink, 1962a) the situation is different. One flagellum, having a very distinct endpiece, always is of the whiplash type, but in *P. aphanider-*

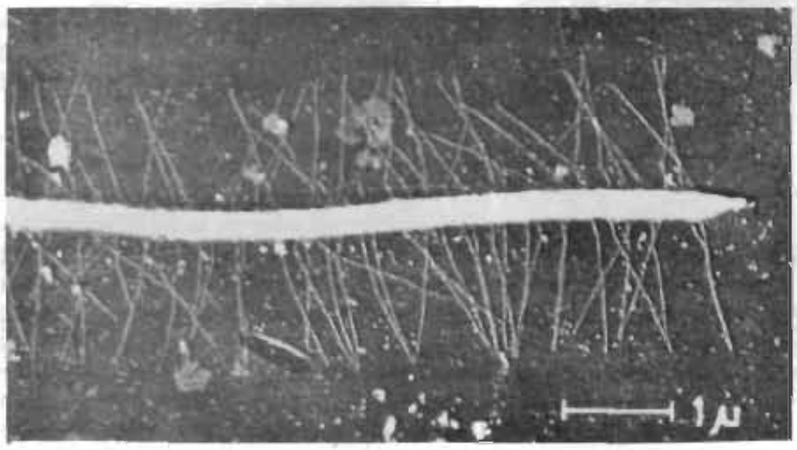

FIG. 5. Tinsel-type flagellum of *Phytophthora infestans*.

matum lateral hairs are never found on the other flagellum; in *P. torulosum* they are sometimes present, sometimes not. When lateral hairs are encountered, they are extremely fine. The absence of lateral hairs in these fungi therefore may be due to their early disintegration. These observations confirm Couch's (1941) statement that the tinsels on the anterior flagellum of *Pythium* sp. are very indistinct when seen with the light microscope.

D. Motile-Cell Inclusions Connected with the Flagellum

Compared with material of zoological origin little is known about the insertion of the fungus flagellum in the body of the motile cell. In contradistinction to the great uniformity in the internal structure of flagella and cilia there is remarkable variation in the structures connected with the insertion of these appendages. There is little point therefore in relating the comprehensive literature on this subject (cf. Fawcett, 1961) to the flagella of fungi.

Knowledge of the blepharoplast and rhizoplast of fungi is largely based on what has been found with the light microscope. In an extensive investigation of motile cells of a number of oomycetes, Cotner (1930) came to the conclusion that the zoospores of the species studied all have a definite basal granule (blepharoplast) at the insertion of each flagellum. This blepharoplast is connected with the nucleus by a definite strand (rhizoplast). According to Cotner the basal apparatus is always of nuclear origin and seems to be composed of several chromatic bodies, one of which

forms the blepharoplast proper. The remaining granules of the basal apparatus are normally retained by, or drawn back into, the nucleus and usually form the tip or beaklike part of that organ during zoospore activity.

In their examination of disintegrated flagella of *Allomyces* and *Olpidium*, Manton *et al.* (1952) found that the fibers at the end of the connection to the cell body are assembled in a characteristic bulbous base, which is presumably internal in the living cell. Koch (1956) in his electron microscope observations of the motile cells of chytrids found that the blepharoplast is also fibrillar and the flagellar fibers and subfibers extend all the way into the functional blepharoplast, double back, and end in a skirt of bulbous tips. A second, nonfunctional or vestigial blepharoplast is found lateral to the functional blepharoplast; it too is composed of fibers and subfibers. The rhizoplast comes out at an angle from the functional blepharoplast and in *Chytridium* ends in a disk-shaped structure which is gridded. The internal structure of motile cells of chytrids has been further investigated by Koch (1958) with light microscopy. The presence of a nonfunctional blepharoplast as well as a functional blepharoplast was confirmed. In several chytrids the rhizoplast comes off of the blepharoplast at an angle. This angled rhizoplast is directed toward a conspicuous lipoidal body and bears an expanded tip. This bulbous tip is probably equivalent to the disk-shaped structure described in Koch's previous paper (1956). Indications were obtained that this enlarged tip may be in contact with the lipoidal body. According to Koch, the lipoidal body may play a direct role in the action of the flagellum.

Ellison (1945) investigating myxomycetes found that two blepharoplasts are always present in both the uniflagellate and biflagellate motile cells. In the biflagellate cells each blepharoplast produces a flagellum. However, in the uniflagellate, as well as in the biflagellate zoospores, only one of the blepharoplasts had a rhizoplast connecting it to the nucleus.

In the myxomycete *Didymium nigripes*, Kerr (1960) found that each flagellum arises from a small granule in the saclike blepharoplast. The blepharoplast is connected to the nucleus by a rhizoplast, which appears as a clear area of cytoplasm looking very dark under phase contrast. The nucleus always remains closely associated with the flagellar apparatus, even when the cell loses its rigid shape to become an amoeboid-flagellate cell. This was also shown by electron micrographs of disintegrated zoosporangial zoospores of *Spongospora subterranea* (Kole and Gielink, 1961b) wherein the two rhizoplasts, each bearing a blepharoplast and a flagellum, emerge from one point on the nucleus and upon further disintegration remain attached to each other.

E. Flagellar Formation

Little is known of the origin of flagella. According to Cotner (1930), the flagella develop as outgrowths from the region of a blepharoplast which has reached a position near the plasma membrane as a result of nuclear activity. From a review of the literature, Cotner concluded that flagella formation in the spores of uniflagellate and biflagellate fungi takes place under entirely different internal conditions. In the uniflagellate group the initiation of flagella formation takes place during very early stages of spore formation. In a number of species in this group, formation of flagella accompanies or follows closely nuclear and cell division when a single flagellum is initiated or formed at each pole of the mother nucleus, which results in a single flagellum for each of the daughter cells. In the biflagellate group this process does not accompany or follow closely nuclear or cell division, but is delayed until later when greater nuclear activity develops. This activity is sufficient to bring about formation of flagella, but not further cell division.

Ritchie (1947) concluded for two reasons that in *Allomyces arbusculus* the flagellum originates late in the development of the zoospores. First, because the nucleolus, which is held in an eccentric position in the mature spore by an intranuclear extension of the rhizoplast, could be seen to be centrally located just before the spores matured; second, premature rupture of the sporangium failed to release any isolated flagella or portions of flagella. In sectioned material, the flagella were not visible until the spores were formed. On the other hand, Blondel and Turian (1960), in electron micrographs of *A. macrogynus,* found flagella to be formed before the cytoplasm of the gametangia begins to differentiate and before the cytoplasmic membranes delimiting the future gametes are formed.

The development of the flagella in Myxomycetes was investigated by Kerr (1960). Upon germination of the spore, nonflagellated myxamoebae are released which move by means of pseudopodia and protoplasmic streaming. Then the amoebae became rounded, and after a series of vigorous eruptions, a flagellum appears. Whereas newly flagellated cells possess a single flagellum, cells which have been flagellated for several hours show two flagella. The flagella continue to lengthen after they first form. Before the formation of the flagellum, the nucleus becomes associated with a specific region of cytoplasm in which the blepharoplast later appears. The flagella, which at first closely resemble pseudopodia, appear quite suddenly.

F. Disappearance of Flagella

The two obvious explanations of the disappearance of the flagella at the end of the motile phase are that they are cast off or that they are resorbed. Disintegration of flagella is not considered here because presumably this is not a natural process. Curtis (1921) investigating in *Synchytrium endobioticum* the fate of the flagella on entry into the host of zoospores and at zygote formation found that the flagella contract and finally are reduced to a knob. Whether this knob is finally thrown off, or withdrawn into the zoospore, could not be determined. According to B. T. Lingappa (1958) in *Synchytrium brownii* the flagella of the zoospores, gametes, and zygotes are gradually absorbed and are not dropped off. After becoming crooked, beaded, or looped, and swollen terminally, the flagellum remains as a short stump and eventually disappears. In *Physoderma pulposum*, according to Y. Lingappa (1959), the whiplash has a bulbous tip as do other chytrids. Electron micrographs indicated that the flagella are absorbed from the tip backward. During absorption the whiplash becomes shorter and the terminal bulb larger. The process of flagellum withdrawal prior to encystment has been further investigated in nine chytrids by Koch (1959, 1961). The flagellum becomes wrapped around the body of the spore, after which it moves coiled up directly through the outer membrane of the spore body. Alternatively a loop or vesicle forms in the flagellum, and this vesicle gradually resorbs the flagellum and finally fuses with the spore body. The coiled, resorbed flagellum, which might show some activity for a while, was clearly seen within stained zoospores.

Data about the fate of the flagella in biflagellate motile cells have also become available. Examination of living zoospores of *Phytophthora capsici* with the phase contrast microscope (Katsura *et al.*, 1956) reveal that a spherical body appears on the middle part of the flagella. Then, flagella suddenly fold in two at this point and seem to wither completely or remain bent back on themselves. The zoospores stop their movement and the flagella, being either spherical or elongated, become detached and float away. Also in some other phycomycetous zoospores (McKeen, 1962), hyaline vesicles, beads, or paddles appear to form at the base of the flagella and glide part or all the way down the flagella before becoming detached. After detachment the ends of the flagella are wrapped around the bead. In one of the fungi the flagellum was withdrawn by being flipped back on the body of the zoospore. According to McKeen, the formation of vesicles or beads occurs just prior to the release of the flagella. Working with *Phytophthora infestans*, Ferris (1954) was of the opinion that the flagella roll up, become detached and float away. Kole and Horstra (1959) found that

zoospores of *P. infestans* suddenly produce a swelling which moves to the end of the flagellum. Generally this first swelling is followed by a second and sometimes even by a third. In the meantime the short flagellum also develops a swelling. Sometimes it can be observed that in flagella with a swelling in the middle the endpiece turns over and settles down on the rest of the flagellum. Ultimately all the swellings are concentrated in a paddle-like structure at the tip of the flagellum. Presently the flagellum disappears by settling against the body of the zoospore. Sometimes a flagellum becomes detached from the zoospore at one of the described stages at the place of its insertion and then floats away. Kole and Horstra explained the paddle-like structures as being protrusions of the sheath, associated with the beginning of the disintegration of the flagella.

Whereas in the motile cells described so far the disappearance of the flagellum means the end of the motile phase, in myxomycetes the unique feature is that motile cells change into myxamoebae by withdrawing their flagella and myxamoebae, in turn, become flagellated under certain conditions (Alexopoulos, 1963).

IV. FLAGELLAR ACTION

The mode of swimming of the motile cells and the action of flagella were examined by Couch (1941) with dark-field microscopy and stroboscopic illumination. According to him the posteriorly uniflagellate motile cells are propelled by the transmission of waves through the flagellum in one plane and in one direction in respect to the spore. The spore may swim in a wide circular orbit or in a straight line not rotating on its axis; or it may swim in a straight line rotating on its axis; or it may swim in a spiral path rotating on its axis. When the spore rotates on its axis, the flagellum presents an alternating single and double image, the single image being formed when the flagellum is undulating in a plane vertical to the observer and the double image when the flagellum is undulating in a plane horizontal or diagonal to the observer. In addition to movement in a straight line and in a spiral path, the chytrid spore also hops and darts about.

Koch (1959, 1961) using dark-field microscopy combined with cinephotomicrography reported that a motile cell usually oscillates while the flagellum undulates and the spore swims forward. The erratic type of swimming is attributed by Koch to frequent changes in direction and frequent starting and stopping. When a swimming cell stops abruptly, it twitches or jerks its flagellum, and this results either in a pivoting of the body or in a pivoting accompanied by a slight change of position or jerking movement. Varying brightness of different parts of the undulating flagellum in a good dark field indicated that flagellar action may be slightly

three-dimensional rather than just two-dimensional. The anteriorly uniflagellate motile cells in Hyphochytridiomycetes have been found by Couch to be pulled forward by undulations which are propagated just back of the tip, traveling toward the body of the zoospore.

Couch's studies have shown that the biflagellate motile cells of the Oomycetes have a common pattern of action. Both flagella are active in the propulsion of the zoospore, each flagellum undulating in a single and different plane. As Couch stated, this is an entirely novel concept, since until then the generally accepted opinion had been that the anterior flagellum propelled the spore while the posterior was dragged along behind as a more or less passive rudder. McKeen (1962) however, is of the opinion that the posterior flagellum propels the zoospore and that the anterior flagellum serves as a rudder. Little is known about the action of the flagella in the fungi having two flagella of the whiplash type. According to Kole (1954, 1955) the zoosporangial zoospores in two plasmodiophoromycetes swim with the short flagellum in front and the long flagellum trailing behind. Additional unpublished observations (Kole and Gielink, 1961a) have revealed that the zoospore moves in a straight line with sudden changes of direction, frequently by tumbling downward or sideward. The front flagellum moves slowly and the hind one undulates irregularly. Miller (1958) observed in another plasmodiophoromycete that before encystment the posterior flagellum is dragged along behind as the anterior flagellum lashes slowly back and forth. McKeen (1962) found that detached flagella frequently continue to move for a short time. According to McKeen, this might provide evidence for active endogenous movement of the flagellum under normal swimming conditions and further indicates that contractions originating within the cell below the flagellar attachment may not be necessary for flagellar action. Little is known as yet about the mechanism on which the movements of the flagella are based, nor of the biochemical processes playing a role. For a survey of present knowledge and current hypotheses we refer to Fawcett (1961).

V. FLAGELLATION AND PHYLOGENY

The number and structure of flagella have contributed greatly to detecting relationships among lower fungi. As Sparrow (1958) states: ". . . the structure *par excellence* which lies at the very base of any natural system of the lower, aquatic Phycomycetes is the zoospore." Mainly because of the type of flagellation the Chytridiales, Blastocladiales, and Monoblepharidales are considered as a closely related group (Chytridiomycetes according to Sparrow, 1958). The same holds for the Saprolegniales, Leptomitales, Lagenidiales, and Peronosporales (Oomycetes). The Hypho-

chytridiomycetes with its anteriorly uniflagellate motile cells is again distinct, as are the Myxomycetes and the Plasmodiophoromycetes.

The phylogeny of the lower fungi is a difficult problem. Bessey (1942) assumed that unicellular heterocont algae, by losing chlorophyll and the anterior flagellum, would have produced the Chytridiomycetes. By their losing the posterior flagellum the Hyphochytridiomycetes would have resulted, and by retention of both the Oomycetes would have evolved. Sparrow (1958), however, is of the opinion that the Phycomycetes is not a homogeneous monophyletic group, but rather an artificial category of coenocytic zoosporic fungi, consisting of four groups which may have come from radically different progenitors. Bessey's concept of the evolution of the lower fungi has become of current interest through Koch's (1956) discovery of the presence of what he calls a second, nonfunctional, vestigial blepharoplast in the chytrid motile cell, suggesting a biflagellate ancestry. This supposition is supported by conclusions from observations on the movement of chytrid zoospores (Koch, 1959, 1961). Koch observed, that the double image of the flagellum formed when the flagellum is undulating in a plane horizontal to the observer, may be asymmetrical, with one side more "humped" than the other. He suggests that this asymmetrical double image originates phylogenetically in a motile cell in which the posteriorly directed flagellum is laterally inserted on the body, so that the primitive or ancestral condition is not the posteriorly attached whiplash flagellum, but rather the laterally attached whiplash flagellum. This, and the second, nonfunctional blepharoplast suggest that the chytrids, and perhaps all posteriorly uniflagellated fungi, have evolved either from biflagellated organisms or from organisms with biflagellated swimming cells. Much has been written about the phylogeny of the Plasmodiophoromycetes and the Myxomycetes, but only little has been stated with certainty. Instead of citing speculative assumptions here, we would rather refer to the literature concerned (Karling, 1942; Martin, 1960).

REFERENCES

Alexopoulos, C. J. (1963). The Myxomycetes II. *Botan. Rev.* 29:1–78.

Bessey, E. A. (1942). Some problems in fungus phylogeny. *Mycologia* 34:355–379.

Blondel, B., and G. Turian. (1960). Relation between basophilia and fine structure of cytoplasm in the fungus *Allomyces macrogynus* Em. *J. Biophys. Biochem. Cytol.* 7:127–134.

Cotner, F. B. (1930). The development of the zoöspores in the Oömycetes at optimum temperature and the cytology of their active stages. *Am. J. Botany* 17:511–546.

Couch, J. N. (1938). Observations on cilia of aquatic Phycomycetes. *Science* 88:476.

Couch, J. N. (1941). The structure and action of the cilia in some aquatic Phycomycetes. *Am. J. Botany* 28:704–713.

Curtis, K. M. (1921). The life-history and cytology of *Synchytrium endobioticum* (Schilb.) Perc., the cause of wart disease in potato. *Phil. Trans. Roy. Soc. London* **B210**:409–478.

Ellison, B. R. (1945). Flagellar studies on zoospores of some members of the Mycetozoa, Plasmodiophorales, and Chytridiales. *Mycologia* **37**:444–459.

Fawcett, D. (1961). *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 217–297. Academic Press, New York.

Ferris, V. R. (1954). A note on the flagellation of *Phytophthora infestans* (Mont.) de Bary. *Science* **120**:71–72.

Fischer, A. (1894). Ueber die Geisseln einiger Flagellaten. *Jahrb. Wiss. Botan.* **26**:187–235.

Gibbons, I. R., and A. V. Grimstone. (1960). On flagellar structure in certain flagellates. *J. Biophys. Biochem. Cytol.* **7**:697–716.

Karling, J. S. (1942). "The Plasmodiophorales." 144 pp. Published by the author, New York City.

Katsura, K., K. Harada, and M. Murakami. (1956). The flagella of zoospores of *Phytophthora capsici* Leonian as viewed with the phase contrast microscope. *Ann. Phytopathol. Soc. Japan* **21**:71–73.

Kerr, N. S. (1960). Flagella formation by myxamoebae of the true slime mold, *Didymium nigripes*. *J. Protozool.* **7**:103–108.

Koch, W. J. (1956). Studies of the motile cells of chytrids. I. Electron microscope observations of the flagellum, blepharoplast and rhizoplast. *Am. J. Botany* **43**:811–819.

Koch, W. J. (1958). Studies of the motile cells of chytrids. II. Internal structure of the body observed with light microscopy. *Am. J. Botany* **45**:59–72.

Koch, W. J. (1959). The motile cell of uniflagellate Phycomycetes. *Abstr. 9th Intern. Botan. Congr.* Montreal, 1959, Vol. 2, p. 196.

Koch, W. J. (1961). *In* "Recent Advances in Botany," pp. 335–339. Univ. of Toronto Press, Toronto, Canada.

Kole, A. P. (1954). A contribution to the knowledge of *Spongospora subterranea* (Wallr.) Lagerh., the cause of powdery scab of potatoes. *Tijdschr. Plantenziekten* **60**:1–65.

Kole, A. P. (1955). Some observations on the zoospores from the zoosporangia of *Plasmodiophora brassicae* Woron. *Tijdschr. Plantenziekten* **61**:159–162.

Kole, A. P. (1957). Elektronenmicroscopische waarnemingen over de zoösporen uit de zomersporangia van *Synchytrium endobioticum* (Schilb.) Perc. *Tijdschr. Plantenziekten* **63**:361–364.

Kole, A. P. (1963). Unpublished data.

Kole, A. P., and A. J. Gielink. (1961a). Unpublished observations.

Kole, A. P., and A. J. Gielink. (1961b). Electron microscope observations on the flagella of the zoosporangial zoospores of *Plasmodiophora brassicae* and *Spongospora subterranea*. *Koninkl. Ned. Akad. Wetenschap., Proc.* **C64**:157–161.

Kole, A. P., and A. J. Gielink. (1962a). Unpublished data.

Kole, A. P., and A. J. Gielink. (1962b). Electron microscope observations on the resting-spore germination of *Plasmodiophora brassicae*. *Koninkl. Ned. Akad. Wetenschap., Proc.* **C65**:117–121.

Kole, A. P., and K. Horstra. (1959). Electron microscope observations on the flagella of the zoospores of *Phytophthora infestans*. *Koninkl. Ned. Akad. Wetenschap., Proc.* **C62**:404–408.

Ledingham, G. A. (1934). Zoospore ciliation in the Plasmodiophorales. *Nature* 133: 534.

Ledingham, G. A. (1935). Occurrence of zoosporangia in *Spongospora subterranea* (Wallroth) Lagerheim. *Nature* 135:394.

Lingappa, B. T. (1958). Sexuality in *Synchytrium brownii* Karling. *Mycologia* 50: 524–537.

Lingappa, Y. (1959). The development and cytology of the epibiotic phase of *Physoderma pulposum*. *Am. J. Botany* 46:145–150.

Loeffler, L. (1889). Eine neue Methode zum Färben der Mikroorganismen, im besonderen ihrer Wimperhaare und Geisseln. *Zentr. Bakteriol., Parasitenk.* 6:209–224.

McKeen, W. E. (1962). The flagellation, movement, and encystment of some phycomycetous zoospores. *Can. J. Microbiol.* 8:897–904.

Manton, I., and B. Clarke. (1952). An electron microscope study of the spermatozoid of *Sphagnum*. *J. Exptl. Botany* 3:265–275.

Manton, I., B. Clarke, and A. D. Greenwood. (1951). Observations with the electron microscope on a species of *Saprolegnia*. *J. Exptl. Botany* 2:321–331.

Manton, I., B. Clarke, A. D. Greenwood, and E. A. Flint. (1952). Further observations on the structure of plant cilia, by a combination of visual and electron microscopy. *J. Exptl. Botany* 3:204–215.

Martin, G. W. (1960). The systematic position of the Myxomycetes. *Mycologia* 52: 119–129.

Miller, C. E. (1958). Morphology and cytology of the zoosporangia and cystosori of *Sorosphaera veronicae*. *J. Elisha Mitchell Sci. Soc.* 74:49–64.

Nagai, M., and T. Takahashi. (1962). Electron microscope observations on the zoospores of *Saprolegnia diclina*. *Mycol. Soc. Japan, Trans.* 3:19–23.

Ritchie, D. (1947). The formation and structure of the zoospores in *Allomyces*. *J. Elisha Mitchell Sci. Soc.* 63:168–205.

Sparrow, F. K. (1958). Interrelationships and phylogeny of the aquatic Phycomycetes. *Mycologia* 50:797–813.

Vlk, W. (1938). Über den Bau der Geissel. *Arch. Protistenk.* 90:448–488.

Vlk, W. (1939). Über die Geisselstruktur der Saprolegniaceenschwärmer. *Arch. Protistenk.* 92:157–160.

CHAPTER 5

The Ultrastructure of Fungal Cells

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I. INTRODUCTION

Much of fungal morphology lies beyond the limits of resolution of the ight microscope. Recently, however, the techniques of electron microscopy ıave permitted its accurate visualization. The intention of this section is to ›ring the various observations of fungal ultrastructure together in an in- erpretation believed compatible with previously known fungal cytology. ncluded is a review of certain aspects of the fine structure of cell and nu- :lear division, sporogenics, interaction of fungi with plant hosts, and of cer- .ain organelles and wall elements.

II. CELL DIVISION

A. Forms of Division

In both the Schizomycota and the Eumycota there occurs a common basic pattern of protoplast division. In outline this is as follows: a peripheral ring or band in the plasma membrane becomes activated and initiates centripetal growth; as the membrane invaginates, new wall material is produced on its infolded opposing faces; cytokinesis is completed by the narrowing aperture closing upon itself to produce two opposing membranes from the single originally infolded one; karyokinesis most frequently is by an apparently nonmitotic process. [This sequence is in contrast to the comparable process in plant and animal cells where cytokinesis is effected by a plane of fusing vesicles, a plasma membrane that is either passive (plants) or invaginates only slightly to form a cleavage furrow (animals), and a mitotically dividing nucleus (cf. Odor and Renninger, 1960; Buck and

Tisdale, 1962; Porter and Machado, 1960; Whaley and Mollenhauer, 1963).] The levels of complexity in this type of cell formation progress from complete cell division and separation seen in representative bacteria and rusts and in some types of spore formation, to complete cell division without separation as seen in the mycelia of actinomycetes and phycomycetes, to the incomplete cell division observed in carpomycete hyphae. These patterns are diagrammed in Fig. 1.

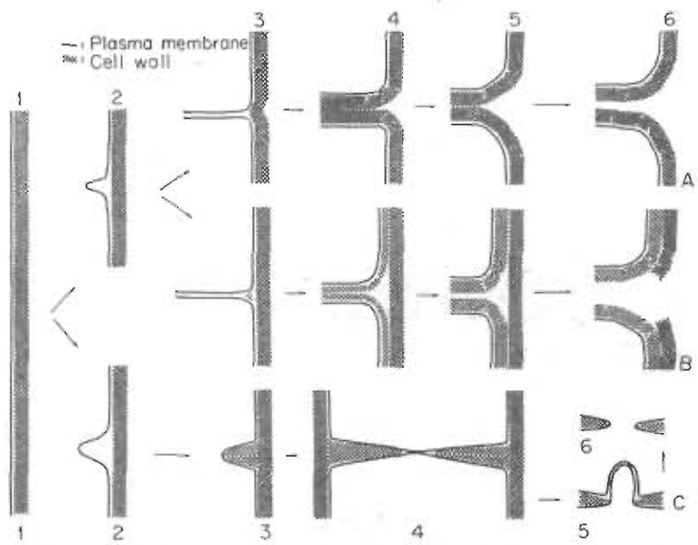


FIG. 1. Interpreted patterns of somatic cell division in the mycota: *A*, that represented by certain bacteria and *Puccinia podophylli* aecia; *B*, that represented by *Streptomyces violaceoruber* and *Coccidioides immitis*; *C*, that represented by ascomycete hyphae. Explanation in text.

B. Complete Cell Division

In assimilative cells of *Bacillus* (Chapman and Hillier, 1953), of an unidentified bacterium (Chapman, 1959), of *Streptomyces* (Moore and Chapman, 1959; Glauert and Hopwood, 1961), of the phycomycetes *Allomyces* (Moore, 1964d), *Coccidioides immitis* (O'Hern and Henry, 1956; Breslau *et al.*, 1961), *Monoblepharis* (Moore, 1964d), and of the rust *Puccinia podophylli* (Moore, 1963b), as well as in the cruciate basidia of *Exidia* (Moore, 1964d; Wells, 1964b), plasma membrane invagination appears to be initiated by a narrow medial ring and consequently the ingrowing sides are approximately parallel and closely appressed; new wall

material is produced over the whole protoplast surface, i.e., along both sides of the newly ingrown membrane across the whole diameter and also upon the pre-existing plasma membrane (Fig. 1, *A*, *B*).

In the bacteria mentioned, matrix material between the new end walls is either absent or readily dissolved, for the walls appear to be noncoherent and upon completion become separated as the daughter cells round up (Fig. 1, *A4–6*). A similar progression has been observed in the accial primordium of *Puccinia podophylli* (Moore, 1963b). Here also the sides of the centripetally invaginating membrane appear nearly parallel, both cytokinesis and cross wall formation go to completion, and the new wall is of constant thickness. As the transverse wall matures it becomes centripetally split. Consequently, the primordial pseudoparenchyma is nonhyphal and composed of a closely packed aggregate of discrete cells.

A homologous sequence of development occurs during spore formation in *Streptomyces* and *Coccidioides immitis* except that the outer hyphal wall maintains its integrity across the zone of invagination. In *Streptomyces* evidence has been presented by Vernon (1955) that for a number of species the spore catenulum is produced within a sheath, and by Glauert and Hopwood (1961) that for *S. violaceoruber* the aerial hyphal "wall thickens and acquires the extra dense layer prior to sporulation." These observations suggest that concomitant with the activation of an invagination ring the whole protoplast surface commences production of new wall material (Fig. 1, *B4*). This would further serve to explain the triangular space, in section, noted by Moore and Chapman (1959) and Glauert and Hopwood (1961) at the intersection of the cross and longitudinal walls; the associated centripetal splitting observed in both these reports would, then, be not the cleaving of a unitary wall, but the progressive separation of two walls already marked out (Fig. 1, *B5*). Thus, after the transverse walls have become completed and split, a process similar to endospore formation in *Coccidioides* (Breslau *et al.*, 1961), the catenulum is still held together by the continuity of the longitudinal wall (Fig. 1, *B5*). The spores break apart mechanically, and each has a single wall across its end and a double wall along its length (Fig. 1, *B6*). This interpretation is complementary to that of Hagedorn (1960) in which the outermost spore wall is regarded as being derived from the parent hypha. On either end of a spore there appears a small collar (Glauert and Hopwood, 1961) that is the broken end of the "supernumerary" outer wall (Fig. 1, *B6*).

A similar process during endospore formation in *Coccidioides immitis* is indicated by Breslau *et al.* (1961). Their Fig. 9 shows a triangular space nearly identical to that mentioned above. Its two internal sides are formed by the inrounded walls of a pair of nearly mature endospores, and its third side is that of the original spherule wall. Further, the walls of the two

endospores where still adjacent show a clear separation line but, at the same time, there is no evidence of separation between the walls of the sporangium and the endospores where these are contiguous. The resultant configuration is virtually the same as that diagrammed in Fig. 1, *B5*. Discharged endospores have not been shown by either Breslau *et al.* (1961) or O'Hern and Henry (1956), and so one may only surmise that such spores probably have double walls over their original outer surfaces and single walls across their formerly juxtaposed inner faces.

Septation is produced when the mature transverse walls develop interstitial coherency so that they neither split nor separate at the point of juncture with the outer wall. Glauert and Hopwood (1961) present evidence for an interstitial wall material in the transverse walls of the hyphae of *Streptomyces violaceoruber;* similar material is also evident in the phycomycetes mentioned above as well as in *Allomyces macrogynus* (Blondel and Turian, 1960), and Dickson (1963) shows it in septa of *Pithomyces chartarum* (Deuteromycetes), and Bracker and Butler (1963) present similar micrographs for the septa of *Rhizoctonia solani* (Mycelia Sterilia) [*Corticium solani*].

C. Incomplete Cell Division

Hyphae of carpomycetes (Ascomycetes and Basidiomycetes) are regularly partitioned by septa that have a single central pore. All available observations show these septa to be tapered toward the center of the cell (e.g., Bracker and Butler, 1963; Dickson, 1963; Girbardt, 1961; Moore,

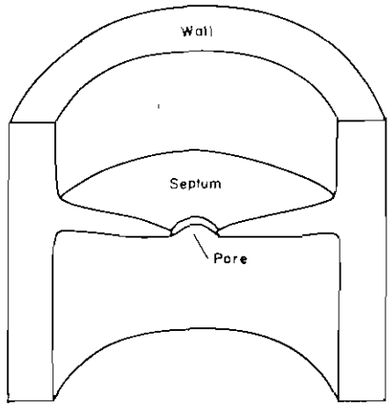


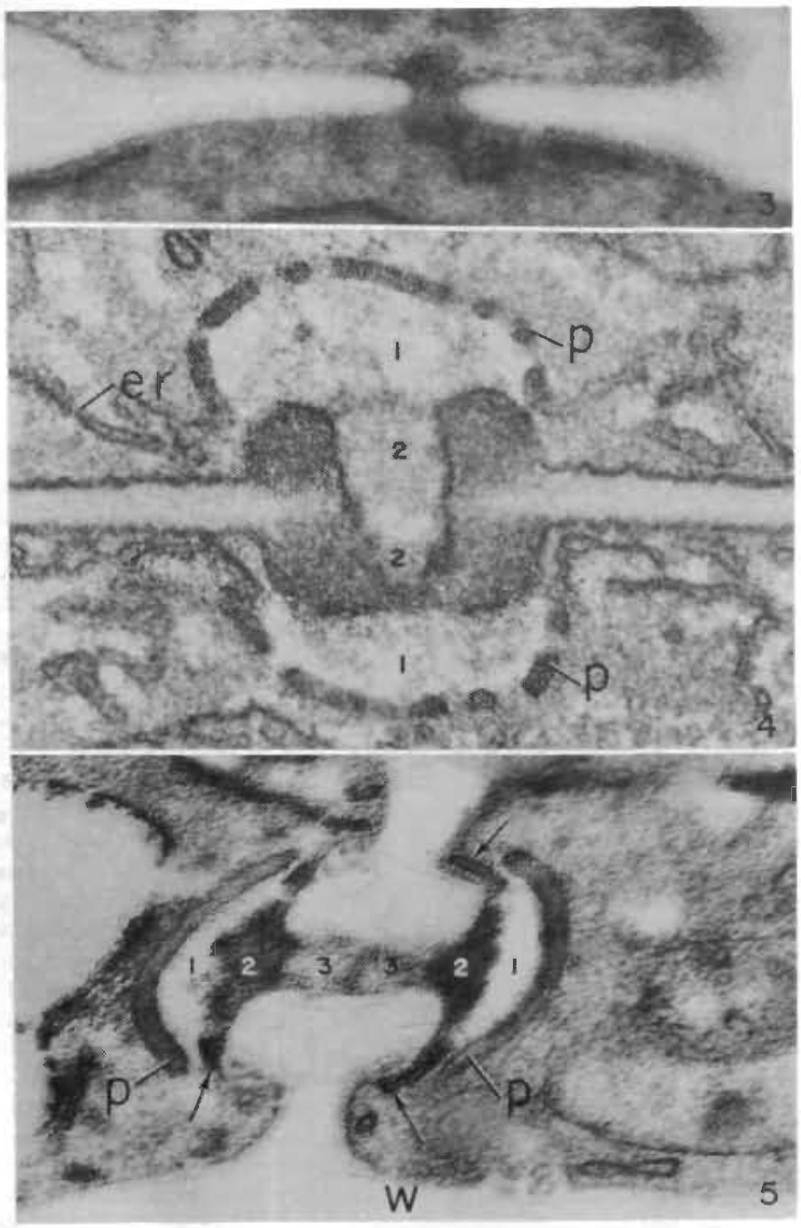
FIG. 2. Interpretation of the ascomycete septum. Explanation in text. Redrawn from Moore and McAlear (1962b).

963a; Moore and McAlear, 1962b; Shatkin and Tatum, 1959; Taplin and Blank, 1961) (Figs. 2–7). If the final form of the septum is a reflection of he mode of invagination, then the septum in this group of fungi would ppear to be initiated by a band rather than a ring in the protoplasm membrane (Fig. 1, *C2*). It would seem that as invagination progresses the thickness of the invagination lip decreases, and consequently the opposing nembrane faces are not parallel but slightly angled (Fig. 1, *C3*).

In ascomycetes it is proposed that when the intersurface distance becomes a critical minimum wall formation ceases (Fig. 1, *C4*). Here, as in he aforementioned examples, invagination and cytokinesis go to completion, but the cessation of the production of wall material leaves a "pore diaphragm" (Moore, 1963a) stretched across a circular gap (Fig. 1, *C4*); this "blows out" (Fig. 1, *C5*), the edges reheal, and longitudinal continuity of the plasma membrane around and through the pore is reestablished (Fig. 1, *C6*). Inclusions and organelles, including nuclei, are capable of migrating through the pore (Moore, 1963a; Shatkin and Tatum, 1959). Thus, the mycelium of ascomycetes is functionally coenocytic. The septa in the ascogenous hyphae, a limited dikaryon, are also of this same general type (Moore and McAlear, 1962b), but, at least in *Dasyscyphus* and *Mollisia* (Fig. 3), a plug of electron-dense material that probably blocks nuclear migration has been frequently observed.

In section the ascomycete septum usually appears homogeneous, but evidence of lamination has been shown in the septa of *Ascodesmis* (Moore, 1963a), *Neurospora* (Kawakami and Nehira, 1958; Shatkin and Tatum, 1959), *Pithomyces* (Dickson, 1963), and *Pseudoplea gaeumannii* (Moore, 1964d).

The micromorphology of the basidiomycete septum, though only recently elucidated, appears to equal or even exceed the basidium in characterizing this class of fungi. A septum of this type has been intensively studied by Bracker and Butler (1963, 1964) in an imperfect strain of *Corticium solani*. In marked contrast to the examples discussed above the centripetally closing lip as it nears the center of the cell becomes suddenly inflated as though further ingrowth had been impeded but membrane increase had continued briefly. This establishes the outline of the dolipore (Moore and McAlear, 1962b). Consequently, it appears that, unlike all previously mentioned examples, cytokinesis is incomplete and that the cell-to-cell continuity of the cytoplasm and the plasma membrane are not interrupted, even briefly. As in *Streptomyces* (Glauert and Hopwood, 1961) and *Exidia* (Wells, 1964a) the wall material deposited between the opposing membrane faces appears as a series of layers. Bracker and Butler present evidence that in their material the substance composing the dolipore differs physically and chemically from the walls (see Fig. 4) and that it is ap-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:ently not rigid and, even in old hyphae, will disperse if the confining ,sma membrane is ruptured.

Both sides of the dolipore in *C. solani* are invested by a membranous ɔtal pore cap—the parenthesome (Moore and McAlear, 1962b)—that is nfluent with portions of the endoplasmic reticulum that lie next to the ıin portion of the septum (Figs. 4 and 6). The parenthesomes observed *C. solani* are perforated (Fig. 6) [as are those in *Polystictus* (Girbardt, '61)] and offer "little, if any, impedance to protoplasmic streaming"; clear migration was not reported. Similar discontinuous parenthesomes .ve been observed in association with the dolipore septa of the non-karyotic hyphae of *Armillaria mellea* rhizmorphs (Fig. 4).

In a number of dikaryons, however, the parenthesome-dolipore system 'esents a different aspect. Observations of the mycelia of a number of uiting bodies (Moore and McAlear, 1962b) suggest a dolipore composed ' wall material and capped by more or less isolated parenthesomes that :nerally lack pores (Figs. 5 and 7) and in *Exidia* sp. (Moore, 1964d) :sides the definitely discrete and entire parenthesomes there is clear /idence of a plug of electron-dense material blocking both openings of the ɔlipore; such plugs are also evident in *Polystictus* (Girbardt, 1961).

The critical difference in these apparently conflicting sets of observations . the karyotic state of the hyphae. Bracker and Butler's imperfect strain of '. *solani* is nondikaryotic and "has not yet been connected with a Basidio-ıycete sexual stage." The material examined by Moore and McAlear was aken from basidiocarps and may be presumed, therefore, to be dikaryotic. 'rom the extensive studies of Snider and Raper (1958) it is known that ıuclei can migrate readily in the monokaryon of *Schizophyllum commune.* n response to Moore and McAlear's contention (1962b) that according to heir interpretation the "basidiomycete septum . . . would appear to pro-ıibit nuclear passage while maintaining humoural continuity," Snider

FIG. 3. Ascomycete septum from ascogenous hyphae in *Mollisia* sp. Note the plug ɔf electron dense material in the pore. Magnification: × 100,000.

FIGS. 4 and 5. Basidiomycete septa. Magnification × 100,000. Fig. 4. *Armillaria mellea* homokaryotic rhizomorph cells. From Roskin and McAlear (1964). Fig. 5. *Dacrymyces deliquescens* var. *minor* dikaryotic basidiocarp cells; from Moore and McAlear (1962b). The parenthesomes (*p*) are perforate in Fig. 4 and show evidence of continuity with the endoplasmic reticulum (*er*); they are entire in Fig. 5, the endoplasmic reticulum (arrows) being represented by small vesicular elements. The dolipore in Fig. 4, unlike the rest of the septum, is composed of an electron-dense material whereas in Fig. 5 it has the same appearance as the walls (*w*). The interparenthesome region has a different appearance than the extraparenthesome cytoplasm: In Fig. 4 there is an electron-light material (*1-1*), the other dark (*2-2*), and a third of intermediate appearance (*3-3*), within the pore. Finally, it is interesting to note that in both these figures there is a central zone in the middle of the pore between (*2-2*) and (*3-3*), respectively.

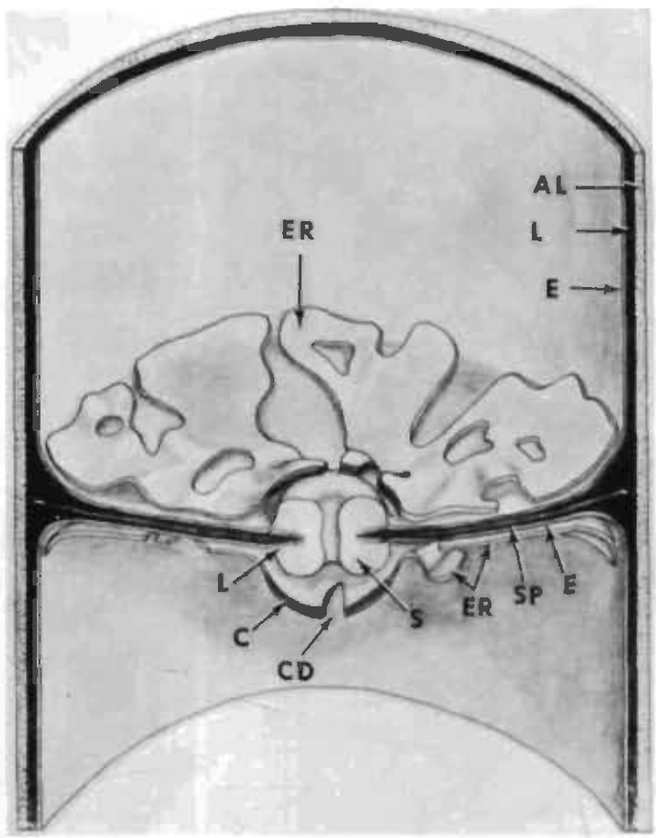


FIG. 6. Interpretation of the septum of *Corticium solani* (*Rhizoctonia* state). Similar septa have been observed in *Armillaria mella* rhizomorphs, *Polystictus versicolor* hyphae, in early mycelial development in *Calvatia gigantea* and *Schizophyllum commune* basidiocarps. From Bracker and Butler (1963). ER, endoplasmic reticulum; AL and L, wall elements; E-plasma membrane; C and CD, pore cap elements; L, S, and SP, septum elements.

(1963) set up a comparable series of exhaustive experiments designed to test the possibility of nuclear migration in *S. commune* dikaryons; his results were negative. Further, Giesy and Day (1965) have studied septal pores of *Coprinus lagopus* heterokaryons in which nuclear migration was thought to be occurring. Their electron micrographs show the apparent conversion of complex dolipore septa to simple septa. They believe that the B mating locus plays a key role in this conversion and that these reduced pores may

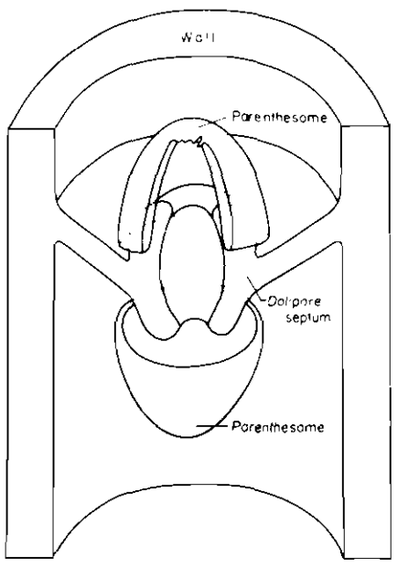


FIG. 7. Interpretation of the basidiomycete septum. Septa of this form have been reported from basidiocarps of *Auricularia, Calvatia, Dacrymyces, Exidia, Merulius,* and *Polyporus* and are suggested in mature *Calvatia gigantea* mycelium. Redrawn from Moore and McAlear (1962b).

facilitate the rapid movement of migrating nuclei. Their micrographs show nuclei and other cytoplasmic components in such pores. Taken together the evidence of these studies would seem to support the hypothesis that there has evolved in the Basidiomycetes a dominant hyphal type, the dikaryon, which is functionally diploid: that the septum in the primary mycelium while of the same general morphology is unstable and subject to being reduced to a form that mimics the ascomycete septum and which readily permits nuclear migration and that the pairing of contrasting nuclei causes the formation of stable dolipore septa in the secondary and tertiary mycelia. This hypothesis proposes that the evolutionary significance of the doliporeparenthesome septal complex is to maintain functional diploidy, particularly as the dikaryon is frequently uniquely characterized by clamp connections whose purported purpose is to ensure the perpetuation of the dikaryotic condition. The strict limitation of clamp connections to dikaryotic hyphae provides clear phenotypic evidence that the pairing of nuclei, which may or may not be of opposite mating types, can effect in these basidiomycetes a profound morphological change such as in unknown for the haploid homokaryon. It seems eminently reasonable, therefore, to sup-

pose that microanatomical changes might also occur in these hyphae promote and sustain the dikaryotic condition. Such potential differences a clearly evident from a comparison of Figs. 4 and 5 and also from Bene (1963).

The above observations on basidiomycete septa concern species of t Tremellales (*sensu* Martin, 1952) and the Holobasidiomycetes. T dolipore–parenthesome septum, however, is still unreported from either t Ustilaginales or the Uredinales (Ehrlich and Ehrlich, 1963; Moore, 196 Moore and McAlear, 1961b).

D. Somatic Division of the Nucleus

The nuclei of fungi lie near the limits of resolution of the light microsc and their behavior during somatic division has engendered consideral discussion as to whether it is classically mitotic. Whether using time-la or regular microscopy it is consistantly described that, with the excepti of *Basidiobolus* (Robinow, 1963), the nuclei behave amorphously and a parently separate like pulled taffy (see Robinow and Bakerspigel, Chap 6).

In the electron microscope mitosis is characterized by the dissociation the nuclear envelope, the condensation of the chromosomes, and the atta ment of spindle fibers to kinetochores (see Harris and Mazia, 1962). Th attributes have not yet been reported for somatically dividing nuclei fungi. Rather, the nuclear envelope is persistant and the electron mic graphs of *Saccharomyces* (Hashimoto *et al.*, 1958, 1959), *Schizos charomyces* (Conti and Naylor, 1960), and *Rhodotorula* (Thyagara *et al.*, 1962) show the nuclei to become dumbbell-shaped as the nucl envelope is drawn out between the separating daughter nuclei (the c relative light micrographs in these reports are quite comparable to simi studies of somatic nuclear division in other fungi). Relating these and otl studies with observations of *Cordyceps militaris*, Moore (1964a) propo a model for what he terms karyochorisis (nuclear sundrance). The electr micrographs of *Cordyceps* nuclei suggest that separation is initiated by invagination of the inner nuclear membrane (Fig. 8d) to partition the n cleoplasm into subunits termed karyomes. These may number two or m and are bounded by the outer element of the nuclear envelope (Fig. 8 Subsequently, the outer nuclear membrane invaginates to separate karyomes into daughter nuclei (Fig. 8f). This same sequence of event believed to part the nexus between separating yeast nuclei (Fig. 8g).

A similar sequence has been reported by Gantt and Arnott (1963) initial stages of chloroplast division in the fern *Matteuccia*, and it may a occur in fungal mitochondria (Moore 1964a). On the basis of these obs

tions Moore (1964a) suggests that karyochorisis "may represent only special example of a general phenomenon of division of double membrane ganelles in primitive cells [and that] if further similar examples of division these organelles can be established they may provide, in lieu of fossil vidence, an insight into early cellular evolution in the protistian cell."

III. SPOROGENESIS

A. Motile Cells

In *Allomyces macrogynus* (Blondel and Turian, 1960) prior to initiation f gamete maturation (the "lipid-crown" stage of light microscopy) the ametangial cytoplasm contains many scattered small vesicles and cisternae, uclei that are surrounded by unbounded lipid inclusions, and an abundance f somewhat irregularly distributed ribosomes. During the first half-hour of amete maturation (initiated by immersing the gametangia in a hypertonic olution) the size and number of vesicles increases, the areas of low bosome density become membrane limited, and within the areas of high bosome density there appear large vesicles. In the next quarter hour iere develops a concentration of lipid inclusions, mitochondria, ribosomes, nd small vesicles in the vicinity of each nucleus. Separating these developing cytoplasmic islands are relatively clear areas of protoplasm bounded y plasma membranes that probably arise from vesicle fusion. By maturity he ribosomes have become aggregated into the nuclear cap—a crescent-haped mass, in section, partially hooding the nucleus and bounded on its ibnuclear side by a double membrane of undetermined origin. Outside of his complex are mitochondria, vesicles, and lipid bodies. Sections of flagella re most frequently observed in the spaces between gametes; their ontogeny s unreported.

A similar sequence of events has been observed during zoospore formation in *A. javanicus* (Moore, 1964c). Cleavage is initiated by the appearance of small aggregates of vesicles of an electron density markedly greater han that of the general endomembranes. The source of these vesicles is resently unknown although the complete absence of a Golgi dictyosome in his fungus eliminates the vesicles of this organelle as one possibility. [The Blastocladiales, unlike other orders of aquatic phycomycetes, show no evidence of a Golgi dictyosome, a deficiency shared by the aplanatae (Moore, 1964d).] These vesicles fuse into planes that in micrographs appear as thick, electron-dense channels filled with some sort of gel and which in their growth progressively segment the cytoplasm. Their continued fusing with one another and eventually with the plasma membrane finally divides the cytoplasm into a number of uninucleate cells. During the final stages of this

cellulation the nuclear cap is formed. Three components go into its synthesis: (1) ER (endoplasmic reticulum) elements at one terminus of which are attached (2) electron-dense filled vesicles, and (3) mitochondria. It appears that the ER elements and the attached vesicles assume a perinuclear orientation, that the ER elements then fuse and the contents of the attached vesicles become the cap matrix. The mitochondria lose their random distribution and become embedded in the cap membrane. This last at maturity is composed of a continuous double membrane, albeit convoluted from the mitochondrial pockets, that is attached at a few circumferential points to the outer membrane of the nuclear envelope. This limited attachment leaves most of the inflated hem of the cap free and consequently the cap contents are contiguous with the general cytoplasm. The function of the mitochondria in this development may be to supply the energy necessary for cap formation. Final zoospore rounding-up is effected by the separation of the membrane sheets between the developing spores, probably by dissolution of the intercellular gel. Flagella formation has not been observed. Unlike during ascospore formation all the sporangial cytoplasm and plasma membrane become incorporated into zoospores, leaving no residuum of particulate material.

Renaud and Swift (1964) have studied the formation of basal bodies and flagella in the gametes of *A. arbuscula*. Though *Allomyces* is uniflagellate they found that at the time the hyphal tip started its differentiation into a gametangium, centrioles were found to exist in pairs. One of these maintains its size while the other elongates distally to more than three times its original length to become the basal body of the future gamete. (These basal bodies are not believed to arise *de novo* as a "small pre-existing centriole," approximately 160 mμ long, was found in an inpocketing of the nuclear membrane in the somatic hyphae.) Transferring cultures in this stage of development to distilled water caused the initiation of gametogenesis and flagella formation. One of the first changes observed was the blebbing of numerous small vesicles from the plasma membrane of the gametangium which apparently migrate into contact with the basal body and there fuse to form a large primary vesicle. They report that the flagellum initiates growth by invaginating into this vesicle and that as the flagellum grows the primary vesicle enlarges by fusing with secondary vesicles until at maturity it becomes the flagellar sheath.

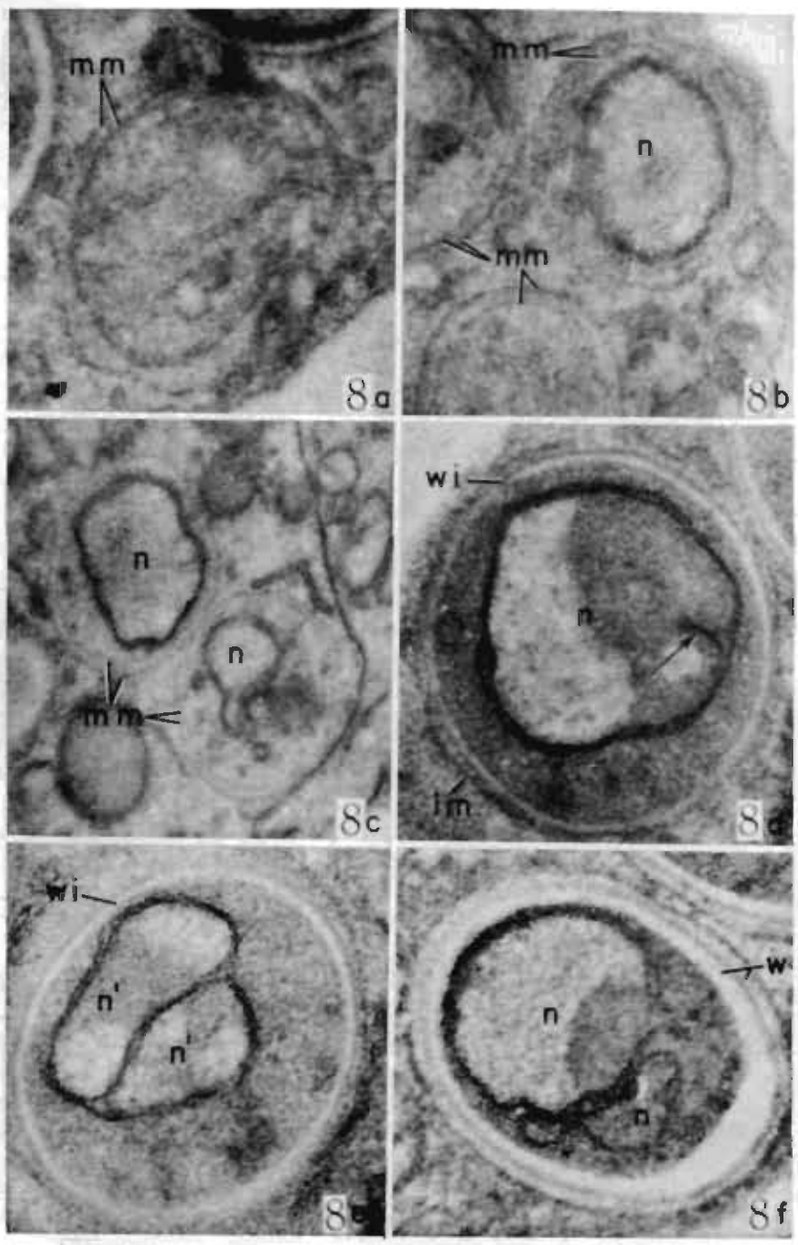
The zoospore of *Blastocladiella emersonii* (Cantino *et al.*, 1963) is similar in the structure and relation of the nucleus, nucleolus, and nuclear cap but differs in possessing a single large eccentric mitochondrion surrounding the base of the flagellum, the presence of small cytoplasmic "gamma" organelles, and a banded apparatus in association with the basal portion of the flagellum. Cantino and co-workers interpret the double

brane bounding the nuclear-cap as being nonporous and continuous the outer membrane of the nuclear envelope. In contrast, the micro-hs of this membrane in the gametes of *A. macrogynus* (Turian and nberger, 1956; Blondel and Turian, 1960) and *A. javanicus* (Moore, b) suggest only partial confluency with the nuclear envelope.

B. Ascospores

ıe sequence of events in ascospore formation may be partially recon-ted from a collation of several studies of Euascomycete ultrastructure. whole ontogeny, initiated by karyogamy, occurs within a single cell, ıscus. The diploid nucleoplasm may appear either homogeneous, as in *desmis* (Moore, 1963a) or, like the somatic nuclei, composed of light dark regions, as in *Dasyscyphus* (Moore and McAlear, 1962a); the ctive ascal cytoplasms of these species display either a number of les, endoplasmic reticulum, and mitochondria, or appear homogeneous; y, in *Ascodesmis* there occur endoplasmic continuities with the nucleus, ıot in *Dasyscyphus*. [Similar continuities have been reported for somatic i in *Mollisia* (Moore and McAlear, 1961c), *Neobulgaria* (Moore and lear, 1963a), and *Stilbum* (McAlear and Edwards, 1959) and sug-d for mitochondria in *Ascodesmis* (Moore, 1963a) and *Uromyces* ore and McAlear, 1963b).] Quite possibly the fusion-nucleus ascus n in *Ascodesmis* represents an earlier stage than the one shown for *scyphus*. Meiosis and a somatic nuclear division immediately follow n. During the division phase as seen in *Dasyscyphus* the nuclear en-ıe dissociates and very little fine structure is evident, after which nuclear lopes, endoplasmic reticulum, and electron-dense inclusions reappear. re-emergent nuclei have light and dark regions, and before separation ısing pores in the respective daughter nuclear envelopes are evident. 8-nucleate phase representing the end of free nuclear division has been in *Ascodesmis*.

ıe development of the ascospore wall is poorly understood. In *Cordy-militaris* the configuration of the endoplasmic reticulum suggests that ay play a role. If endoplasmic reticular elements were to form the ix membranes (*mm*, Fig. 8a–c) between whose surfaces wall material oduced (similar to the opposing membrane faces during septum forma-), it would explain the origin of both the plasma membrane and the in-ng membrane (*im*, Fig. 8d) (and seen also in *Ascodesmis*) of the erializing ascospore. The developing spore walls (*wi*, Fig. 8d, e) rapidly ten, apparently as a series of smooth shells, for, again like the septa, appear layered in section (*w*, Fig. 8f); six such layers have been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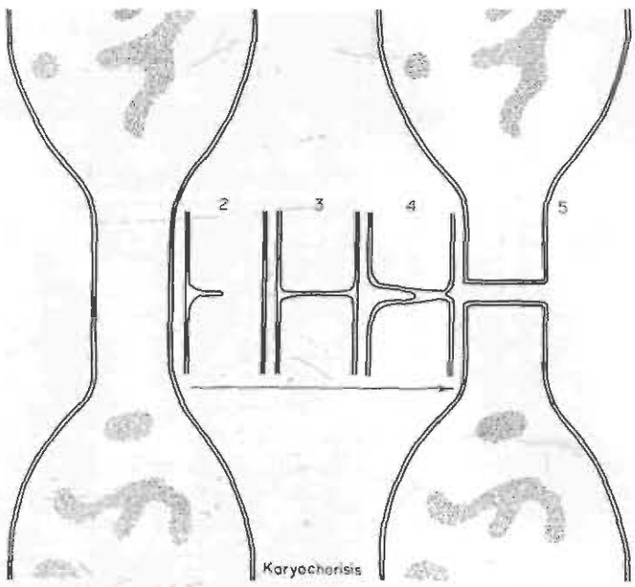
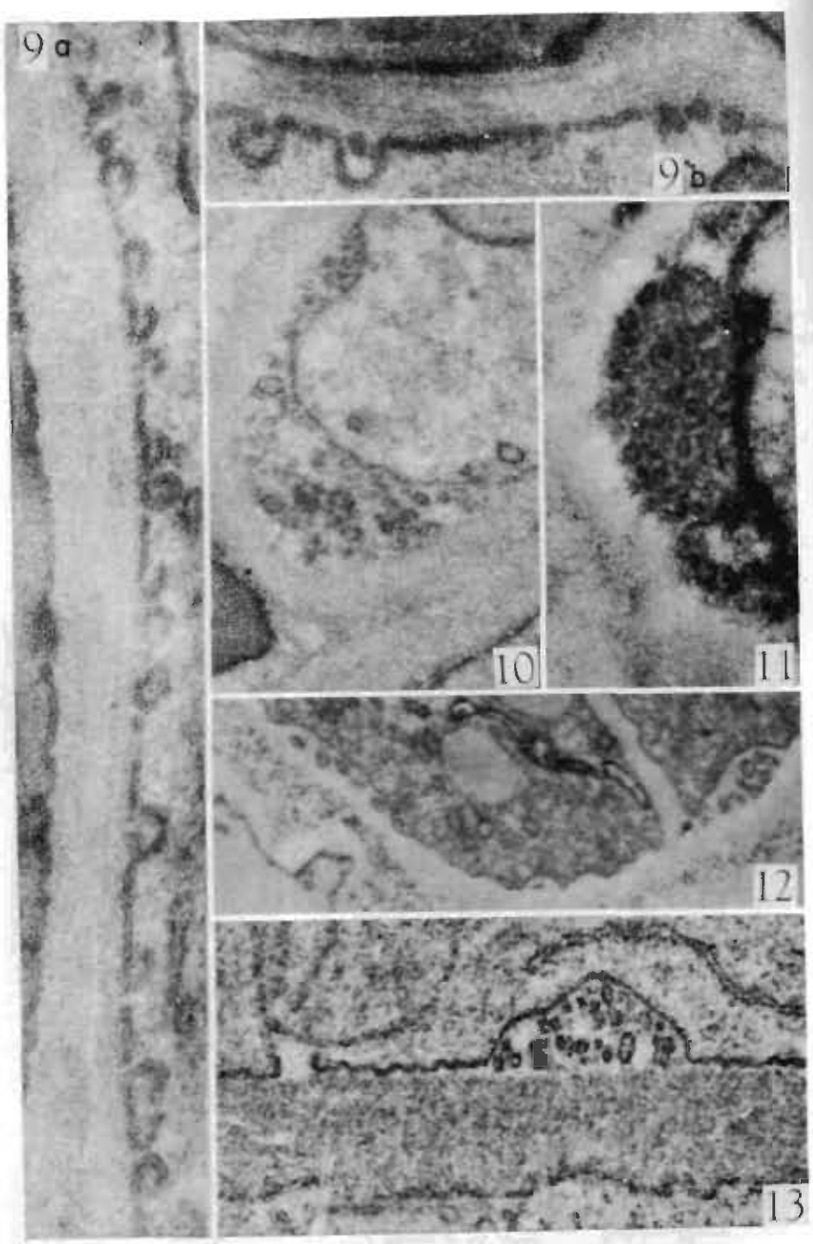


FIG. 8g(1–5). Karyochorisis—interpretation of fungal somatic nuclear division. 1. Isthmus between separating daughter nuclei. Prior to this time or during it the chromatin material (shaded) has replicated and separated and the nucleus is briefly in a genetically $n + n$ state. 2. Initiation of invagination of the inner nuclear membrane. 3. Completion of inner nuclear membrane invagination partitions the nucleoplasm into karyomes that are geneticaly n but still contained within the common boundry of the outer nuclear membrane. 4. Invagination of the outer nuclear membrane initiates karyome separation. 5. Completion of outer nuclear membrane invagination separates the karyomes into daughter nuclei. (From Moore, 1964a.)

counted in *Ascodesmis*. In *Ascodesmis* a secondary, electron-opaque, sculptured wall is produced. The material composing this outer spore coat passes out through the primary wall and accumulates in a reticular pattern on the surface beneath the investing membrane. The available evidence suggests

FIG. 8. Ascosporogenesis and karyochorisis in *Cordyceps militaris*. In (a–c) a membrane matrix (*mm*) forms the outline of the new ascospores; in (d and e) wall initials (*wi*) have appeared and in (d) the investing membrane (*im*) is evident; in (f) the new wall (*w*) is prominent and at this stage is composed of two layers. Nuclear division is initiated (d) by an invagination of the inner membrane of the nuclear envelope (*arrow*) which proceeds (e) to divide the nucleoplasm into karyomes (*n'*, *n'*); finally (f) the outer membrane invaginates to complete karyochorisis and produce the daughter nuclei (*n*, *n*). Figs. 8d and e are from Moore (1964a). Magnifications: a, × 75,000; b, × 60,000; c, × 40,000; d, × 90,000; e, × 90,000; f, × 60,000.


9a
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12
13

hat the surface pattern may be a reflection of an endoplasmic reticular net ressed against the underlying plasma membrane.

The epiplasm, which is the enucleated ascal cytoplasm left after the scospore nuclei and other organelles are encapsulated, breaks down as he spores mature. In *Ascodesmis* it appears to contain only a suspension of ibosomes by the time the spores are ready to be discharged.

C. *Aeciospores*

In basidiomycetes we have some information on aeciospore formation in *Puccinia podophylli* (Moore, 1963b). The aeciospores are produced in the outer region of the aecium in a manner similar to cell division in the primordium (Section II, B) but unlike the primordial cells they display no evidence of Golgi membranes (Moore, 1963c). However the spore walls are much thicker than the primordial cell walls and are composed of an electron-light, apparently microfibrillar, material within which are embedded protruding pegs that also are electron-light but homogeneous. Between the pegs there is an electron-dense material that appears similar to some of the intercellular material observed in the primordium. Similar observations have been made in *Uromyces* (Moore and McAlear, 1961b). The disjunctors, like the spores, are binucleate, probably disk-shaped, and appear to be cut out of developing spore cells secondarily. The reason for their eventual breakdown is unknown. Also, within the aeciospores there is a much greater quantity of electron-dense storage material than within the primordial cells.

IV. LOMASOMES

Lomasomes (Figs. 9–13) occur between the cell wall and plasma membrane and may be found on any part of the hyphal wall including septa (e.g., Bracker and Butler, 1963). They were first observed by Girbardt

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FIGS. 9–13. Lomasomes.

FIG. 9. Lomasome initiation in *Armillaria mellea:* (a) the plasma membrane has fragmented and shows various degrees of inrolling; (b) a somewhat later stage in which (left) the plasma membrane has re-formed, isolating particles outside the protoplast. Magnification: × 108,000. From Roskin and McAlear (1964).

FIG. 10. *Puccinia podophylli.* Magnification: × 50,000.

FIG. 11. *Merulius tremellosus.* From Moore and McAlear (1961a). Magnification: × 100,000.

FIG. 12. *Neobulgaria pura.* From Moore and McAlear (1963a). Magnification: × 40,000.

FIG. 13. *Schizophyllum commune.* From Moore and McAlear (1961a). Magnification: × 50,000.

(1961) in the basidiomycete *Polystictus versicolor*; the term, meaning "border body," was coined by Moore and McAlear (1961a) following observations of their occurrence in all classes of the true fungi. Subsequent reports have considerably enlarged their range of distribution in the Eumycota (Beneke, 1963; Berlin and Bowen, 1964; Bracker and Butler, 1963; Hawker and Abbott, 1963; Peyton and Bowen, 1963) (Figs. 9–13). In addition Bouck (1962) illustrates "various pockets and spheres" at intervals along the walls of the red alga *Lomentaria* and Manocha and Shaw (1964) report lomasomes in mesophyll cells of rust resistant "Khapli" wheat.

Lomasomes appear to be composed of a matrix of electron-light material, similar in appearance to that of the wall, within which appear vesicles (Moore and McAlear, 1961a; Girbardt, 1961) or tubules (Peyton and Bowen, 1963) of electron-dense material. In yeasts their three dimensional appearance is that of regular ingots scattered over the inner wall surface Moor and Muhletholer (1963). The mechanism of lomasome formation is not known, nor is it known whether all structures that appear lomasome-like are homologous. Moore and McAlear (1961a) proposed a secretion process of lomasome formation whereby vesicles move out of the cytoplasm and fuse with the plasma membrane and Manocha and Shaw (1964) conjecture "Whether or not the rapidity and efficiency with which foreign substances can be excreted from mesophyll cells via vesicles have any connexion with rust resistance. . . ." Girbardt (1961) believes that their function is to increase surface area. Recent micrographs by Cunningham (1963) of *Collybia velutipes* and by Roskin and McAlear (1964) of *Armillaria mellea* show portions of the plasma membrane becoming segmented and partially inrolled (Fig. 9a,b). McAlear (1964) postulates that the segments curl up and close on themselves and that subsequently the plasma membrane re-forms beneath the so-formed vesicles, effectively isolating them to the outside of the protoplast. This latter interpretation would agree with Peyton and Bowen's (1963) belief that lomasomes are elaborations of the plasma membrane.

From the available evidence of a limited but widely representative sample it appears that lomasomes are characteristic of fungal cells. Our present ignorance of their function, composition, or mode of formation forbids more than speculation as to their phylogenetic significance.

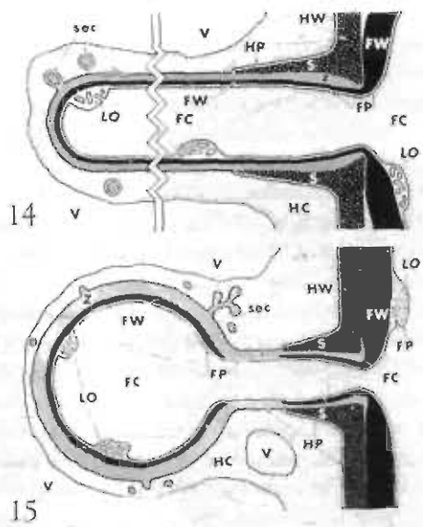
V. INTERACTION WITH HOST PLANTS

The union of fungal and plant cells, whether parasitic or saprobic, is achieved most commonly by penetration of the host cell wall by hyphae. *Peronospora manshurica* (Peyton and Bowen, 1963), the downy mildew of soybeans, forms a ramifying, branched, coenocytic intercellular hyphal

system in the host leaf tissue. Haustoria develop at the points of contact with the host cells. These penetrate the cell wall but do not rupture the plasma membrane even though they ramify extensively. The inviolability of the plasma membrane has also been observed in the host/parasite relationship of *Uromyces caladii/Arisaema triphyllum* (Moore and McAlear, 1961b) and *Puccinia podophylli/Podophyllum peltatum* (Moore, 1964d); it is also suggested in *P. graminis/Triticum* vars. (Ehrlich and Ehrlich, 1963), *Plasmodiophora brassicae/Brassica chinensis* (Yukawa, 1957), and the lichen *Cladonia cristatella* (Moore and McAlear, 1960). The haustorium wall is generally continuous (Fig. 14), but an apparent exception is noted in *Albugo* (Fig. 15). At the point of entry of the haustorium a sheath develops that is apparently an ingrowth of the host cell wall (Figs. 14 and 15). Enveloping the whole haustorium is a relatively dense layer that becomes thicker in the region between the haustorial wall and sheath and which Peyton and Bowen term the "zone of apposition." A comparable investing layer is evident around the haustoria of *Uromyces caladii* (Moore and McAlear, 1961b) and *Albugo candida* (Fig. 15). Similar to these relationships are those suggested in micrographs of *Puccinia graminis* haustoria in *Triticum* cells (Ehrlich and Ehrlich, 1963) and of the mycobiont-phycobiont interaction in certain lichens (Moore and McAlear, 1960).

In both *Peronospora* and *Puccinia,* vesicles have been observed in the region of the host plasma membrane that bounds the haustorium and some have been noted to be continuous with it. Peyton and Bowen (1963) interpret these as derived from the host cytoplasm and fusing with the plasma membrane to release material into the zone of apposition, the same interpretation being applied to *Albugo* (Figs. 14,15). Ehrlich and Ehrlich (1963), however, believe that they are pinched off the plasma membrane and carry material from the "encapsulation" (= zone of apposition) into the host cytoplasm. No choice between these hypotheses can be made as yet.

The effect of *Peronospora* on its host is to cause a progressive reduction in chloroplast size and in number of photosynthetic lamellae. In advance stages of infection the chloroplasts appear necrotic and disorganized and contain a quantity of lipid inclusions. In late infection of *Arisaema* (Moore and McAlear, 1961b) the ground cytoplasm virtually disappears, probably through lysis, and the mitochondria and Golgi complex appear disrupted even though the cytoplasm and organelles of the *Uromyces* haustoria appear normal. Further, the quantity of ribosomes and amount of smooth endoplasmic reticulum is higher in infected cells (Peyton and Bowen, 1964). In contrast to these changes is the effect on algal cells by the fungus partner in lichens (Moore and McAlear, 1960) in which the "invading" haustoria do not adversely affect the host cytoplasm. The small micromorphological difference between the modes of parasitic and symbiotic interaction strengthens the idea that the latter has evolved as a result of a



FIGS. 14 and 15. Diagrams of host–parasite interface in haustorial region: Fig. 14, *Peronospora manshurica* infection of *Glycine max;* Fig. 15, *Albugo candida* infection of *Raphanus sativus*. Intercellular hyphae are on the right and host mesophyll cells on the left. Fungal cytoplasm (*FC*) is bounded by the fungal plasma membrane (*FP*) and lomasomes (*LO*). The fungal cell wall (*FW*) is continuous in *Peronospora* but is interrupted in *Albugo*. The relative positions of the host cell vacuole (*V*), host cytoplasm (*HC*), and host plasma membrane (*HP*) are indicated. The host cell wall (*HW*) terminates in a sheath (*S*). The zone of apposition (*Z*) separates the haustorium from the host plasma membrane. Invaginations of the host plasma membrane and vesicular host cytoplasm are considered evidence for host secretory activity (*soc*). Figure 14 is from Peyton and Bowen (1963); Fig. 15 is from Berlin and Bowen (1964).

balance being reached between susceptibility and resistance in a parasitic relationship.

VI. RÉSUMÉ

Some aspects of cell and nuclear division, lomasomes, sporogenesis organelles and the interaction with plants have been examined. The foregoing summary has served to point out that in the fungi, within the bounds of our present knowledge, cell division, including cross-wall formation, is a product of plasma membrane activity. As one progresses from phycomycetes to carpomycetes there is a shift from sparse septation, which is primarily abscissional, to regular septation with central pores, and in the

ransition from ascomycetes to basidiomycetes the septa become highly laborated and the shift is from functional coenocytes to functional di-loids. Further, the plasma membrane appears to play an active role in the ormation of lomasomes—structures presently limited, with two exceptions, o the true fungi and perhaps of phylogenetic significance. Moreover, the lectron microscopy of dividing nuclei offers support to the hypothesis that ungal nuclei can divide by a nonmitotic process, a process described by the erm karyochorisis. Our information on the fine structure of sporogenesis s very sketchy, but even the little available evidence suggests develop-nental changes unknown in other major groups. Finally, the few studies of parasitism and saprobism intimate that these processes differ not so much n microhistology as in the degree of adaptive resistance of the plant partner.

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References

Beneke, E. S. (1963). *Calvatia*, calvacin, and cancer. *Mycologia* **55**:257–270.

Berlin, J. D., and C. C. Bowen. (1964). The host-parasite interface of *Albugo candida* on *Raphanus sativus*. *Am. J. Botany* **51**:445–452.

Blondel, B., and G. Turian. (1960). Relation between basophilia and fine structure of cytoplasm in the fungus *Allomyces macrogynus* Em. *J. Biophys. Biochem. Cytol.* 7:127–134.

Bouck, G. B. (1962). Chromatophore development, pits, and other fine structure in the red alga, *Lomentaria baileyana* (Harv.) Farlow. *J. Cell Biol.* **12**:553–569.

Bracker, C. E., and E. E. Butler. (1963). The ultrastructure and development of septa in hyphae of *Rhizoctonia solani*. *Mycologia* **55**:35–58.

Bracker, C. E., and E. E. Butler. (1964). Function of the septal pore apparatus in *Rhizoctonia solani* during streaming. *J. Cell Biol.* **21**:152–157.

Breslau, A. M., T. J. Hensley, and J. O. Erickson. (1961). Electron microscopy of cultured spherules of *Coccidioides immitis*. *J. Biophys. Biochem. Cytol.* 9:627–637.

Buck, R. C., and J. M. Tisdale. (1962). An electron microscopic study of the development of the cleavage furrow in mammalian cells. *J. Cell Biol.* 13:117–125.

Cantino, E. C., J. S. Lovett, L. V. Leak, and J. Lythgoe. (1963). The single mitochondrion, fine structure, and germination of the spore of *Blastocladiella emersonii*. *J. Gen. Microbiol.* **31**:393–404.

Chapman, G. B. (1959). Electron microscope observations on the behavior of the bacterial cytoplasmic membrane during cellular division. *J. Biophys. Biochem. Cytol.* 6:221–224.

Chapman, G. B., and J. Hillier. (1953). Electron microscopy of ultra-thin sections of bacteria. I. Cellular division in *Bacillus cereus*. *J. Bacteriol.* **66**:362–373.

Conti, S. F., and H. B. Naylor. (1960). Electron microscopy of ultrathin sections of *Schizosaccharomyces octosporus*. II. Morphological and cytological changes preceding ascospore formation. *J. Bacteriol.* **79**:331–340.

Cunningham, W. P. (1963). Personal communication.

Dickson, M. R. (1963). A study of the fine structure of the spores of *Pithomyces chartarum*. *N. Zealand J. Botany* **1**:381–388.

Ehrlich, H. G., and M. A. Ehrlich. (1963). Electron microscopy of the host-parasite relationships in stem rust of wheat. *Am. J. Botany* **50**:123–130.

Gantt, E., and H. J. Arnott. (1963). Chloroplast division in the gametophyte of the fern *Matteuccia struthiopteris* (L.) Todaro. *J. Cell Biol.* **19**:446–448.

Giesy, R. M., and P. R. Day. (1965). The septal pores of *Coprinus lagopus* (Fr.) sensu Buller in relation to nuclear migration. *Am. J. Botany* **52**(3): in press.

Girbardt, M. (1961). Licht- und elektronenmikroskopische Untersuchungen an *Polystictus versicolor*. II. Die Feinstruktur von Grundplasma und Mitochondrien. *Arch. Mikrobiol.* **39**:351–359.

Glauert, A. M., and D. A. Hopwood. (1961). The fine structure of *Streptomyces violaceoruber* (*S. coelicolor*). III. The walls of the mycelium and spores. *J. Biophys. Biochem. Cytol.* **10**:505–516.

Hagedorn, H. (1960). Elektronenmikroskopische Untersuchungen an *Streptomyces griseus* (Krainsky). *Zentr. Bakteriol., Parasitenk., Abt. II* **113**:234–253.

Harris, P., and D. Mazia. (1962). The finer structure of the mitotic apparatus. *In* "The Interpretation of Ultrastructure" (R. J. C. Harris, ed.), Vol. 1, pp. 279–305. Academic Press, New York.

Hashimoto, T., S. F. Conti, and H. B. Naylor. (1958). Fine structure of microorganisms. III. Electron microscopy of resting and germinating ascospores of *Saccharomyces cerevisiae*. *J. Bacteriol.* **76**:406–416.

Hashimoto, T., S. F. Conti, and H. B. Naylor. (1959). Studies of the fine structure of microorganisms. IV. Observations on budding *Saccharomyces cerevisiae* by light and electron microscopy. *J. Bacteriol.* **77**:344–354.

Hawker, L. E., and P. McV. Abbott. (1963). Fine structure of young vegetative hyphae of *Pythium debaryanum*. *J. Gen. Microbiol.* **30**:401–408.

Kawakami, N., and T. Nehira. (1958). Electron-microscopy of fungi (VIII). Intracellular structure of *Neurospora*. *J. Ferment. Technol.* **36**:421–424.

McAlear, J. H. (1964). Personal communication.

McAlear, J. H., and G. A. Edwards. (1959). Continuity of plasma membrane and nuclear membrane. *Exptl. Cell Res.* **16**:689–692.

Manocha, M. S., and M. Shaw. (1964). Occurrence of lomasomes in mesophyll cells of 'Khapli' wheat. *Nature* **203**:1402–1403.

Martin, G. W. (1952). Revision of the North Central Tremellales. *Iowa State Univ., Studies Nat. Hist.* **19**: No. 3, 1–122.

Meor, H., and K. Mühlethaler. (1963). Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**:609–628.

Moore, R. T. (1963a). Fine structure of mycota. 1. Electron microscopy of the discomycete *Ascodesmis*. *Nova Hedwigia* **5**:263–278.

Moore, R. T. (1963b). Fine structure of mycota. 10. Thallus formation in *Puccinia podophylli* aecia. *Mycologia* **55**:633–642.

Moore, R. T. (1963c). Fine structure of mycota. XI. Occurrence of the Golgi dictyo-

some in the heterobasidiomycete *Puccinia podophylli*. *J. Bacteriol.* **86**:866–871.

Moore, R. T. (1964a). Fine structure of mycota. 12. Karyochorisis—somatic nuclear division—in *Cordyceps militaris*. *Z. Zellforsch.* **63**:921–937.

Moore, R. T. (1964b). The nuclear cap of the phycomycete *Allomyces*. *J. Cell Biol.* **23**:62A.

Moore, R. T. (1964c). Zoospore formation in the phycomycete *Allomyces*. *J. Cell Biol.* **23**:108A–109A.

Moore, R. T. (1964d). Personal observations.

Moore, R. T., and G. B. Chapman. (1959). Observations of the fine structure and modes of growth of a streptomycete. *J. Bacteriol.* **78**:878–885.

Moore, R. T., and J. H. McAlear. (1960). Fine structure of mycota. 2. Demonstration of the haustoria of lichens. *Mycologia* **52**:805–807.

Moore, R. T., and J. H. McAlear. (1961a). Fine structure of mycota. 5. Lomasomes—previously uncharacterized hyphal structures. *Mycologia* **53**:194–200.

Moore, R. T., and J. H. McAlear. (1961b). Fine structure of mycota. 8. On the aecidial stage of *Uromyces caladii*. *Phytopathol. Z.* **42**:297–304.

Moore, R. T., and J. H. McAlear. (1961c). Fine structure of mycota. 3. Reconstruction from skipped serial sections of the nuclear envelope and its continuity with the plasma membrane. *Exptl. Cell Res.* **24**:588–592.

Moore, R. T., and J. H. McAlear. (1962a). [Fine structure of mycota. 6.] Observations on ascospore initiation in the discomycete *Dasyscyphus*. *J. Gen. Microbiol.* **28**:211–218.

Moore, R. T., and J. H. McAlear. (1962b). Fine structure of mycota. 7. Observations on septa of Ascomycetes and Basidiomycetes. *Am. J. Botany* **49**:86–94.

Moore, R. T., and J. H. McAlear. (1963a). Fine structure of mycota. 4. The occurrence of the Golgi dictyosome in the fungus *Neobulgaria pura* (Fr.) Petrak. *J. Cell. Biol.* **16**:131–141.

Moore, R. T., and J. H. McAlear (1963b). Fine structure of mycota. 9. Fungal mitochondria. *J. Ultrastructure Res.* **8**:144–153.

Odor, D. L., and D. F. Renninger. (1960). Polar body formation in the rat oocyte as observed with the electron microscope. *Anat. Record* **137**:13–23.

O'Hern, E. M., and B. S. Henry. (1956). A cytological study of *Coccidioides immitis* by electron microscopy. *J. Bacteriol.* **72**:632–645.

Peyton, G. A., and C. C. Bowen. (1963). The host-parasite interface of *Peronospora manshurica* on *Glycine max*. *Am. J. Botany*. **50**:787–797.

Porter, K. R., and R. D. Machado. (1960). Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip. *J. Biophys. Biochem. Cytol.* **7**:167–180.

Renaud, F. L., and H. Swift. (1964). The development of basal bodies and flagella in *Allomyces arbuscula*. *J. Cell Biol.* **23**:339–354.

Robinow, C. F. (1963). Observations on cell growth, mitosis, and division in the fungus *Basidiobolus ranarum*. *J. Cell Biol.* **17**:123–152.

Roskin, P. S., and J. H. McAlear. (1964). Personal communication.

Shatkin, A. J., and E. L. Tatum. (1959). Electron microscopy of *Neurospora crassa* mycelia. *J. Biophys. Biochem. Cytol.* **6**:423–426.

Snider, P. J. (1963). Personal communication.

Snider, P. J., and J. R. Raper. (1958). Nuclear migration in the basidiomycete *Schizophyllum commune*. *Am. J. Botany* **45**:538–546.

Taplin, D., and H. Blank. (1961). Microscopic morphology of *Trichophyton rubrum*. *J. Invest. Dermatol.* 37:523–528.

Thyagarajan, T. R., S. F. Conti, and H. B. Naylor. (1962). Electron microscopy of *Rhodotorula glutinis*. *J. Bacteriol.* 83:381–394.

Turian, G., and E. Kellenberger. (1956). Ultrastructure du corps paranucleaire, des mitochondries et de la membrane nucleaire des gametes d'*Allomyces macrogynus*. *Exptl. Cell Res.* 11:417–422.

Vernon, T. R. (1955). Spore formation in the genus *Streptomyces*. *Nature* 176:935–936.

Wells, K. (1964a). The basidia of *Exidia nucleata*. I. Ultrastructure. *Mycologia* 56: 327–341.

Wells, K. (1964b). The basidia of *Exidia nucleata*. II. Development. *Am. J. Botany* 51:360–370.

Whaley, W. G., and H. H. Mollenhauer. (1963). The Golgi apparatus and cell plate formation—a postulate. *J. Cell Biol.* 17:216–221.

Yukawa, Y. (1957). Electron microscopic studies on the club-root tissues of crucifers. *Bull. Fac. Agr., Yamaguti Univ.* 8:659–664.

CHAPTER 6

Somatic Nuclei and Forms of Mitosis in Fungi[1]

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I. INTRODUCTION

Resting nuclei of fungi are bounded by a membranous envelope of the usual kind, which is more distinct in electron micrographs than it is during life or in optimally preserved and stained specimens. After fixation the chromatin of resting nuclei appears finely or coarsely granular or filamentous. In most, but not all, instances it is not organized in the form of identifiable chromosomes. Resting fungal nuclei commonly, though again not invariably, contain a single relatively large nucleolus.

It is generally taken for granted that mitosis in fungi follows the same course as in higher organisms, but cytologically acceptable evidence for this belief is hard to find. The majority of fungal cytologists have to this day been mainly interested in the details of meiosis, though lately work is beginning to be done on *mitosis* as well. Thus, Käfer (1961) says, "No analysis of mitosis in *Aspergillus nidulans* has been carried out so far," and a recent book observes, "The details of nuclear division in vegetative hyphae [of *Neurospora*] are rather hard to make out" (Fincham and Day, 1963). Recent work has already taught us that fungal mitoses are of several different kinds and have peculiarities that set them off from the better known ordinary forms of nuclear division. Phase-contrast microscopy now makes fungal nuclei readily visible in living hyphae. Before the advent of this method of examination nuclei were rarely seen, or at least not recorded, in illustrations accompanying studies of living mycelia. It is inter-

[1] The original photomicrographs in this article were supplied by C. F. Robinow.

esting to find that the general recognition of the presence of nuclei in cells of plants and animals preceded recognition of their universal presence in fungi by nearly forty years, despite the fact that since 1858 hematoxylin had been available to help in the search, and even then some materials, such as vegetative cells of ascomycetes, proved refractory (Schmitz, 1879). It took even longer for the mode of division of fungal nuclei to be understood. In some instances that problem continues to resist even phase-contrast microscopy, as for example in *Thanatophorus cucumeris* (Flentje *et al.*, 1963) and *Penicillium* (Robinow, 1963a), were the nuclei fade from view as they enter division.

What follows is conceived as an introduction to several current problems of the cytology of the somatic (vegetative) nuclei of fungi. We are aware that it is premature to attempt a "review" of a subject which is only now beginning to acquire a basis of useful morphological work.

Today it appears to us that fungal cytology faces three principal problems: (1) the basis of the commonly encountered low affinity of somatic fungal chromosomes for ordinary stains if these are used in ordinary ways; (2) the meaning of the peculiar associations which the chromosomes of some species form at metaphase; (3) the nature of the forces or devices which separate sister chromosomes at anaphase. In other words, the frequent absence of a demonstrable spindle apparatus.

II. CYTOCHEMISTRY

Little definite information can be given at present under this heading. The small amount of useful literature on mitosis in fungi is sufficient proof that fungal somatic chromosomes are not easily studied. It has become common practice to treat fungal specimens with hydrochloric acid (1*N* at 60°C) before trying to stain somatic chromosomes with acetoorcein. They stain poorly with hematoxylin, as suggested in a recent paper on cytological studies of *Penicillium notatum* (Pauli, 1956), which is silent about mitotic figures and essentially illustrates only nucleoli. Bromophenol blue-mercuric chloride (Mazia *et al.*, 1953), which stains ordinary chromosomes distinctly, is not helpful in demonstrating them in fungi, and the method of Alfert and Geschwind (1953) for the detection of basic proteins has also given negative results. In all instances it is only, or principally, the nucleoli that accept the stains.

It is clearly desirable that fungal nuclei be isolated so that their chemistry can be properly investigated. It is said that the nuclei of the septaless phycomycetes are easily obtainable by breaking their hyphae in a Waring blendor (Foster, 1956).

III. BEHAVIOR OF CHROMOSOMES AT MITOSIS

A. Chromosome Association

The search for evidence of spindle devices in mitosis, which has given rise to a number of contentious papers, will presumably soon be brought to an end with the help of the electron microscope. But there remains another aspect of mitosis in fungi that has so far received little attention. In many instances metaphase chromosomes seem to enter relationships not easily accounted for in terms of ordinary mitotic behavior. The observed irregularity is most simply expressed by saying that where n is the number of chromosomes in a resting nucleus, only that number of seemingly single chromosomes is found at late metaphase, instead of $2n$. Thus 16 chromosomes ought to be visible at late metaphase of mitosis in *Aspergillus nidulans,* but we are not aware that this number has, so far, been seen. Our experience with other aspergilli and several *Penicillium* species suggests that a smaller number of separately visible elements will eventually be found. Three examples will be given. In *Neurospora* (Bakerspigel, 1959b,c) rosettes or asterisks are found at metaphase which are composed of 6 or 7 arms instead of the expected 12 or 14. In *Penicillium,* where many micrographs have been collected (by Robinow, unpublished), *rings* are formed early in metaphase. Each ring consists of a pair of concave chromatids facing each other. Later, contact is lost at one end, the two chromatids open out and stretch while remaining in touch at the other end. A filament results which at first glance suggests a single long chromatid. At anaphase the thread breaks at the point where end-to-end contact between the members of the original pair had until now been retained. This accounts for the puzzling fact that late in metaphase four seemingly single chromatids are found although that is the number received by *each* daughter nucleus at telophase (for a tentative diagram of this process see Robinow, 1963a). In *Lipomyces* (Robinow, 1961), there is a similar decline of the number of separately visible chromatids during mitosis. Ten to twelve may be counted at early metaphase. By late metaphase these have been "reduced" to 5 or 6, presumably again by temporary associations of sister chromatids which are broken only in late anaphase. Discussion of the implications of these findings must be deferred until they have been more completely documented.

There is a further reason why the behavior of the chromosomes at metaphase deserves close attention. In several species, including *Saccharomyces* (McClary *et al.,* 1957), *Allomyces* (Wilson, 1952), *Aspergillus nidulans* (Elliott, 1960), and most recently *Pythium debaryanum* (Sansome, 1963),

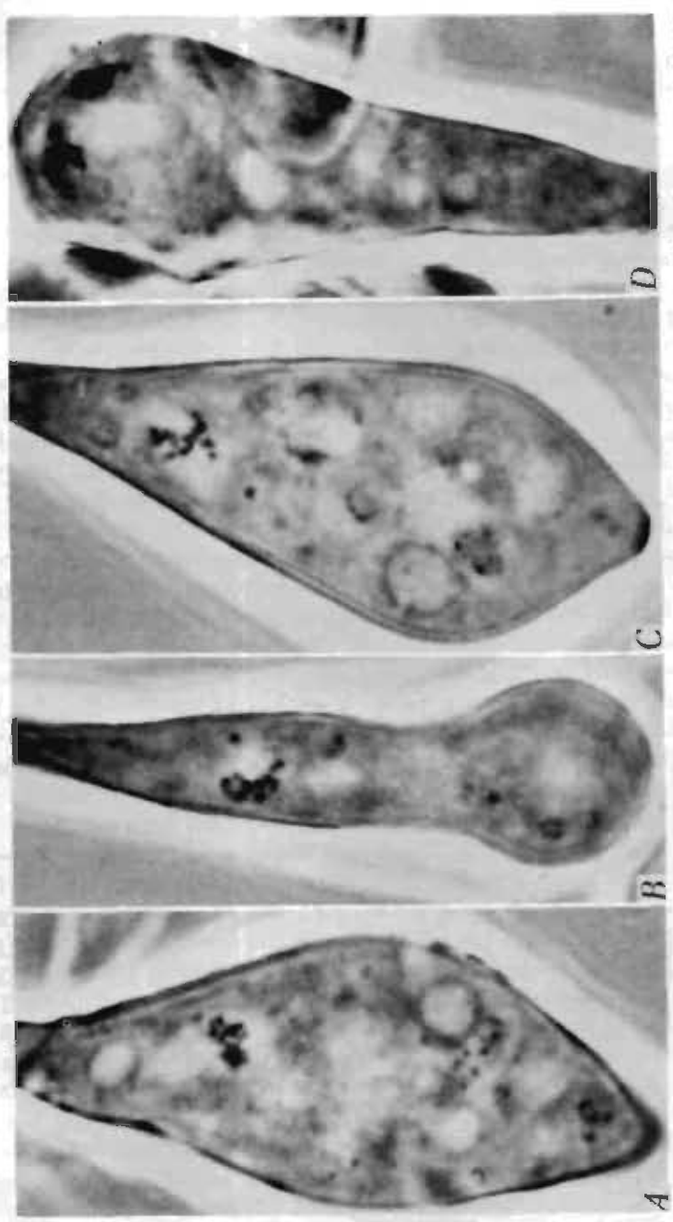


FIG. 1. (A–D) Three metaphases and one anaphase of somatic mitoses of *Allomyces macrogynus*. The chromosome patterns are not unlike those found by Wilson (1952) in meiotic nuclei. Helly, Feulgen, acetocarmine, phase contrast.

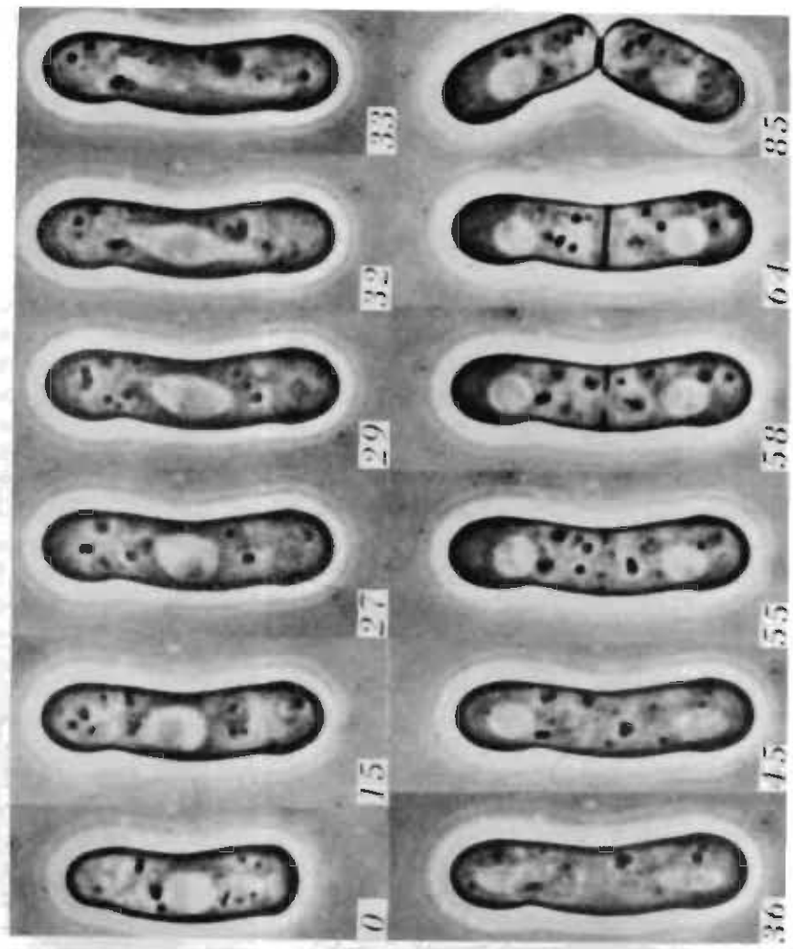
neiotic configurations have been described in conventional terminology, ılthough the behavior of the chromosomes of the same species at mitosis ıas not (or had not then) been described. Where information on both ɔhases is available, as in *Allomyces,* a surprising similarity between them s apparent (Fig. 1A–D). This is not to suggest that there is no meiosis in *Allomyces* at the point of the life cycle where it is now believed to take ɔlace (Wilson, 1952), but it opens the possibility that the process of neiosis in *Allomyces* may not be an entirely conventional one. On the norphological plane this still appears to be so in bakers' yeast, which is dealt with in Section VII of this chapter.

B. Chromosome Movements

(See also Notes Added in Proof on p. 139)

Behind the tentative, dotted, stippled rays that converge on metaphase chromosomes in many illustrations lies uncertainty about the true nature of the mitotic apparatus. The present situation is poignantly foreshadowed in two papers by Olive (1906, 1907), concerned with mitosis in *Empusa* and *Basidiobolus.* Both studies were carried out by the best methods current at the time (Flemming fixation, paraffin sections, triple staining), but they gave different results. No mitotic configurations, and no spindle apparatus, could be detected in dividing nuclei of *Empusa,* whereas spindles and metaphase plates of conventional distinctness were easily seen in *Basidiobolus.* The care with which this work was done compels us to take its failures and its successes equally seriously. Today we are facing the same dilemma. The mitotic nucleus of *Empusa* is still reported to be without a spindle (Robinow, 1963b). None has been found in dividing hyphal nuclei of *Mucor* (Robinow, 1957), *Saprolegnia* (Bakerspigel, 1960; Smith, 1923), *Conidiobolus* (Robinow, 1963b), *Neurospora* (Bakerspigel, 1959a; Somers *et al.,* 1960; Ward and Ciurysek, 1962), *Aspergillus* (Robinow, 1964), *Penicillium* (Robinow, 1964), *Lipomyces* (Robinow, 1961), or *Helminthosporium* (Robinow, 1964) (where spindles are well developed during ascospore formation) (Knox-Davies and Dickson, 1960).

Autonomous chromosome movements are not unknown to cytologists. They have been reported in the radiolarian *Aulacantha* (Grell, 1953) and, many times, in *Euglena* (Leedale, 1958). However, those who feel that the unequivocal demonstration of centromeres (by genetic means) logically demands the presence of spindle fibers, will welcome the fact that clear morphological evidence of spindle devices has now been found (or rediscovered) in somatic mitoses of certain fungi and that techniques of electron microscopy are now available that may settle before long the question of the mechanics of mitosis also in species which at present seem to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ack spindles. Three examples of spindles have recently been described:

1. In *Polystictus,* a basidiomycete, Girbardt's (1962) meticulous investigations have established the emergence of a thin Feulgen-negative cord "Zentralstrang") by the side of the clumped Feulgen-positive metaphase hromosomes. The chromosomes appear to become adsorbed to the cord nd to invest it completely at anaphase. A Feulgen-positive cord results in vhich chromosomes can no longer be separately recognized. This stretches it anaphase and is broken into two pieces in the manner recently described n *Schizophyllum* (Bakerspigel, 1959a). Numerous illustrations in the iterature content themselves with a rendering of this breaking cord as the ole token of mitosis, but true cytology asks for completeness. An electron *nicrograph of a section of the "Zentralstrang," kindly sent to the writers by* Dr. M. Girbardt, shows this organelle to be fibrous. To our knowledge this is he first time that a fibrous mitotic motor device in a fungus has been seen n the electron microscope. Recently Robinow discovered essentially the same kind of motor cord in the fission yeast *Schizosaccharomyces versatilis* (Fig. 2). First seen in a living, dividing nucleus with phase-contrast microscopy, the cord was later demonstrated also in nuclei of that yeast

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FIG. 2. Time-lapse photomicrographs of mitosis and cell division in *Schizosaccharomyces versatilis.* Numbers indicate minutes since the first picture was taken. Phase contrast microscopy of a slide culture in 21% gelatin containing 0.5% yeast extract and 2.0% glucose. Magnification: × 1870.

Note that the cell has grown during the interval between the first and the second picture. At minute 27 the nucleus is larger than it was at the start and a straight fiber is seen by the side of the nucleolus. In the next two pictures the fiber becomes longer and appears to push the poles of the nucleus apart from the inside. The disintegrating nucleolus cannot follow this movement as rapidly as the rest of the nuclear contents. This accounts for the spindle shape of the expanding nucleus. However, the inertia of the nucleolar material is soon largely overcome. Observations on numerous other dividing nuclei, including both living and stained ones, have shown that for a brief moment after the stage of minute 32 + the nucleus, including most of the nucleolar material, is pulled out into a long narrow shape with two masses of nucleolar material at each end and one immovable remainder in the middle (Fig. 3). Immediately after this transformation, and with elongation continuing, the nucleus snaps in two. The images of the daughter nuclei at minute 33 are blurred because of the speed with which the nuclei were moving apart at this moment. The four pictures covering the time from minute 27 to minute 33 were taken as quickly as was compatible with refocusing and changing of negatives by hand after each exposure. The intervals between the inner members of this series are therefore not known with precision. Note that the many dark granules in the cell have changed their relative positions but little during the interval between the last two pictures of this sequence although their constellations in any other two pictures are widely dissimilar. This is either because the interval between these pictures was especially short or that the cytoplasm had become stiffer, as it does at metaphase in the fungus *Basidiobolus* (Robinow, 1963b).

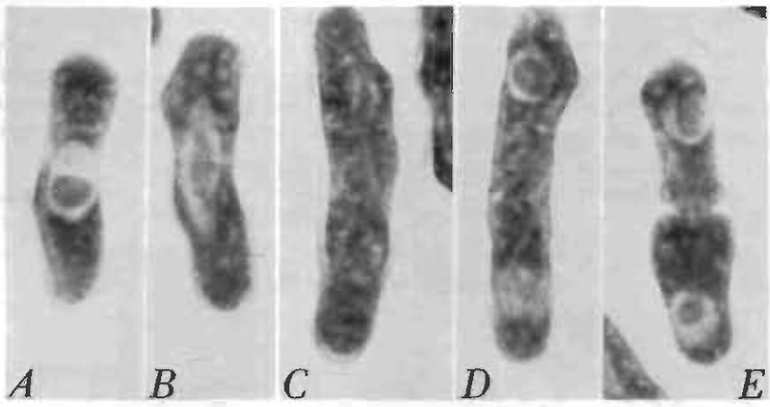

FIG. 3. *Schizosaccharomyces versatilis.* (A–C) The behavior of the nucleolus during mitosis. (D and E) The emergence of the daughter nuclei and the beginning of cell division. Helly, acetocarmine. The technique used here is unsuitable for the demonstration of the spindle fiber in dividing nuclei of this yeast.

with the help of the bromophenol blue-mercuric chloride stain. It closely resembles the spindle of meiosis in *S. octosporus* that was well illustrated by Guilliermond (1917). *S. versatilis* resembles *S. pombe*, and it is likely that a recent, otherwise accurate, account (Schopfer *et al.*, 1963) which re-

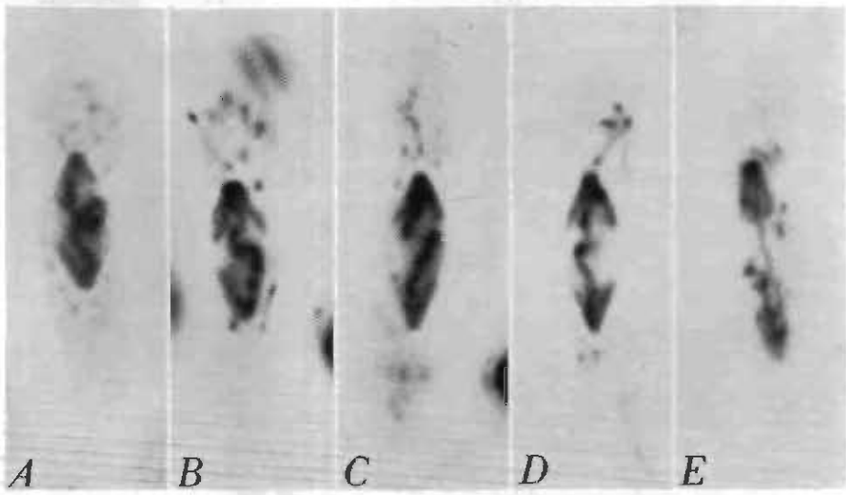

FIG. 4. *Schizosaccharomyces versatilis.* Disengaging chromosomes at metaphase and anaphase of mitosis. Helly, HCl, Giemsa. Magnification: × 3600.

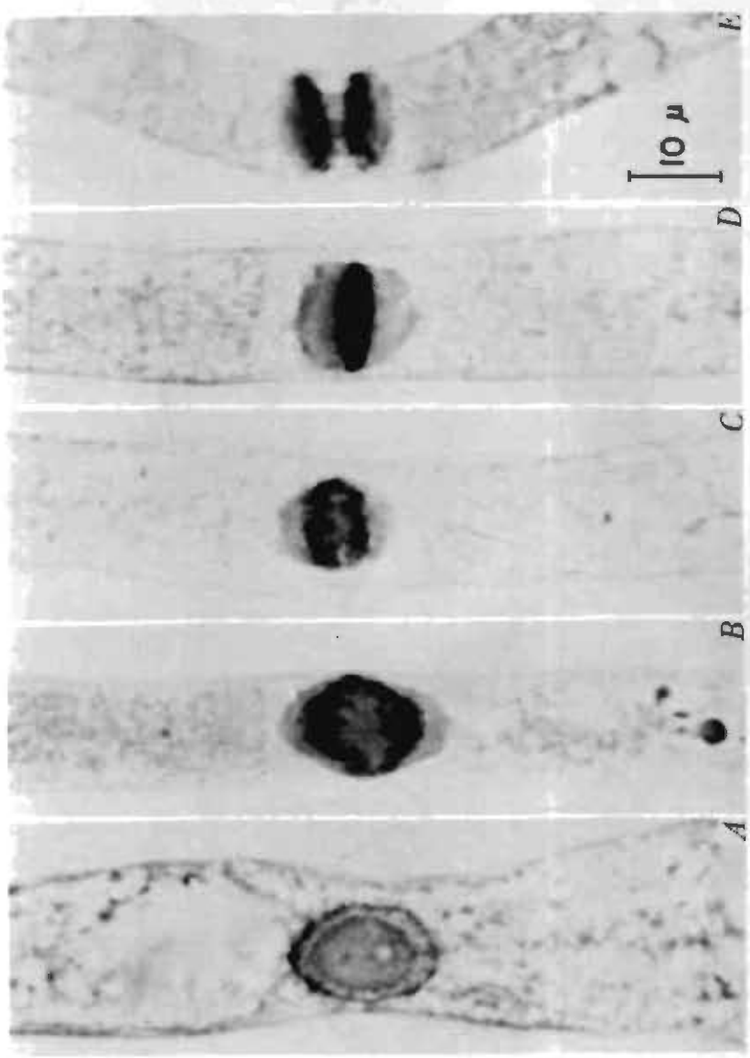

FIG. 5. *Basidiobolus ranarum* (isolated in London, Ontario). Helly, HCl, Giemsa. (A) Resting nucleus with large nucleolus. (B) Early and (C) late prophase. The chromosomes have moved into the nucleolar material. (D) Metaphase, seen sideways. (E) Anaphase.

ports no spindle in the former species, will have to be amended. *S. versatilis*, like *S. pombe*, has distinct chromosomes (or a chain of them) (Fig. 4), but their relationship to the spindle fiber has not yet been established.

2. Since the work of Raciborski (1896), Fairchild (1897), and Olive (1907), *Basidiobolus* has been known to be remarkable in several ways, not only because of the large size of its nuclei and their visibility in the living cell by ordinary microscopy, but because it has a large barrel-shaped spindle at mitosis (Figs. 5 and 6). Robinow (1963a,b) has rein-

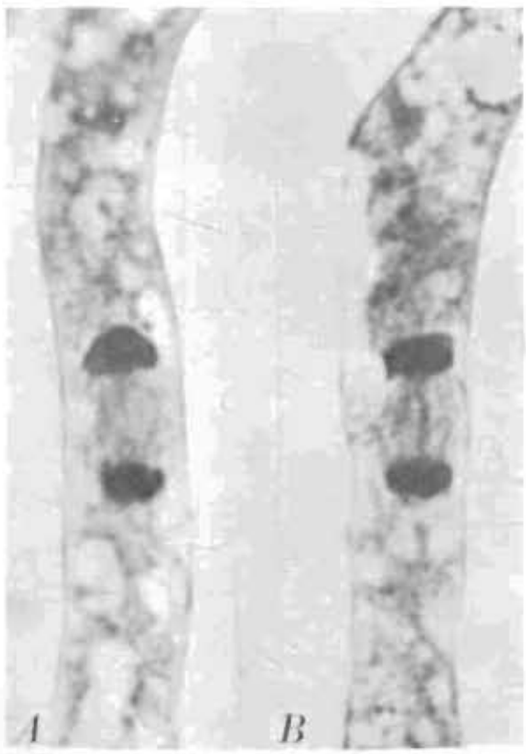

FIG. 6. A, B. *Basidiobolus ranarum* (isolated in Brisbane, Australia). Spindles of anaphase. Helly bromophenol blue, mercuric chloride. Magnification: × 1800.

vestigated mitosis in this fungus and has confirmed the old descriptions and presented evidence that the spindle is derived from some of the material of the former nucleolus. Electron microscopy of thin sections (Robinow and Marak, 1963) has confirmed this impression. All spindle fibers

seen were well within the region filled with finely granular nucleolar material and none were seen outside the nucleus. The apparent absence of centrioles in *Basidiobolus* is puzzling.

3. An intranuclear spindle, diverging from extranuclear centrioles at opposite poles of dividing nuclei, has recently been seen in electron micrographs of *Allomyces* (Robinow and Marak, 1963). This work was stimulated by the earlier demonstration (Renaud and Swift, 1964) of the existence of typical centrioles in mitosporangia of *Allomyces,* examined at various phases of the process of differentiation of zoospores (Fig. 7a). The history of mitosis in *Allomyces* is checkered. Kniep (1930) complained that *Allomyces* is not a favorable object for the study of mitosis, and this was borne out by much subsequent work. Hatch (1938) depicted intranuclear spindles in nuclei of the first postzygotic divisions. Turian (1959), on the other hand, ascribed to the nuclei of *A. macrogynus* the direct mode of division by constriction which had by that time been rediscovered for *Mucor* (Robinow, 1957), and other phycomycetes.

Robinow (1962) could not confirm either Hatch's or Turian's findings. He found neither spindles, nor division by elongation and constriction. According to him the nucleolus breaks down at the height of division and new ones are formed by the daughter nuclei. It now appears (1963) that there is truth in all three descriptions. There *is* an intranúclear spindle. Phase-contrast microscopy has revealed that early in mitosis the nucleolus *is* stretched out and develops a narrow waist. It has also been shown that the nucleolus later *contracts again* and thereafter quickly disappears from view. Electron microscopy has now revealed the mitotic spindle of *Allomyces* (Fig. 7b).

The somatic chromosomes of *A. arbuscula* were probably first seen by Geerts and Stumm (1960), who regarded their behavior as conventional. In Robinow's (1962) Feulgen preparations of the same species, the chromosomes are also distinct, but no metaphase plates were found, and the mechanism of mitosis was not elucidated. More conventional patterns have since been found in *A. macrogynus* (Fig. 1A–D). Indirect evidence of the participation of a spindle in mitosis was provided by Sost (1955), who achieved several levels of polyploidization with colchicine.

The technical basis of the many unpublished observations referred to in this chapter cannot be discussed here, but one useful point may be made. The centriole and intranuclear spindle of *Allomyces* were discovered with the help of a procedure for which one of the writers (C.F.R.) and Mr. John Marak are indebted to their colleagues Professor Robert C. Buck and Dr. N. Krishan. The fungus was grown on the surface of coverslips. Dividing nuclei were first identified (either in life or after fixation, unstained) with the light microscope and photographed at low power and

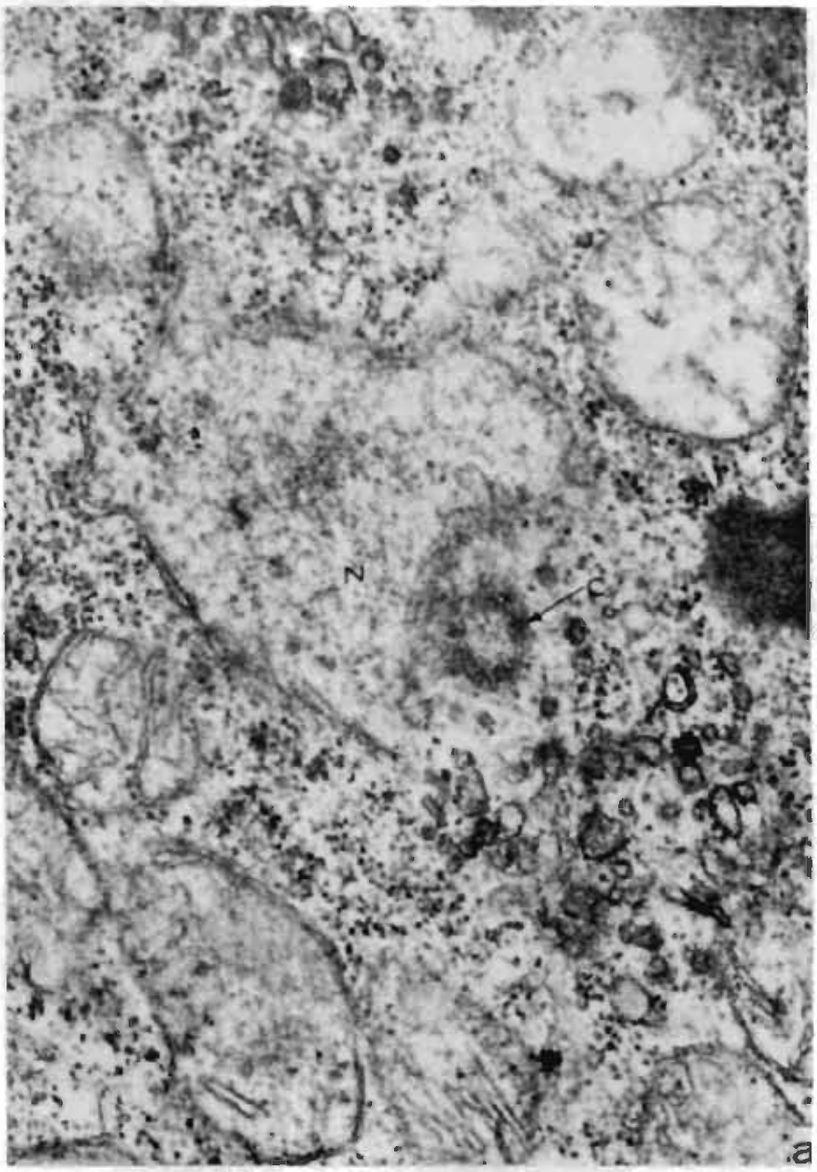

FIG. 7a. Section of hypha of *Allomyces arbuscula* showing nucleus (*N*) and centriole (*C*). This print, which appears in Renaud and Swift (1964), was furnished through the kindness of these authors. Magnification: Approximately × 70,000.

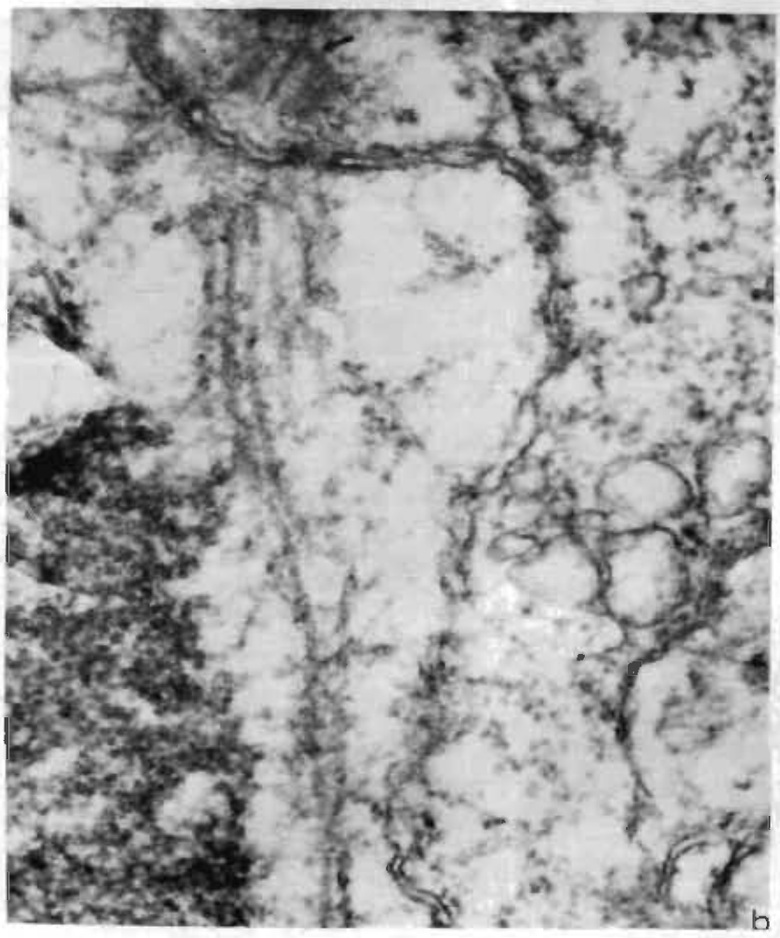

FIG. 7b. Part of a dividing nucleus in a growing sporophyte germling of *Allomyces macrogynus*. The centriole (arrow) lies in a depression of the nuclear envelope. Spindle tubules run the length of the picture. At lower left is part of the nucleolus. Robinow and Marak (1963) unpublished. Magnification × 79,500.

marked in such a manner that their position in the hypha, and the precise localization of the hypha on the glass slip, remained known throughout the procedure of preparation for electron microscopy, and after the embedded specimen had been detached from the glass slip. The face presented to the knife was a minute rectangle with the selected hypha at the center. A procedure of this kind ought, in principle, to be able to resolve many of the remaining questions of fungal karyology.

Still under study is a fourth example of a spindle recently detected by Bakerspigel (1965) in *Trichophyton mentagrophytes*. Although none of the dividing vegetative nuclei in this dermatophyte has as yet been examined in the electron microscope, stained preparations strongly suggest the existence of a spindle apparatus. This spindle is not barrel-shaped nor does it appear to be connected with the nucleolus (Robinow, 1963b). It does appear to be strandlike (whether it is composed of one or more strands is not yet clear), resembling the "Zentralstrang" described by Girbardt (1962) in *Polystictus* and the solitary spindle fiber of *S. versatilis* (Fig. 2). During metaphase the chromosomes align themselves on this spindle and appear to cover it. Thus, in Feulgen-stained preparations a Feulgen-positive complex may be found. However, as the chromosomes separate the spindle emerges as a delicate, strandlike structure which eventually separates at or near its midregion.

IV. BEHAVIOR OF THE NUCLEOLUS AT MITOSIS

In many fungi the nucleolus plays an important role during mitosis, whereas in others it takes no part in that process. In the first class are species of *Mucor* (Robinow, 1957), *Phycomyces* (Robinow, 1957), *Saprolegnia* (Bakerspigel, 1960; Smith, 1923), *Blastocladiella* (Turian and Cantino, 1960), *Empusa* (Robinow, 1963b), *Conidiobolus* (Robinow, 1963b, *Gelasinospora* (Bakerspigel, 1959c), *Schizosaccharomyces versatilis* (Fig. 3), and, standing by itself, *Basidiobolus* (Robinow, 1963b). In all but the last two of these the nucleolus becomes longer during division and is divided between the daughter nuclei. The process has been followed during life and is undoubtedly part of the normal, regular mode of division of the nuclei of many phycomycetes. The mechanism of this form of mitosis is still obscure. It will be remembered that no spindle apparatus has yet been found in these directly dividing nuclei. Some light is thrown on this matter by observations on *S. versatilis* and *Basidiobolus*. In the former, as illustrated in Fig. 3, the originally round nucleolus becomes adsorbed somehow during mitosis onto the solitary intranuclear spindle fiber described above, is stretched out by it, and partly passed on to the daughter nuclei. The inert middle third of it remains for a while in the center of the cell and is later dissolved. To one unaware of the participation of an elongating fiber, mitosis in *S. versatilis* resembles the direct division of *Mucor* nuclei. This is one reason for suspecting that in *Mucor* nuclei, and others of the same type, the elongating nucleolus may harbor an internal elongating fibrous axis. Electron microscopy, which can now solve this problem, has not yet been done on suitably fixed material. The popular $KMnO_4$ is not helpful here but has helped to show that in *Mucor* the nuclear membrane remains intact during division (Robinow, 1963).

Basidiobolus provides further reasons for suspecting that fibrous structures may be at work in the elongating *Mucor*-type nucleolus, because in *Basidiobolus* the nucleolus is largely transformed at mitosis into a barrel-shaped mass of spindle fibers (Robinow, 1963b). Those portions of it which are not so transformed pass to the daughter nuclei which apparently make their new nucleolus partly from old material (as has been described for *Spirogyra* (references in Robinow, 1963b).

Among phycomycetes with nucleoli suspected of being partly fibrous is *Sorosphaera* (Miller, 1958) (and doubtless many other members of the Plasmodiophorales). Its "cruciform" divisions appear to be accomplished solely by the lengthening of the nucleolus. But they are followed by other divisions in which the nucleoli disappear during mitosis and distinct *fibrous spindles* are seen in their place.

In many species the nucleolus is "cast out" altogether from the division figure during mitosis and soon disappears in the cytoplasm, as in *Schizophyllum* (Bakerspigel, 1959a), *Penicillium* (Robinow, 1963), *Trichophyton* (Bakerspigel, 1965), *Lipomyces* (Robinow, 1961), and *Allomyces* (Robinow, 1962). In the latter fungus the nucleolus is first stretched by the intranuclear spindle but later slips off, collapses again, and disintegrates (Robinow, 1963).

V. THE NUCLEAR MEMBRANE

A nuclear membrane has been demonstrated surrounding vegetative nuclei in several fungi (Agar and Douglas, 1957; Breslau *et al.*, 1961; Edwards and Edwards, 1960; O'Hern and Henry, 1956; Tsuda, 1956). Other recent examples were those shown in *Saccharomyces cerevisiae* (Koehler, 1962; Vitols *et al.*, 1961) and in blastospores of *Candida albicans* (Bakerspigel, 1964). These envelopes appear to be composed of at least two, closely apposed unit membranes pierced by a number of pores. During division the membrane remains intact until the chromosomes have contracted and become deeply stainable (Bakerspigel, 1962).

VI. NUCLEAR MIGRATION AND MOTILITY

It was Buller (1931) and Dowding and Buller (1940) who clearly demonstrated that nuclei migrate in ascomycetes and basidiomycetes. They showed that nuclear migration occurred relatively rapidly and in most instances the migrating nucleus of one genotype could colonize the mycelium of another genotype. Observations made on several fungi have also revealed that for a given fungal species the migration rate is several times the maximal linear growth of the mycelium. In their report on *Schizo-*

phyllum commune, Snider and Raper (1958) described the effects of incompatibility and temperature upon the rate of nuclear migration and showed that the migratory rate for *S. commune* at 27°C, in radiate residents, was at least 10 times faster than hyphal tip growth, which was only 0.13 mm per hour. In addition, they argued that the high Q_{10} of 6.0 for migration indicates that the rate is limited by an unknown chemical, rather than a physical process.

In a more recent report, Swiczynski (1961) summarized the basic facts concerning migration of nuclei in tetrapolar basidiomycetes. He proposed a model based mainly on genetic evidence since cytological and biochemical evidence is still scarc. Swiczynski based his hypothesis on two assumptions: (1) that nuclei will migrate only if a force attracting them is present and (2) that the attraction is directed; i.e., if one nucleus is attracted t other the reverse may not be true. Obviously this kind of model c proved only if the factor responsible for the attraction of nuclei is den strable and if it will function in a predicted manner. Thus, even thou there is ample genetic evidence for nuclear migration within several fungal species, there is still no cytological evidence which demonstrates the mechanics responsible for these migrations.

During their investigations of nuclear migration in several species of fungi, Dowding and Bakerspigel (1954, 1956) had suggested that nuclei in *Gelasinospora tetrasperma* are not necessarily carried by the cytoplasm as it streams through the mycelium but that they may remain anchored to the cell wall. These nuclei could migrate through the mycelium while in an expanded, contracted, or serpentine form. When mitochondria surrounded a nucleus they took on a spherical form; and when in the nuclear vacuole, they stretched into beaded filaments. Such filaments appeared to coalesce into a single lashing whip which joined the nucleus, and together the two formed a violently moving "ophioplast" which, when extended, reached a length about one-third that of the cell. When nuclei of *G. tetrasperma* became freed from the cell wall to which they appeared to be attached, they might be capable of independent motility. In a later report on nuclear streaming in *Gelasinospora,* Dowding (1958) described nuclei carried by the streaming cytoplasm at speeds as high as 40 mm per hour. On the basis of these observations eight nuclear migrations were envisaged. These might now be described as follows:

1. Migration of one or more nuclei from a conidiophore into an attached conidium. This occurs either following a nuclear division in the conidiophore or by the migration of a mature, interdivisional nucleus from the conidiophore into the conidium.

2. Migration of a nucleus out of a germinating conidium into the neck of the germ tube in preparation for division in that region.

3. Migration of a sister nucleus back into a conidium following the first division in the neck of the germ tube. The other sister nucleus may remain stationary or migrate farther down the tube. Following this a septum may be laid down separating the nuclei in the conidial remnant from those in the growing germ tube.

4. Migration of one or more nuclei within a growing, unicellular hypha. This migration may be toward the tip or in a reverse direction.

5. Migration of nuclei from one cell to another through septal pores in multicellular hyphae.

6. Migration of one or more nuclei from a unicellular or multicellular primary hypha into lateral branches. These nuclei may be interdivisional or the product of a recent division.

7. Migration of nuclei in hyphae of the same species that have fused with each other. A good example of this can be found during the formation of fruiting heterokaryons obtained by mating plus and minus strains of *G. tetrasperma.*

8. Migration within a hypha appears to be sporadic. Only one of several nuclei may migrate while the others remain stationary.

There is probably no doubt that fungal nuclei are carried by streaming cytoplasm. Whether these nuclei do indeed have independent motility has not yet been definitely demonstrated. It is, however, interesting to note that during migration of nuclei from one cell to another the chromatin migrates ahead of the nucleolus suggesting that the nucleolus might play some role in the mechanics of migration. Furthermore, it is also known that mitochondria attach themselves to nuclear membranes and that such nuclei appear to migrate with lashing motion, from one region in a hypha to another.

VII. KARYOLOGY OF YEAST

(See also Notes Added in Proof on p. 139)

After many years of controversy, the literature on the karyology of bakers' yeast (*Saccharomyces cerevisiae*) is beginning to converge (Ganesan, 1959; McClary *et al.,* 1957; Nagel, 1946; Pontefract and Miller, 1962; Ramirez and Miller, 1962; Robinow, 1961), although much remains to be done. The essentials of the situation seem to be these:

1. The nucleus of cells in the growing, budding phase divides directly by elongation and "étranglement." This is the inevitable conclusion from the phase-contrast microscopy of living cells (Fig. 8), from stained preparations (since the days of Guilliermond's early work, 1910) (Fig. 9) and from the electron microscopy of sections (Hashimoto *et al.,* 1959).

2. What happens inside the dividing nucleus? There is no doubt that it

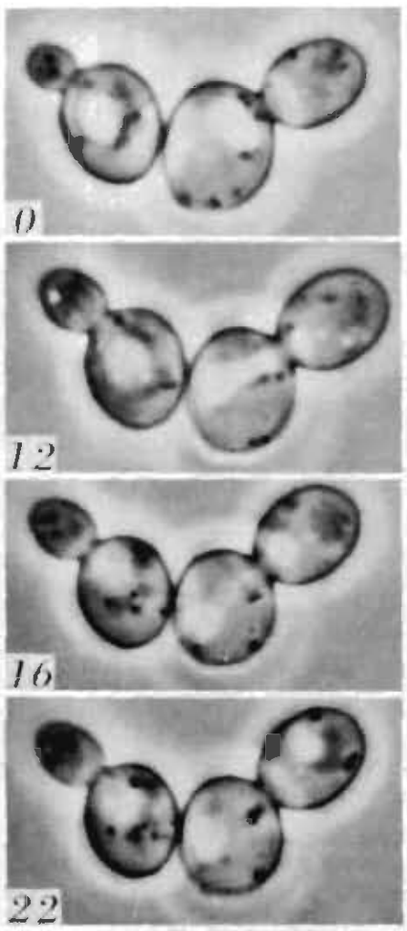

FIG. 8. Successive stages of the division of the nucleus of a living yeast cell (*Saccharomyces cerevisiae*). Slide culture in 20% gelatin containing 1% yeast extract and 1% glucose. Numbers give minutes since the taking of the first photograph. At *0* the nucleus, an angular body of low density is at the base of the bud of the cell on the right. In the pictures that follow the nucleus is stretched into a ribbon which later thins in the middle and breaks into two daughter nuclei. Phase contrast microscopy. Magnification: approximately × 3000.

contains chromosomes *in some form*, but it is equally certain that their cycle of duplication and separation does not resemble the events of an ordinary mitosis. Beads and granules have often been described and perhaps they represent chromosomes (Ramirez and Miller, 1962). But they are very small and their movements are not yet sufficiently understood to

FIG. 9. *Saccharomyces cerevisiae* A–F and H–K illustrate commonly encountered stages in the direct division of the nucleus. The nucleolar moiety of the nucleus is not stained in these preparations. Helly, HCl, Giemsa. Squashed after staining.

permit description in terms of a mitotic process. According to Moor and Mühlethaler (1963) the nucleus of the living yeast cell contains no elements that are larger than 100 Å except in one direction. An interesting exception is provided by the yeast *Lipomyces lipofer*, a member of the Saccharomycetaceae, in which chromosomes are distinct and countable even in nuclei of the vegetative, budding phase (Robinow, 1961).

3. Distinct and more or less countable chromosomes do appear in yeast cells about to form ascospores. First clearly shown by McClary *et al.* (1957), their emergence has been confirmed by Pontefract and Miller

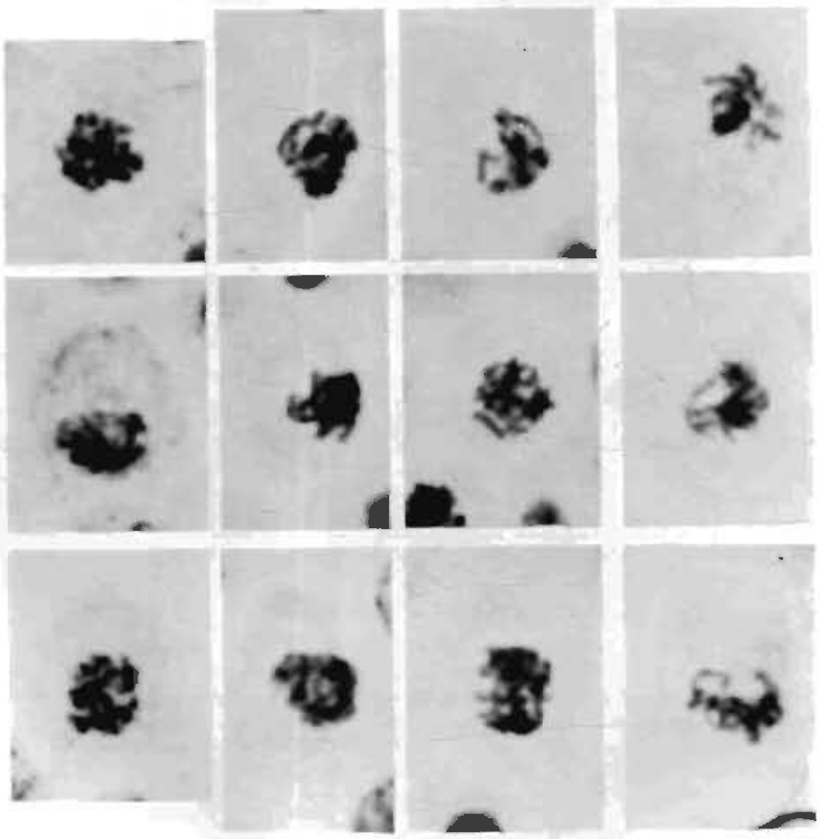

FIG. 10. Same strain of yeast as in Fig. 9. Emergence of chromosomes during preparation for the first sporogenous division. Fixed with Helly or half-strength Schaudinn. HCl, Giemsa. Magnification: × 4500. Similar pictures have been obtained by McClary *et al.* (1957) and Pontefract and Miller (1962).

(1962) and one of the writers (C. F. R.) (Fig. 10). It is important to realize that during sporogenesis the chromosomes are rendered visible by the same methods that fail to show them distinctly in budding cells. A real change in the physical and chemical nature of the chromosomes appears to be involved.

4. Separation of the chromosomes is not yet good enough to make reliable counts possible, but it is our impression that the number of chromosomes demonstrable at the anaphase of the first sporogenous division is smaller than that demanded by the results of the analysis of recombinants (Hawthorne and Mortimer, 1960).

NOTES ADDED IN PROOF

Knowledge of spindles in somatic mitoses has been extended by demonstrations given at the 10th International Congress of Botany at Edinburgh, August 3–13, 1964. Electron micrographs of dividing nuclei in hyphae of two species of *Albugo,* shown by C. C. Bowen and his associates at Iowa University, have revealed clear-cut centrioles and intranuclear spindles resembling those found simultaneously in *Allomyces* by Robinow and Marak (Fig. 7B of the present article). [See also J. D. Berlin and C. C. Bowen, *Am. J. Botany* **51**, 650 (1964).]

Girbardt has extended his work on *Polystictus* to include different species of Basidiomycetes. A "Zentralstrang" has been found in dividing nuclei of all of them. Electron micrographs of glutaraldehyde-fixed material reveal the existence of bundles of spindle tubules not only among the chromosomes between a pair of highly modified centrioles but also radiating backwards from each of the centrioles far out into the cytoplasm.

A spindle fiber composed of a small number of parallel microtubules with diameters around 150 Å stretched out between disklike centrioles attached to the intact nuclear envelope has recently been discovered in dividing nuclei of *Saccharomyces cerevisiae* (Robinow and Marak, 1965).

REFERENCES

Agar, H. D., and H. C. Douglas. (1957). Studies on the cytological structure of yeast: Electron microscopy of thin sections. *J. Bacteriol.* 73:365–375.

Alfert, M., and I. I. Geschwind. (1953). A selective staining method for the basic proteins of cell nuclei. *Proc. Natl. Acad. Sci. U.S.* 39:991–999.

Bakerspigel, A. (1959a). The structure and manner of division of the nuclei in the vegetative mycelium of the basidiomycete *Schizophyllum commune. Can. J. Botany* 37:835–842.

Bakerspigel, A. (1959b). The structure and manner of division of the nuclei in the vegetative mycelium of *Neurospora crassa. Am. J. Botany* 46:180–190.

Bakerspigel, A. (1959c). The structure and manner of division of the nuclei in the vegetative mycelium of *Gelasinospora tetrasperma*. *Can. J. Microbiol.* **5**:125–130.

Bakerspigel, A. (1960). Nuclear structure and division in the vegetative mycelium of the Saprolegniaceae. *Am. J. Botany* **47**:94–100.

Bakerspigel, A. (1965). Unpublished data.

Bakerspigel, A. (1962). Further observation on the vegetative nuclei of *Neurospora crassa*. "Neurospora Newsletter," No. 2, p. 16.

Bakerspigel, A. (1964). Some observations on the cytology of *Candida albicans*. *J. Bacteriol.* **87**:228–230.

Breslau, A. M., T. J. Hensley, and J. O. Erickson. (1961). Electron microscopy of cultured spherules of *Coccidioides immitis*. *J. Biophys. Biochem. Cytol.* **9**:627–637.

Buller, A. H. R. (1931). "Researches on Fungi," Vol. IV, pp. 329. Longmans, Green, New York.

Dowding, E. S. (1958). Nuclear streaming in *Gelasinospora*. *Can. J. Microbiol.* **4**: 295–301.

Dowding, E. S., and A. Bakerspigel. (1954). The migrating nucleus. *Can. J. Microbiol.* **1**:68–78.

Dowding, E. S., and A. Bakerspigel. (1956). Poor fruiters and barrage mutants in *Gelasinospora*. *Can. J. Microbiol.* **4**:295–301.

Dowding, E. S., and A. H. R. Buller. (1940). Nuclear migration in *Gelasinospora*. *Mycologia* **32**:471–488.

Eckstein, B. (1958). Karyologische Untersuchungen an einer Wildhefe. *Arch. Mikrobiol.* **32**:65–80.

Edwards, G. A., and R. M. Edwards. (1960). The intracellular membranes of *Blastomyces dermatitidis*. *Am. J. Botany* **47**:622–632.

Elliott, C. G. (1960). The cytology of *Aspergillus nidulans*. *Gen. Res. Cambridge* **1**:462–476.

Fairchild, D. G. (1897). Ueber Kerntheilung und Befruchtung bei *Basidiobolus ranarum* Eidam. *Jahrb. Wiss. Botan.* **30**:285–296.

Fincham, J. R. S., and P. R. Day. (1963). "Fungal Genetics," 300 pp. Blackwell, Oxford.

Flentje, N. T., H. M. Stretton, and E. J. Hawn. (1963). Nuclear distribution and behaviour throughout the life cycles of *Thanatophorus*, *Waitea* and *Ceratobasidium* species. *Australian J. Biol. Sci.* **16**:450–467.

Foster, J. W. (1956). Personal Communication.

Ganesan, A. T. (1959). The cytology of *Saccharomyces*. *Compt. Rend. Trav. Lab. Carlsberg* **31**:149–174.

Geerts, S. J., and C. Stumm. (1960). Mitosen bei *Allomyces arbuscula* Butl. *Naturwissenschaften* **47**:211.

Girbardt, M. (1962). Licht- und elektronenmikroskopische Untersuchungen an *Polystictus versicolor*. VIII. Faerberische Analyse der vegetativen Kernteilung. *Planta* **58**:1–21.

Grell, K. G. (1953). Die Chromosomen von *Aulocantha scolymantha* Haeckel. *Arch. Protistenk* **99**:1–54.

Guilliermond, A. (1910). Remarques sur les recents travaux parus sur la cytologie des levures et quelques nouvelles observations sur ce groupe de champignons. *Zentr. Bakteriol., Parasitenk., Abt. II* **26**:577–589.

Guilliermond, A. (1917). Sur la division nucléaire des levures. *Ann. Inst. Pasteur* **31**:107–113.

Hartmann, R. T. (1964). Nuclear division in *Alternaria tenuis*. *Am. J. Botany* **51**: 209–212.

Hashimoto, T., S. F. Conti, and H. B. Naylor. (1959). Studies of the fine structure of microorganisms. IV. Observations on budding *Saccharomyces cerevisiae* by light and electron microscopy. *J. Bacteriol.* **77**:344–354.

Hatch, W. R. (1938). Conjugation and zygote germination in *Allomyces arbuscula*. *Ann. Botany (London)* [N. S.] **2**:583–614.

Hawthorne, D. C., and R. K. Mortimer. (1960). Chromosome mapping in *Saccharomyces:* centromere linked genes. *Genetics* **45**:1085–1110.

Käfer, E. (1961). The processes of spontaneous recombination in vegetative nuclei of *Aspergillus nidulans*. *Genetics* **46**:1581–1609.

Kniep, H. (1930). Über den Generationswechsel von *Allomyces*. *Z. Botan.* **22**:433–441.

Knox-Davies, P. S., and J. G. Dickson. (1960). Cytology of *Helminthosporium turcicum* and its ascigerous stage, *Trichometasphaeria turcica*. *Am. J. Botany* **47**: 328–338.

Koehler, J. K. (1962). The nuclear envelope of *Saccharomyces cerevisiae*. *J. Ultrastruct. Res.* **6**:432–436.

Leedale, G. F. (1958). Nuclear structure and mitosis in the Euglenineae. *Arch. Mikrobiol.* **32**:32–64.

Lu, B. C. (1964). Chromosome cycles of the basidiomycete *Cyathus stercoreus* (Schw.) de Toni. *Chromosoma* **15**:170–184.

McClary, D. O., M. A. Williams, C. C. Lindegren, and M. Ogur. (1957). Chromosome counts in a polyploid series of *Saccharomyces*. *J. Bacteriol.* **73**:360–364.

Mazia, D., P. A. Brewer, and M. Alfert. (1953). The cytochemical staining and measurement of protein with mercuric bromphenol blue. *Biol. Bull.* **104**:57–67.

Miller, C. E. (1958). Morphology and cytology of the zoosporangia and cystosori of *Sorosphaera veronicae*. *J. Elisha Mitchell Sci. Soc.* **74**:49–64.

Moor, H., and K. Mühlethaler. (1963). Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**:609–628.

Nagel, L. (1946). A cytological study of yeast (*Saccharomyces cerevisiae*). *Ann. Missouri Botan. Garden* **33**:249–281.

O'Hern, E. M., and B. S. Henry. (1956). A cytological study of *Coccidioides immitis* by electron microscopy. *J. Bacteriol.* **72**:632–645.

Olive, E. W. (1906). Cytological studies on the Entomophthoraceae. II. Nuclear and cell division of *Empusa*. *Botan. Gaz.* **41**:229–261.

Olive, E. W. (1907). Cell and nuclear division in *Basidiobolus*. *Ann. Mycol. (Berlin)* **5**:404–418.

Pauli, F. (1956). Heidenhain's hematoxylin in cytological studies of *Penicillium notatum*. *Stain Technol.* **31**:91–93.

Pontefract, R. D., and J. J. Miller. (1962). The metabolism of yeast sporulation. IV. Cytological and physiological changes in sporulating cells. *Can. J. Microbiol.* **8**: 573–584.

Raciborski, M. (1896). Study a mykologiczne. I. Karyokinese bei *Basidiobolus ranarum* (and other fungi). *Bull. Intern. Acad. Sci. Cracovie* p. 377.

Ramirez, C., and J. J. Miller. (1962). Observations on vegetative nuclear division in *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **8**:603–608.

Renaud, F. L., and H. Swift. (1964). The development of basal bodies and flagella in *Allomyces arbusculus*. *J. Cell Biol.* **23**:339–354.

Robinow, C. F. (1957). The structure and behavior of the nuclei in spores and growing hyphae of Mucorales. *Can. J. Microbiol.* **3**:771–789, 791–798.

Robinow, C. F. (1961). Mitosis in the yeast *Lipomyces lipofer*. *J. Biophys. Biochem. Cytol.* **9**:879–892.

Robinow, C. F. (1963–1965). Unpublished data.

Robinow, C. F. (1962). Some observations on the mode of division of somatic nuclei of *Mucor* and *Allomyces*. *Arch. Mikrobiol.* **42**:369–377.

Robinow, C. F. (1963a). "Lectures on Theoretical and Applied Aspects of Modern Microbiology, 1962–1963," No. 2. Univ. of Maryland Press, College Park, Maryland.

Robinow, C. F. (1963b). Observations on cell growth, mitosis, and division in the fungus *Basidiobolus ranarum*. *J. Cell Biol.* **17**:123–152.

Robinow, C. F., and J. Marak. (1963). Unpublished data.

Sansome, E. R. (1963). Meiosis in *Pythium debaryanum* Hesse and its significance in the life-history of the Biflagellatae. *Brit. Mycol. Soc. Trans.* **46**:63–72.

Schmitz, F. (1879). Ueber den Zellkern der Thallophyten. *Sitzber. Niederrh. Ges.* Aug. 4.

Schopfer, W. H., D. Wustenfeld, and G. Turian. (1963). La division nucléaire chez *Schizosaccharomyces pombe*. *Arch. Mikrobiol.* **45**:304–313.

Smith, F. E. V. (1923). On direct nuclear divisions in the vegetative mycelium of *Saprolegnia*. *Ann. Botany* (*London*) **37**:63–73.

Snider, P. J., and J. R. Raper. (1958). Nuclear migration in the basidiomycete *Schizophyllum commune*. *Am. J. Botany* **45**:538–546.

Somers, C. E., R. P. Wagner, and T. C. Heu. (1960). Mitosis in vegetative nuclei of *Neurospora crassa*. *Genetics* **45**:801–810.

Sost, H. (1955). Über die Determination des Generationswechsels von *Allomyces arbuscula* (Butl.). *Arch. Protistenk.* **100**:544–564.

Swiezynski, K. M. (1961). Migration of nuclei in tetrapolar Basidiomycetes. *Acta Soc. Botan. Polon.* **30**:529–534.

Tsuda, S. (1956). Electron microscopical studies of ultrathin sections in *Penicillium chrysogenum*. *J. Bacteriol.* **71**:450–453.

Turian, G. (1959). Mitose végétative et action polyploidisante de l'acide borique chez *Allomyces macrogynus*. *Rev. Cytol. Biol. Vegetales* **21**:63–69.

Turian, G., and E. C. Cantino. (1960). A study of mitosis in the mold *Blastocladiella* with a ribonuclease-acetoorcein staining technique. *Cytologia* (*Tokyo*) **25**:101–107.

Vitols, E., R. J. North, and A. W. Linnane. (1961). Studies on the oxidation metabolism of *Saccharomyces cerevisiae*. I. Observations on the fine structure of the yeast cell. *J. Biophys. Biochem. Cytol.* **9**:689–699.

Ward, E. W. B., and K. W. Ciurysek. (1962). Somatic mitosis in *Neurospora crassa*. *Am. J. Botany* **49**:393–399.

Wilson, C. M. (1952). Meiosis in *Allomyces*. *Bull. Torrey Botan. Club* **79**:139–160.

Yoneda, Y. (1963). Karyological studies on yeasts. *Cytologia* **28**:131–145.

CHAPTER 7

Nuclear Behavior during Meiosis

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I. INTRODUCTION

With relatively few exceptions, the only diploid cell in the life cycle of a fungus is the zygote. It is in the zygote that nuclear fusion occurs, and when the diploid fusion nucleus divides, it does so meiotically. Thus, there is no equational division of a diploid nucleus in the life cycle. There are some exceptional fungi, such as certain species of *Allomyces* and yeasts, that have an alternation of haploid and diploid generations or even the predominance of a diploid vegetative phase.

The fungi have developed a great variety of methods for bringing together the pairs of nuclei that fuse in the zygotes. Such divergent processes as gametic union, the fusion of sex organs, and the fusion of vegetative cells all occur, but regardless of what type of plasmogamy is involved the criteria for a complete sexual cycle must include both nuclear fusion and meiosis. The meiotic prophase nucleus is the largest nucleus in the life cycle, and it is from studies of this nucleus in the process of division that the most detailed information on fungal cytology has been derived.

Probably the greatest impetus to earlier investigations of meiosis in the fungi came from Harper's publications on the powdery mildews (1897, 1905). Harper's descriptions and illustrations of the meiotic process still stand as a model of clarity and accuracy. Most of the earlier studies were made on sectioned material stained with Heidenhain's hematoxylin, gentian violet, or Flemming's triple stain. In recent years the stimulus to a renewed interest in fungal cytology is clearly traceable to McClintock's remarkably successful application of the orcein squash technique to the asci of *Neurospora* (1945). The great advantage of this technique is that it permits the observation of whole stained nuclei and the more accurate study of chromosome morphology during meiosis.

In this chapter a synthesis of some of the more lucid reports on meiosis will be attempted. For a more general treatment of meiosis in the fungi, the reader is referred to the reviews of Tischler (1951) and Olive (1953).

II. A GENERALIZED DESCRIPTION OF MEIOSIS IN FUNGI

The true fungi show consistent similarities in certain basic features of the meiotic process. Two successive nuclear divisions are involved, and in all species that have received adequate genetic study, separation of homologous kinetochores has been found to occur in the first meiotic division, with

FIG. 1. Asci of *Sordaria fimicola* heterozygous for a spore color marker, showing first and second division segregation. Magnification: × 435.

segregation of genetic markers occurring in either of the two divisions, depending upon whether crossing over has occurred (Fig. 1). In fact, there appears to be no essential difference between the fungi and most other organisms in the basic cytological features and genetic implications of the meiotic process. Some of the cytological differences of possibly secondary importance include the intranuclear origin of the spindle and the relatively small size of the spindle and chromosomes. Polar centrosomes, with or without astral rays, have been reported in a number of fungi.[1] The single nucleolus degenerates near the end of prophase or sometimes later. The nuclear membrane usually degenerates also by the end of prophase. At metaphase the chromosomes may appear irregularly distributed on the spindle or, less often, in a distinct equatorial plate arrangement, and disjunction at anaphase is typically asynchronous. Interphase stages are of short duration, and uncoiling of chromosomes is incomplete. Cytologically, the second division figures resemble the first, but the spindles are smaller, as the components of the dyads separate to opposite poles.

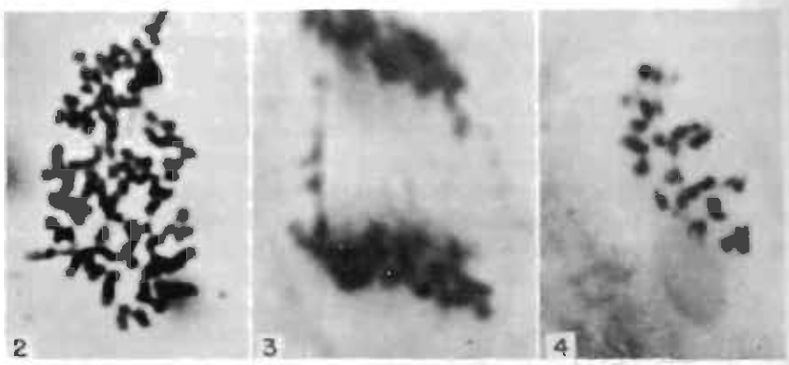
Haploid chromosome counts in the fungi range mostly from 3 to 28 (up to 90 in the myxomycetes), with numbers of 8 or less predominating. Most earlier reports of 2 appear to have resulted from misinterpretation of telophase figures in sectioned material.

III. MEIOSIS IN THE MAJOR GROUPS OF FUNGI

A. Lower Fungi

Because of the small size of the nuclei and the general lack of genetic information, there are few reliable data on meiosis in the lower fungi. Often it has not been possible to establish convincingly just where in the life cycle meiosis occurs, although it has generally been thought to occur at germination of the zygote. In certain myxomycetes, which can hardly be considered true fungi, Wilson and Ross (1955), using aceto-orcein to stain whole nuclei, obtained preparations indicating the occurrence of meiosis in the last two divisions prior to spore formation in the sporangia (but in the sporoid bodies in *Ceratiomyxa*). Recent genetic studies (Collins, 1961) have supported this interpretation. Haploid chromosome numbers are reported to range from 8 in *C. fruticulosa* to about 90 in *Hemitrichia vesparium* (Figs. 2 and 3). The latter number is much higher than any reported for the true fungi.

[1] J. D. Berlin and C. C. Bowen [*Am. J. Botany* **51**:650–652 (1964)], with electron microscopy, have demonstrated for the first time in fungi that paired extra-nuclear centrioles typical in structure are present in *Albugo candida*. In the zoospores they give rise to the flagella.



FIGS. 2-4. Meiosis in lower fungi. Fig. 2. Metaphase II in *Hemitrichia vesparium*. Fig. 3. Late anaphase I in same species, showing chromosome bridge. Fig. 4. Prometaphase I in *Allomyces javanicus* var. *javanicus* (× 3600). Figs. 2 and 3 from Wilson and Ross (1955); Fig. 4 from Wilson (1952).

In the Plasmodiophorales, which some consider to be parasitic relatives of the myxomycetes, the position of karyogamy and meiosis in the life cycle is still so uncertain as to discourage further discussion here.

Probably the largest nucleus reported for any of the fungi is that found in the mature thallus of *Synchytrium*, where it reaches a diameter as great as 80 μ (Olive, 1953). Kusano (1930) described the division of this nucleus in the resting sporangia of *S. fulgens* as being meiotic. During vegetative growth of the thallus and later during prophase of nuclear division, there is a copious discharge of chromatic material into the cytoplasm from the large nucleolus and possibly other parts of the nucleus. One is tempted to suggest that this is actually the passage of abundant amounts of ribonucleic acid from nucleus to cytoplasm in association with the high rate of metabolism of a rapidly enlarging thallus destined for the formation of a large number of zoospores. As the primary nucleus enters metaphase, its spindle and chromosomes are seen to be quite small.

Allomyces is one of the few phycomycetous genera in which both cytological and genetic information has clearly established the position of meiosis. With the aid of aceto-orcein, C. M. Wilson (1952) was able to recognize meiotic stages in the germinating resting sporangia (Fig. 4) and to establish the basic chromosome numbers (n = 14, 16, 28) for three species. Polyploidy was found in two species. These observations are supported by the genetic findings of Emerson and Wilson (1954). The haploid zoospores from the resting sporangia, produced on diploid individuals, give

rise to the gametophytic phase, and fusing gametes form zygotes that reproduce the diplophase.

It has generally been thought that meiosis in oomycetes occurs at germination of the zygote (oospore), but Sansome and Harris (1962), in recent cytological studies of species of *Achlya, Pythium,* and *Phytophthora,* have revived an earlier concept that it occurs in the sex organs, which would also mean that the vegetative thallus is diploid. But these cytological studies are not convincing. The earlier studies of Couch (1926) on *Dictyuchus monosporus* indicate that genetic recombination and, therefore, meiosis occur in the germinating oospore. Neither the limited amount of genetic data nor the inconclusive cytological observations on these small nuclei can provide a final answer to this question. Most urgently needed at the moment are further genetic studies.

Among zygomycetes, cytological and genetic studies have established in several species the occurrence of both karyogamy, typically involving several pairs of nuclei, and meiosis in the zygospores (Fig. 5). The studies of Burgeff (1915, 1929) on *Phycomyces nitens* and *P. blakesleeanus* strongly indicate that, although many nuclear pairs fuse in the zygospore while others fail to do so, only one diploid nucleus survives to divide meiotically and produce all the haploid nuclei for the spores of the meiosporangium. With the aid of several genetic markers, it was shown that the meiosporangia might contain progeny of from one to four of the four possible genotypes that could be expected. This was concluded to be the result of varying degrees of survival among the members of the tetrad of nuclei resulting from meiosis.

Köhler (1935) germinated zygospores of *Mucor mucedo* heterozygous for two loci and showed that only one of the four possible genotypes appeared in the progeny of any one meiosporangium, though all four types of meiosporangia could be found. These discoveries, in conjunction with the cytological observations of Sjöwall (1946) on *Rhizopus stolonifer* [*R. nigricans*], have shown that, in these two species, only one diploid nucleus among many in the zygospore survives, and only one member of the tetrad resulting from meiosis survives to produce, by successive mitoses, the haploid nuclei for all the spores of a single meiosporangium (Fig. 5).[2] This would, of course, prohibit inbreeding among the progeny of the same meiosporangium.

B. Ascomycetes

The most detailed and accurate accounts of meiosis in the fungi have come from studies of pyrenomycetes and discomycetes, especially the

[2] W. L. Gauger [*Am. J. Botany* 48:427–429 (1961)] found that some meiosporangia of this species contain spores of both mating types.

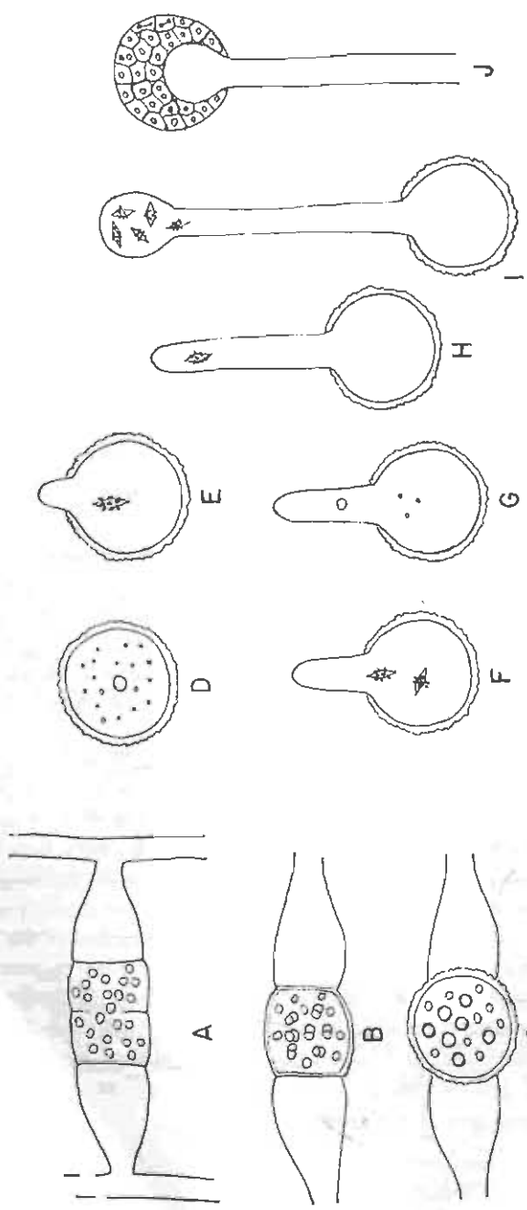


FIG. 5. Diagrammatic representation of karyogamy and meiosis in a mucor such as *Rhizopus stolonifer*: (A) Plasmogamy; (B) karyogamy; (C) diploid and haploid nuclei in zygospore; (D) degeneration of all but one diploid nucleus; (E) first meiotic division; (F) second meiotic division; (G) degeneration of all except one of the tetrad nuclei; (H) and (I) mitotic divisions of haploid nuclei; (J) mature meiosporangium.

former. McClintock's introduction (1945) of the orcein and carmine smear techniques to fungal cytology made it possible for the first time to study chromosome morphology in considerable detail and to make accurate chromosome counts. Singleton (1953), after studying with McClintock, published for *Neurospora crassa* the most detailed and best illustrated account of meiosis that has yet appeared for any fungus, in which McClintock's earlier findings were confirmed (Figs. 7–18). Singleton's paper will, therefore, furnish the main basis for the step-by-step description of meiosis in this chapter. The publications of other investigators, especially those of Harper (1905) on powdery mildews (Figs. 20–25), will be referred to whenever they can further elucidate any aspect of the process. Unfortunately, space does not permit discussion of all of the excellent papers on this subject. Stages in the cytology of ascus development are diagrammed in Fig. 6.

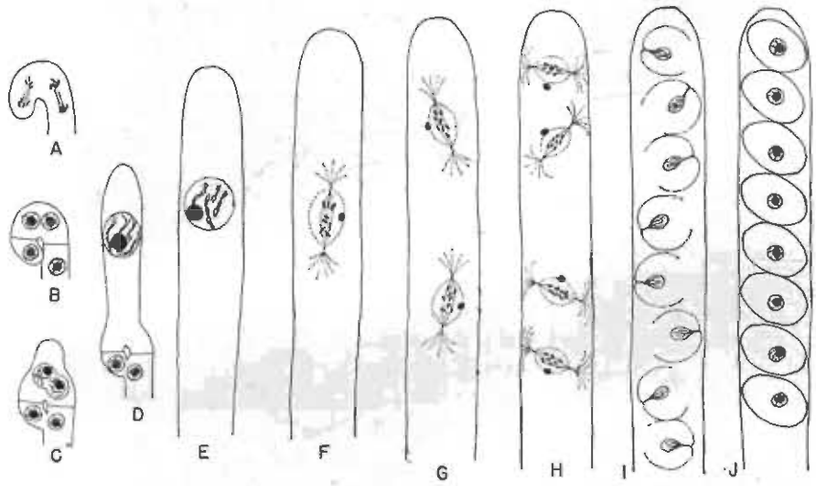
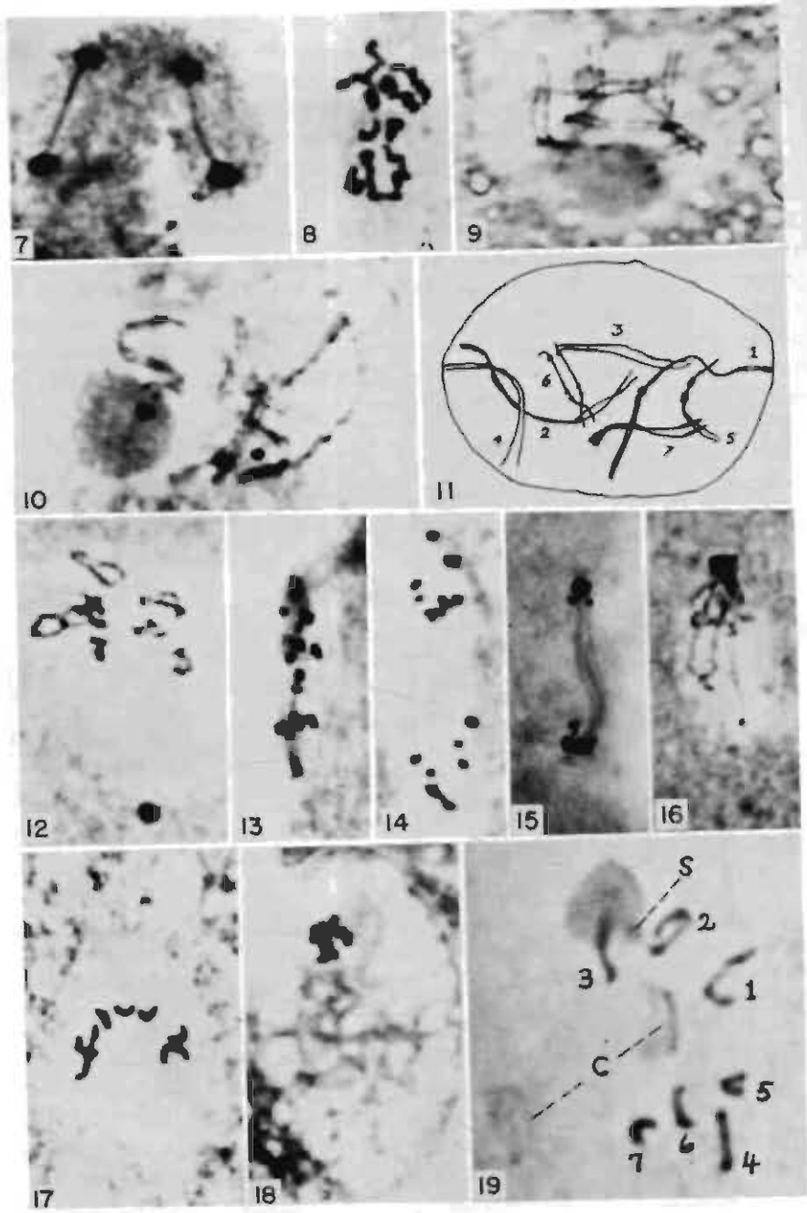


FIG. 6. Diagrammatic representation of ascus development in a higher ascomycete: (A) and (B) crozier development; (C) karyogamy; (D) synapsis; (E) diplotene; (F) anaphase I; (G) anaphase II; (H) anaphase III; (I) ascospore delimitation; (J) 8-spored ascus.

Singleton observed that the conjugate premeiotic nuclear divisions in the croziers (Fig. 7) are mitotic, with a set of chromosomes (7 in *Neurospora*) passing to each spindle pole. Both he and Harper (Fig. 20) noted that the prophase chromosomes of the prefusion and early fusion nuclei are gathered together at a common deeply staining spot on the nuclear membrane. Harper called this spot the "central body," recognizing that it had some of the characteristics of a centriole. Singleton considered it an area in which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the chromosomes are held together by the aggregation of the heterochromatic regions of the chromosomes, the orientation of the chromosomes from this point representing a "relic anaphase orientation" from the previous division. He found this to be a common chromosomal arrangement at all interphase stages in the ascus, while Harper found it to persist in reorganizing nuclei at all stages in the life cycle of powdery mildews.

In *Neurospora,* karyogamy occurs in the binucleate penultimate cell of the crozier, the chromosomes being at this time somewhat diffuse. Very soon after nuclear fusion the two nucleoli merge into a single larger one, while the chromosomes, persisting for a time in two groups of 7 each, undergo contraction (Fig. 8). Prior to the beginning of synapsis, they reach a state of contraction equal to or greater than that of the previous metaphase in the crozier. This surprising development, which thus far appears to be unique to the fungi, has been repeatedly observed in various pyrenomycetes and is probably a characteristic feature of these fungi. Harper (1905) seems to have been the first to report this stage, of which he states: "It is clear that this contracted stage of the chromatin elements is identical with the synapsis stage in the spore mother cells of the higher plants." Harper believed, probably mistakenly, that contraction occurred after synapsis had begun. He considered the moving together of the two "central bodies" as being responsible for bringing the two sets of chromosomes into contact (Fig. 21).

In *Neurospora* synapsis commences as the contracted chromosomes begin to elongate, pairing usually starting at the ends. Now the ascus and nucleus enlarge greatly, while the chromosomes continue to elongate until they reach what is generally recognized in the fungi as pachytene. The nucleus now reaches its maximum size (about $10 \times 20\ \mu$ in *N. crassa*). In good preparations at this stage it is possible to see clearly the two strands

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FIGS. 7–19. Meiosis in pyrenomycetes. Figs. 7–18. *Neurospora crassa.* Fig. 7. Conjugate mitoses in crozier. Fig. 8. Condensed presynaptic chromosomes (14) in early fusion nucleus. Fig. 9. Pachytene bivalents, showing chromomere pattern. Fig. 10. Pachytene, showing chromosome 2 attached to nucleolus (black spot on nucleolus due to a defect in negative). Fig. 11. Diagram of pachytene chromosomes identified by number. Fig. 12. The 7 bivalents at diakinesis. Fig. 13. Anaphase I. Fig. 14. Late anaphase I, showing 7 dyads near each end of dividing nucleus. Fig. 15. Early telophase II. Fig. 16. Nucleus at interphase II. Fig. 17. Prometaphase III, showing 7 univalents. Fig. 18. Prometaphase IV in ascospore. Fig. 19. *Sordaria fimicola.* Prometaphase III, showing the 7 morphologically distinct chromosomes, the nucleolus organizing satellite (*s*) of chromosome 2, and the centrosomes (*c*). (All photographs $\times$ 2400, except Fig. 13, $\times$ 1350; Fig. 15, $\times$ 2000; Fig. 19, $\times$ 4150.) Figs. 7, 8, 10–18, from Singleton (1953); Fig. 9, courtesy of Dr. Edward Barry; Fig. 19, from Carr and Olive (1958).

of each bivalent, with many small chromomeres in series (Figs. 9[2] and 10). In *Neurospora* each chromosome also shows one or more large chromomeres, while an achromatic gap near the largest chromomere in each of several chromosomes indicates the location of the centromere. At this time the chromosomes may be identified by length and major chromomere pattern (Fig. 11). The largest chromosome of *N. crassa* reaches a length of 22.4 μ at pachytene. The second longest is the nucleolus organizing chromosome with its small satellite at the end of the short arm. An extreme degree of heteropycnosis has been reported at pachytene and diplotene in the discomycete *Pyronema confluens* [*P. omphalodes*] (McIntosh, 1954), at which time the 12 bivalents usually appear as many smaller chromosomes.

St. Lawrence (1952) was able for the first time in a fungus to identify specific chromosomes with known linkage groups. Using X-ray mutants of *N. crassa* carrying translocations, she found that chromosomes 1 and 2 were broken close to their respective centromeres, the long arm of chromosome 1 being translocated to the short arm of chromosome 2 and the long arm of the latter being translocated to the short arm of chromosome 1. It was concluded that chromosome 1 carries the factors of linkage group II and that chromosome 2 carries linkage group IV. In addition, chromosome 6 was found to carry linkage group 1, including the compatibility locus.

Contraction of the chromosomes follows pachytene, and at diplotene and diakinesis chiasmata become apparent (Fig. 12). It is at this time that chromosome counts may be made most readily. In the ascomycetes most haploid chromosome counts range from 4 to 16, numbers of 8 or less predominating. The studies of Fincham (1949) and others have established that 7 is the common haploid number throughout the genus *Neurospora*. Probably because of the relatively small dimensions of the bivalent chromosomes, their 4-stranded nature is almost never apparent at any time during prophase or metaphase. However, I. M. Wilson (1937) in a study of *Aleuria rutilans,* which has unusually large chromosomes for a fungus, was able to observe the double nature of each of the two paired homologs at diplotene and later the double nature of the dyads on the anaphase spindle.

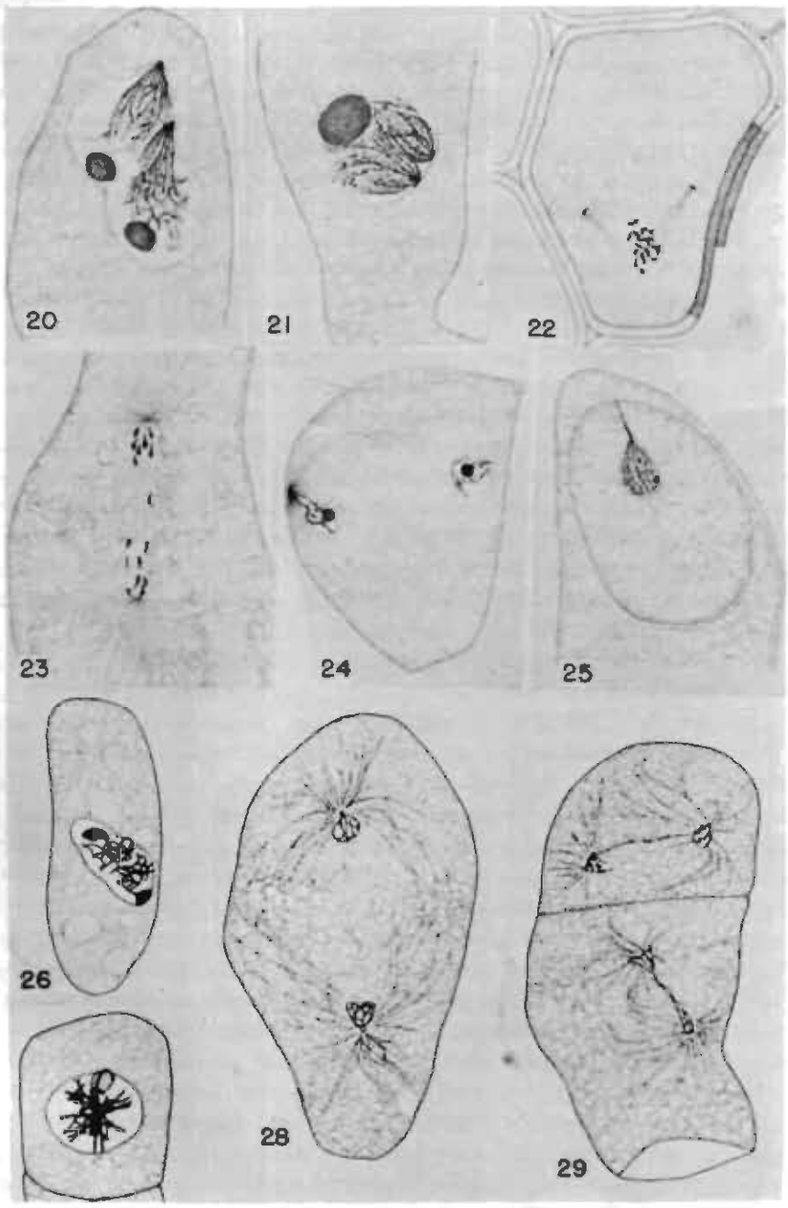
Recent genetic studies on *Sordaria fimicola* (Olive, 1959; Kitani *et al.,* 1962) indicate that the completely paired meiotic prophase bivalent should be considered as four chromatids, each in turn composed of two strands that are equivalent to the two strands comprising the double helix of the Watson-Crick model. Such a structure is strongly indicated by the occurrence of aberrant 5:3 and 4:4 ratios in asci heterozygous for a spore color factor. These aberrant tetrads apparently result from "gene conver-

sion" and are difficult to explain without acceptance of an 8-strand meiotic prophase model. The exact time of chromosome replication is not known.

With the orcein technique, Singleton was not able to determine the fate of the nuclear membrane throughout his studies on *Neurospora*. It has generally been reported that the nuclear membrane breaks down at late prophase or metaphase. At the same time, the nucleolus, which has already dwindled in size, may degenerate in the nucleus or later in the cytoplasm, where it becomes detached from the nucleus. The spindle is of intranuclear origin. The orcein and carmine techniques are not suitable for detailed observations on either the spindle or the astral rays. However, with the aid of the earlier techniques with sectioned material, both Harper (1897, 1905) and I. M. Wilson (1937) reported that a "central body" or centromere, positioned on the inside of the nuclear membrane with spindle fibers radiating back from it into the nucleus, divides near the termination of prophase and that the two daughter bodies, each with a spindle cone, move to opposite sides of the nucleus (Fig. 22). This carries the two cones of spindle fibers into the same axis, thus forming a complete spindle with the chromosomes arranged around the mid-region. In many fungi astral rays emanate from the spindle poles into the cytoplasm, but there has been little agreement on whether the poles are provided with true centrosomes. This subject will be discussed again in connection with ascospore delimitation.

Rarely are distinct metaphase plate stages described in fungi. Typically, the short, stumpy bivalents are scattered irregularly over the central part of the spindle, possibly conforming to space requirements on a small spindle. At this time, the longest chromosome of *N. crassa* measures only about 1.7 μ in length. At anaphase, disjunction of the homologs is typically asynchronous, a factor which interferes with chromosome counts during this stage (Figs. 13, 14, 23). The dyads pass to each pole and the telophase spindle elongates (Fig. 15). Interphase I follows, during which the chromosomes elongate somewhat. At this and subsequent interphases in *Neurospora* and a number of other species, the nucleolus reappears, only to degenerate at the onset of each new division. In some species there is no evidence that the nucleoli reappear during the interphases. Also, a number of investigators have reported that there is a reorganization of the nuclear membrane during interphase, while others have failed to substantiate this.

Interphase I is rather brief and is followed by contraction of the chromosomes during prophase II. The spindles of the second meiotic division typically appear in tandem arrangement in the ascus. They resemble those of the first division but are smaller. A set of univalents passes to each pole.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Following telophase II, four nuclei in a row are produced. In *N. crassa* and most other species with similar cylindric asci, there is little if any spindle overlap in the ascus. In a few species, however, it is known to occur fairly frequently, in which case the segregation pattern is superficially disturbed. However, Whitehouse (1957) has shown how such a pattern may be very simply reinterpreted.

In *N. crassa* the second interphase is of somewhat longer duration than the first, and the chromosomes become more elongated (Fig. 16). Upon contracting, the chromosomes become very distinct and their relative sizes and morphology may again be readily studied (Fig. 17). This is also true of the related *Sordaria fimicola* (Carr and Olive, 1958), whose chromosome complement is similar to that of *Neurospora* (Fig. 19).

At prophase of the third or equatorial division in the ascus of *Neurospora* and many other higher ascomycetes, structures which almost certainly may be considered centrosomes become quite distinct. Singleton described two triangular ones associated with each nucleus and believed that they arose by division of the centrosome at each pole of the anaphase II spindle. He did not determine whether the centrosome is of intranuclear or extranuclear origin. Carr and Olive (1958) observed two of them presumably inside the prophase III nucleus of *S. fimicola,* where they appeared as flat, nearly rectangular structures (Fig. 19). At metaphase they become situated at the spindle poles, where they appear beaklike and have astral rays emanating from their tips out into the cytoplasm. Subsequent developments in spore formation have been best described by Harper in the powdery mildews (Figs. 24 and 25) and by Dodge (1927) in *Neurospora.* After the haploid chromosome complement has passed to each pole, the telophase spindle elongates and the astral rays recurve in the cytoplasm, finally intersecting on the side opposite their origin and thereby cutting out around each nucleus a mass of cytoplasm. Harper stated that the astral rays coalesce laterally to delimit the spore membrane, following which the spore wall is laid down outside the membrane. Dodge (1927) has described the unique manner in which pairs of nuclei cooperate in cutting out binucleate

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FIGS. 20–25. Stages in ascus development in powdery mildews. Figs. 20 and 21. Stages in karyogamy in *Phylactinia corylea.* Fig. 22. Spindle formation at anaphase I in same species. Fig. 23. Anaphase I in *P. corylea,* with 8 chromosomes (dyads) passing to each pole. Fig. 24. Ascospore delimitation in *Erysiphe cichoracearum.* Fig. 25. Late stage in spore delimitation in *P. corylea,* from Harper (1905).

FIGS. 26–29. Stages in meiosis in *Coleosporium vernoniae.* Fig. 26. Presynaptic fusion nucleus. Fig. 27. Synapsis. Fig. 28. Late telophase I, showing astral rays participating in delimitation of basidial septum. Fig. 29. Anaphase (below) and early telophase (above) of second meiotic division, showing recurving astral rays. Magnification: × 1410, from Olive (1949).

spores heterokaryotic for mating type in such tetrasporic species as *N. tetrasperma.*

In *N. crassa* and *S. fimicola,* as well as some other species of pyrenomycetes, an equational nuclear division in the spore makes it binucleate at maturity. At prophase of this division, Singleton, as well as Carr and Olive, observed distinctly the haploid complement of 7 chromosomes (Fig. 18). To complete this picture, Somers *et al.* (1960) and Ward and Ciurysek (1962) have demonstrated in *N. crassa* the presence of 7 chromosomes at nuclear division in the vegetative hyphae.

Both Lindegren (1934) and I. M. Wilson (1937) observed in *N. crassa* and *Aleuria rutilans,* respectively, that akaryotic ascospores are occasionally delimited. Lindegren suggested that "the mechanism governing the production of spore walls enjoys a certain amount of autonomy." This concept was later fully substantiated by Heslot (1958), whose cytological studies of certain mutants of *S. macrospora* revealed that centrosomes detached from the nuclei were able to cut out enucleate spores, while chromosome groups lacking centrosomes were unable to delimit spores. In one mutant it was found that centrosomes which had become isolated in the cytoplasm were able to divide once or twice before delimiting enucleate sporoid bodies. Like Harper and others, Heslot also considered these structures to be spindle organizers.

Both cytological and genetic studies of recent years have repeatedly demonstrated that no credence can be given to Gwynne-Vaughan's concept of two different nuclear fusions in the life cycle of Ascomycetes (a concept first proposed by Harper), followed by typical meiosis and then a "brachymeiotic" division in the ascus. Also, the cytological studies of El-Ani (1956) have demonstrated that the existence of strains of *Hypomyces solani* showing different sexual potentialities (Hirsch, 1949) cannot be explained on the basis of differences in chromosome numbers, since all strains were found to have 4 chromosomes. Nor has it been possible thus far to demonstrate in any other fungus the existence of sexual differentiation based on morphological or numerical differences in the chromosome complements.

C. Basidiomycetes

Most students of phylogeny of the fungi seem to agree that the basidiomycetes evolved from the ascomycetes. The two groups clearly have much in common. In spite of the fact that meiospores are produced exogenously in the basidiomycetes and endogenously in the ascomycetes, it is apparent that clamp connections, basidia, and basidiospores are homologous with croziers, asci, and ascospores, respectively. It is, therefore, not surprising that stages in karyogamy and meiosis in the basidium (Figs. 30–34) are

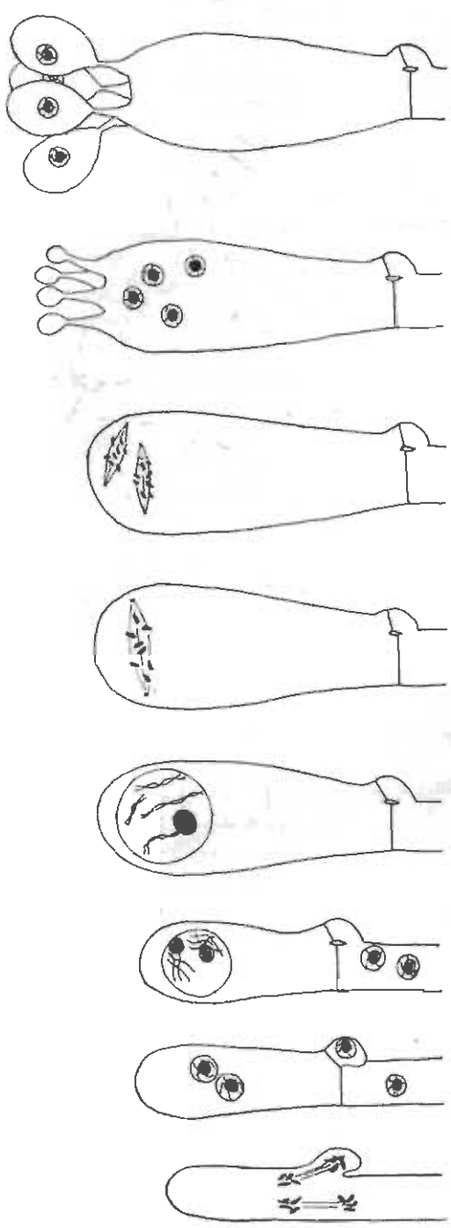


FIG. 30. Diagrammatic representation of basidial development in an autobasidiomycete: (A) Conjugate mitoses associated with clamp formation; (B) young binucleate basidium; (C) early fusion nucleus; (D) early diplotene; (E) and (F) meiotic divisions; G) basidium with a tetrad of haploid nuclei; (H) basidium with 4 haploid basidiospores.

essentially the same as those in the ascus. Since basidia are generally smaller than the asci of most ascomycetes that have been studied cytologically and their nuclei are correspondingly smaller, there is little in the way of important detail that can be added here to the description of meiosis just presented. Therefore, the discussion with respect to basidiomycetes will be less extensive.

Among the lower basidiomycetes, detailed descriptions of the meiotic process have been presented for various species of the rust genus *Coleosporium* (Holden and Harper, 1902; Olive, 1949; Sanwal, 1953), whose fusion nuclei are unusually large for a basidiomycete. In *C. vernoniae* they attain a diameter of 8.5–13 μ. Stages in meiosis proceed very much as in *Neurospora*. Again, the presynaptic chromosomes are more condensed than is characteristic of higher organisms (Fig. 26). After synapsis gets under way (Fig. 27), the chromosomes elongate markedly. Both here and in the higher ascomycetes, the prolonged stage of chromosomal elongation is probably associated with the metabolic function of the nucleus during enlargement of the ascus or basidium. In *C. vernoniae*, the chromosomes finally contract into 8 short prometaphase bivalents. Disjunction of chromosomes at anaphase is asynchronous. At telophase of each of the meiotic divisions in the basidium, polar radiations recurve in the cytoplasm and participate in the delimitation of a septum at right angles to each spindle axis (Figs. 28 and 29), thus producing a 4-celled basidium. This function of the astral rays in *Coleosporium* is probably indicative of phylogenetic relationship with the process of ascospore delimitation by astral rays (Olive, 1949). Haploid chromosome numbers in heterobasidiomycetes range from 3 to 8.

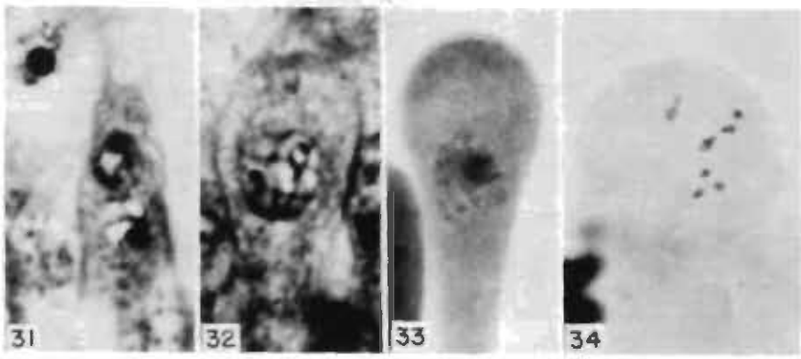
Among the more illuminating earlier studies of meiosis in sectioned material in higher basidiomycetes are those of Wakayama (1930, 1932) and Colson (1935), but the best of these studies can add little of importance to what has already been presented. There is little concise information on the details of meiotic prophase. Wakayama and certain other investigators have reported centrosomes at the poles of the meiotic spindles.

Several investigators have recently applied the carmine and orcein squash techniques to meiotic studies of higher basidiomycetes. Such methods have enabled Evans (1956) and Hughes (1961), in a study of *Agaricus campestris*, to determine that the haploid chromosome number in the 2-spored cultivar and in the 4-spored wild type is 12.[3]

Dr. Robert Lowry of the University of Michigan has generously permitted the publication here for the first time of several of his photographs

[3] B. C. Lu [*Am. J. Botany* **51**:343–347 (1964)] has reported $n = 12$ in the gasteromycete, *Cyathus stercoreus*, a species believed to have evolved by allotetraploidy.

showing meiosis in *Amanita fulva* (Figs. 31–34) in sectioned material as well as in whole basidia stained by the carmine squash technique. The figures emphasize the similarity to meiosis in higher ascomycetes. Figure 33 probably represents the first clear photograph of pachytene bivalents in an



Figs. 31–34. Stages in basidial development in *Amanita fulva*. Fig. 31. Prefusion nuclei in young basidium. Fig. 32. Prophase in fusion nucleus. Fig. 33. Late pachytene, showing bivalent chromosomes. Fig. 34. Prometaphase I. (× 2000). Figs. 31 and 32, from hematoxylin-stained sections; Figs. 33 and 34, from acetocarmine squash. Courtesy of Dr. Robert J. Lowry.

autobasidiomycete. Lowry has found the haploid chromosome number to be 8. Chromosome counts in the autobasidiomycetes vary from 2 to 12, the unusual number of 3 having been reported in *Schizophyllum commune* by Ehrlich and McDonough (1949).

In conclusion, it may be stated that, while meiosis in fungi differs from that in most higher forms in such details as the onset of synapsis while the chromosomes are in a much contracted state, the production of an intranuclear spindle, and the asynchronous disjunction of the chromosomes at anaphase, there is much more in common than at variance with what occurs in higher forms. In addition, genetic studies further emphasize a fundamental similarity between the meiotic process in fungi and that in higher forms.

REFERENCES

Burgeff, H. (1915). Untersuchungen über Variabilität, Sexualität und Erblichkeit bei *Phycomyces nitens* Kuntze. *Flora (Jena)* 108:353–448.

Burgeff, H. (1929) Variabilität, Vererbung und Mutation bei *Phycomyces blakesleeanus* Bgff. *Z. Induktive Abstammungs-Vererbungslehre* 49:26–94.

Carr, A. J. H., and L. S. Olive. (1958). Genetics of *Sordaria fimicola*. II. Cytology. *Am. J. Botany* 45:142–150.

Collins, O. R. (1961). Heterothallism and homothallism in two myxomycetes. *Am. J. Botany* 48:674–683.

Colson, B. (1935). The cytology of the mushroom, *Psalliota campestris*. *Ann. Botany (London)* 49:1–18.

Couch, J. N. (1926). Heterothallism in *Dictyuchus*, a genus of water moulds. *Ann. Botany (London)* 40:848–881.

Dodge, B. O. (1927). Nuclear phenomena associated with heterothallism and homothallism in the ascomycete *Neurospora*. *J. Agr. Res.* 35:289–305.

Ehrlich, H. G., and E. S. McDonough. (1949). The nuclear history in the basidia and basidiospores of *Schizophyllum commune* Fries. *Am. J. Botany* 36:360–363.

El-Ani, A. S. (1956). Ascus development and nuclear behavior in *Hypomyces solani* f. *cucurbitae*. *Am. J. Botany* 43:769–778.

Emerson, R., and C. M. Wilson. (1954). Interspecific hybrids and cytogenetics and cytotaxonomy of *Euallomyces*. *Mycologia* 46:393–434.

Evans, H. J. (1956). Chromosomes of the cultivated mushroom. *Nature* 178:1005–1006.

Fincham, J. R. S. (1949). Chromosome numbers in species of *Neurospora*. *Ann. Botany (London)* [N. S.]13:23–26.

Harper, R. A. (1897). Kernteilung und freie Zellbildung im Ascus. *Jahrb. Wiss. Botan.* 30:249–284.

Harper, R. A. (1905). Sexual reproduction and the organization of the nucleus in certain mildews. *Carnegie Inst. Wash. Publ.* 37:104 pp.

Heslot, H. (1958). Contribution à l'étude cytogénétique et génétique des Sordariacées. *Rev. Cytol. Biol. Vegetales* 19:1–209.

Hirsch, H. E. (1949). The cytogenetics of sex in *Hypomyces solani* f. *cucurbitae*. *Proc. Natl. Acad. Sci. U.S.* 33:268–270.

Holden, R. J., and R. A. Harper. (1902). Nuclear divisions and nuclear fusion in *Coleosporium sonchi-arvensis* Lév. *Trans. Wisconsin Acad. Sci.* 14:63–82.

Hughes, D. T. (1961). Chromosomes of the wild mushroom. *Nature* 190:285–286.

Kitani, Y., L. S. Olive, and A. S. El-Ani. (1962). Genetics of *Sordaria fimicola*. V. Aberrant segregation at the *g* locus. *Am. J. Botany* 49:697–706.

Köhler, F. (1935). Genetische Studien an *Mucor mucedo* Brefeld. *Z. Induktive Abstammungs-Vererbungslehre* 70:1–54.

Kusano, S. (1930). Cytology of *Synchytrium fulgens* Schroet. *J. Coll. Agr., Imp. Univ. Tokyo* 10:347–388.

Lindegren, C. C. (1934). The genetics of *Neurospora* VI. Bisexual and akaryotic ascospores from *Neurospora crassa*. *Genetics* 16:315–320.

McClintock, B. (1945). *Neurospora*. I. Preliminary observations of the chromosomes of *Neurospora crassa*. *Am. J. Botany* 32:671–678.

McIntosh, D. L. (1954). A cytological study of ascus development in *Pyronema confluens* Tul. *Can. J. Botany* 32:440–446.

Olive, L. S. (1949). Karyogamy and meiosis in the rust *Coleosporium vernoniae*. *Am. J. Botany* 36:11–54.

Olive, L. S. (1953). The structure and behavior of fungus nuclei. *Botan. Rev.* 19: 439–586.

Olive, L. S. (1959). Aberrant tetrads in *Sordaria fimicola*. *Proc. Natl. Acad. Sci. U.S.* 45:727–732.

St. Lawrence, P. (1952). The association of particular linkage groups with their respective chromosomes in *Neurospora crassa*. Ph.D. thesis, Columbia University.

Sansome, E. R., and B. J. Harris. (1962). Use of camphor-induced polyploidy to determine the place of meiosis in fungi. *Nature* **196**:291–292.

Sanwal, B. D. (1953). The development of the basidium in *Coleosporium sidae*. *Bull. Torrey Botan. Club* **80**:205–216.

Singleton, J. R. (1953). Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. *Am. J. Botany* **40**:124–144.

Sjöwall, M. (1946). Über die zytologischen Verhältnisse in dem Keimschlauchen von *Phycomyces blakesleeanus* und *Rhizopus nigricans*. *Botan. Notiser* pp. 331–334.

Somers, C. E., R. P. Wagner, and T. C. Hsu. (1960). Mitosis in vegetative nuclei of *Neurospora crassa*. *Genetics* **45**:801–810.

Tischler, G. (1951). Allgemeine Pflanzenkaryologie. 2. Hälfte: Kernteilung und Kernverschmelzung. In "Handbuch der Pflanzenanatomie" Vol. 2, pp. 254–278.

Wakayama, K. (1930). Contributions to the cytology of fungi. I. Chromosome number in Agaricaceae. *Cytologia (Tokyo)* **1**:369–388.

Wakayama, K. (1932). Contributions to the cytology of fungi. IV. Chromosome number in Autobasidiomycetes. *Cytologia (Tokyo)* **3**:260–284.

Ward, E. W. B., and K. W. Ciurysek. (1962). Somatic mitosis in *Neurospora crassa*. *Am. J. Botany* **49**:393–399.

Whitehouse, H. L. K. (1957). Use of tetratype frequencies for estimating spindle overlapping at the second division of meiosis in 'ordered' tetrads. *Nature* **179**:162–163.

Wilson, C. M. (1952). Meiosis in *Allomyces*. *Bull. Torrey Botan. Club* **79**:139–159.

Wilson, C. M., and I. K. Ross. (1955). Meiosis in the myxomycetes. *Am. J. Botany* **42**:743–749.

Wilson, I. M. (1937). A contribution to the study of the nuclei of *Peziza rutilans* Fries. *Ann. Botany (London)* [N. S.] **1**:655–671.

CHAPTER 8

Chemical Constituents of the Fungal Cell

1. Elemental Constituents and Their Roles

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I. INTRODUCTION

Chemical analyses to have meaning should be related to the species, age, and type of cells analyzed, the composition of the medium in which the cells were grown, and environmental factors. Fungus cells do not have an unvarying composition.

Relatively few complete analyses of the mineral elements (ash) found in fungus cells have been reported. Richards and Troutman (1940) made a spectroscopic examination of the mineral elements present in the ash of yeast cells grown in various media. They found: Ba, Bi, B, Ca, Cr, Cu, Au, Fe, La, Pb, Mg, Mn, P, Pt, K, Ag, Na, Tl, Sn, and Zn. Recently, Grant and Pramer (1962) analyzed quantitatively five samples of yeast extract and found Al, Ba, Cd, Co, Cr, Cu, Fe, Ga, Mg, Mn, Mo, Ni, Pb, Sn, Sr, Ti, V, and Zn. Some of these elements may not have been present in the original yeast cells.

The composition of fungus cells may be made to vary widely. Conway and Moore (1954) subjected yeast cells to repeated fermentations in a medium containing glucose and sodium citrate; 98% of the potassium was replaced by sodium after seven 2-hour fermentations. By fermenting a "sodium yeast" in a glucose-magnesium acetate medium most of the sodium and potassium was replaced by magnesium. The magnesium yeast contained 3, 2.6, and 292 meq/kg wet yeast of potassium, sodium, and magnesium, respectively (Conway and Beary, 1962).

II. ESSENTIAL ELEMENTS

At least a third, perhaps half, of the elements have been found in fungus cells. Analysis in itself does not reveal which elements are essential, or the functions of essential elements in the living cell. An essential element is one that is indispensable. Nicholas (1957, 1961) would replace the term "essential element" by "metabolic nutrient." More importantly, he distinguishes between the classical and the functional methods of determining essentiality.

A. The Classical Method

The classical method uses a highly purified medium, complete in all respects save one. When the missing essential element is added to such a medium, the test fungus grows. In addition, the response of the test fungus should be proportional, within limits, to the concentration of the essential element in question.

There is no difficulty in showing that those elements required in high concentrations are essential. These include carbon, nitrogen, phosphorus, potassium, and magnesium. Difficulties arise when low concentrations of an element are required. These elements are called variously micro, trace, or minor elements and include iron, zinc, manganese, copper, calcium, and possibly others.

The primary problem in the classical method is to remove completely, or to reduce to very low levels, the metallic contamination in the medium. Only then can the increased growth be attributed to the element under study.

B. Various Methods of Removing Metallic Contamination

Steinberg (1935) autoclaved the complete medium with calcium carbonate, which was then filtered off. Nicholas (1952) used a variety of methods including precipitation with hydrogen sulfide (Cu and Mo), complex formation with 8-hydroxyquinoline followed by extraction with chloroform (Zn, Fe, and Ga), and coprecipitation of copper and manganese. Media so purified still contained traces of metals: copper, 1 μg/l; iron, zinc, manganese, and gallium, 0.2 μg/l; and molybdenum, 0.1 m μg/l. See Donald *et al.* (1952) for a comparison of 15 methods of removing metallic contamination from media.

The early investigators were hampered by the solubility of the chemical glassware then available (Javillier, 1914). Quartz vessels are considered

o be the most desirable, but Pyrex glass is satisfactory. Plastic culture vessels have been used; presumably polypropylene vessels which can be autoclaved would be satisfactory.

There is an extensive literature on the metal ion requirements of fungi, ncluding reviews by Steinberg (1939b), Foster (1939, 1949), Perlman 1949), Lavollay (1955), Lilly and Barnett (1951), Hawker (1950), and Cochrane (1958).

C. The Functional Method

The basic approach of the functional method is to show that an element plays an indispensable role in some enzyme system isolated from a fungus (or other organism). The classical method is usually employed first; the more sophisticated method can then be used to answer questions that the classical method cannot. If it can be shown that a fungus contains an essential enzyme system that requires a specific metal as a constituent or activator, this is excellent proof that the metal is essential (see pages 171–173).

The use of electron paramagnetic resonance spectroscopy to study the function of the transition metals in biological systems is relatively new. Nicholas *et al.* (1962) used this method to study iron, manganese, molybdenum, and copper complexes in bacteria. Presumably, the method is applicable to fungi as well.

III. BIOLOGICAL SUBSTITUTION

If one element completely replaces another the concept of an essential element is in need of modification and amplification. The total replacement of one element by another has rarely been reported in fungi. Ingraham and Emerson (1954) reported that strontium completely replaced calcium in the nutrition of *Allomyces arbuscula,* strain Burma IDb. However, some ten times as much strontium was required as calcium. Partial biological substitution of sodium for potassium in *Aspergillus niger* was reported by Steinberg (1946). No evidence was presented to indicate that sodium could completely replace potassium. In nature, or on complex natural media, partial biological substitution may be more common than usually thought (see the interesting discussion by MacLeod and Snell, 1951).

IV. ION ANTAGONISM

The concentration of an essential ion required may depend upon the concentration of other ions in the medium. While this phenomenon is of

great interest in cultural studies, it may be assumed that the composition of the cells is also affected. MacLeod and Snell (1951) found competition between potassium and sodium ions in the growth of *Saccharomyces carlbergensis*. As the concentration of sodium ions was increased it was necessary to increase the concentration of potassium ions to obtain an equivalent amount of growth. Abelson and Aldous (1950) found the amounts of cobalt and nickel accumulated by cells of *Torula* [*candida*] *utilis* to be dependent on the magnesium concentration of the medium.

V. ESSENTIAL NONMETALLIC ELEMENTS

The nonmetals hydrogen, carbon, nitrogen, oxygen, phosphorus, and sulfur are essential for all fungi. Some fungi metabolize, or accumulate, arsenic, selenium, chlorine, silicon, and other nonmetals.

A. Hydrogen

Hydrogen is the most abundant element in the universe, both on a weight and on an atom per cent basis. On the atom per cent basis, hydrogen is the most abundant element in the cells of fungi and other organisms. However, most analytical data are reported on the basis of weight per cent, a method that obscures the relative numbers of different atoms found in cells.

Hydrogen is a structural and functional element. This element is contained in all the organic compounds found in cells. For some of the transformations of organic compounds in the living cell see Chapters 10 to 12.

The hydrogen content of *dry* cells is seldom reported. From the standpoint of proximate analysis, fungus cells are mainly carbohydrate, protein, fat, and ash. Presumably, an average value for fungus cells would lie between 6 and 8%. Hilpert *et al.* (1937) reported *Aspergillus niger* mycelium to contain 6.7% hydrogen.

1. Water

Fungus cells (excepting some spores) are mainly water. Hawker (1950) cites data on the water content of various sporophores which ranged from 73.7% (*Pleurotus ostreatus*) to 93.85% (*Amanita rubescens*). The water content of mycelium grown in liquid medium is difficult to measure exactly because it is difficult to judge when the surface water has been removed.

Spores, in general, contain less water than mycelium. Yarwood (1950) determined the water content of the asexual spores of 9 species of fungi:

Penicillium digitatum, 6.0%; *Uromyces appendiculatus*, 12%; *Aspergillus niger*, 13%; *Peronospora destructor* and *Botrytis cinerea*, 17%; *Monilinia (Sclerotina) fructicola*, 25%; *Erysiphe cichoracearum*, 52%; *E. polygoni*, 72%; and *E. graminis*, 75%. The high water content of spores of *Erysiphe* spp. is not typical of most others.

2. Bound Water

Water exists in fungus cells in two forms. Free water is that removed from cells by drying at room temperature over a desiccant in a vacuum. Bound water is that removed by additional drying at elevated temperatures (80°–110°C). Todd and Levitt (1951) measured the bound water content of mycelium of *Aspergillus niger* cultivated in media containing from 50 to 410 g of glucose per liter. The bound water content increased about tenfold as the glucose concentration increased. Presumably, bound water is essential in the life processes of fungi, and the concentration varies with the environment.

B. Carbon

Raistrick *et al.* (1931) made extensive studies of *Aspergillus*, *Penicillium*, and *Fusarium*. The extreme carbon values for *Aspergillus* were 56.6% (*A. sydowii*) and 43.5% (*A. parasiticus*); for *Penicillium*, 62.9% (*Scopulariopsis brevicaulis*) and 45.3% (*Penicillium viridicatum*); and for *Fusarium*, 61.2% (*F. rhizophilum*) and 49.0% (*F. lini*).

Whitaker (1951) determined the carbon content of the mycelium of 40 species, representing 21 genera of wood-rotting fungi. In general, the mycelium of this group of fungi contained less carbon than those studied by Raistrick *et al.* (1931). The effects of continuous and restricted aeration on the carbon content of the mycelium were compared for a number of species. The carbon content of *Peniophora gigantea* under restricted aeration was 44.1%, and under continuous aeration 40.0%. On the other hand, the mycelium of *Fomes fomentarius* contained 40.7% carbon under restricted aeration, and 44.3% under continuous aeration.

C. Nitrogen

The nitrogen content of fungus cells is variable and depends on species, age, and composition of the medium. Nielsen and Schneider (1957) grew *Rhodotorula gracilis* in media containing 1 and 10 g of asparagine per liter. The nitrogen content of the cells was 1.34 and 9.72% in the low and high asparagine media, respectively. Similar results were obtained with media

containing 1 and 10 g of ammonium sulfate per liter. The nitrogen content was 2.94 and 7.00% in the low and high ammonium sulfate media, respectively [see Cochrane (1958) for additional citations].

D. Oxygen

No direct analyses of mycelium for oxygen appear to have been published. An estimate, based on carbohydrate, protein, and fat content, would lie in the range of 25–35%. Most of the organic compounds found in cells contain oxygen. See Chapter 12 for information on the role of oxygen in respiration.

E. Phosphorus

This element is usually the most abundant nonmetallic element found in fungus ash. Analytical results in the literature are often expressed as percentages of phosphorus pentoxide (P_2O_5 contains 43.64% P). The literature has been reviewed by Foster (1949) and Cochrane (1958).

Rennerfelt (1934) studied the salt uptake of *Aspergillus niger* under a variety of nutritional conditions. The phosphorus content of mycelium grown in medium containing 1/2500 mole KH_2PO_4 per 100 ml was 0.014 mmole/gm; when the phosphate content of the medium was increased tenfold, the mycelium contained 0.095 mmole of phosphorus per gram. Increasing the phosphate content of the medium to still higher levels increased the phosphorus content of the mycelium slightly. Bajaj *et al.* (1954) found inorganic and organic phosphates to be easily leached from the spores of *A. niger*. The phosphorus content of spores was 3–4 times that of the mycelium on which they were borne (Rennerfelt reported that spores of *A. niger* contained 1.4 times as much phosphorus as mycelium). Young mycelium was found to contain more phosphorus than old; in one experiment the phosphate content of 3-, 6-, and 9-day-old mycelium was 12.7, 4.9, and 2.4 μg/mg, respectively.

The roles of phosphorus in metabolism and energy transfer are discussed in Chapter 10.

F. Sulfur

The sulfur content of fungus cells is variable; it is dependent on the concentration of this element in the medium, or substrate, and on other factors. Nielsen and Lundin (1955) found the nitrogen content of the medium to influence the sulfur content of the cells of *Rhodotorula gracilis*. Presumably, this was due in part to the increased protein content of the

cells, since most proteins contain methionine and cysteine. Cells from the high nitrogen medium contained 0.53% sulfur; those from the low nitrogen medium contained 0.11%. These investigators also found that the sulfur content of the medium influenced that of the cells. When the medium contained 0.5 g of sulfur per liter, the cells contained 0.21% sulfur; and when the sulfur content of the medium was reduced to 0.0025 g/l, the sulfur content of the cells was 0.10%.

The sulfur-containing compounds found in mycelium include enzymes and other proteins, the amino acids cysteine and methionine, the tripeptide glutathionine, and two vitamins, thiamine and biotin.

VI. OTHER NONMETALLIC ELEMENTS

Fungi metabolize, or accumulate, nonessential elements. Holzapfel and Engel (1954) cultured *Aspergillus niger* on a special medium containing high concentrations of silicon (280–330 mg SiO per liter). The surface mycelium contained about 2%, and the submerged mycelium contained about 9%, silica. Selenium appears to be metabolized by *A. niger* (Weissman and Trelease, 1955); the organic selenium compounds formed were not identified. *Scopulariopsis brevicaulis* synthesizes arsine from arsenic trioxide (Challenger *et al.*, 1954). A number of fungi synthesize organic chlorine compounds, e.g., griseofulvin. For a review, see Foster (1949).

VII. ESSENTIAL METALLIC ELEMENTS

The following metallic elements appear to be essential for all fungi tested: potassium, magnesium, iron, zinc, manganese, copper, and molybdenum. Calcium is essential for some, but not all, fungi. Restricted evidence is available for the essentiality of a few other metals.

Many of the essential metals may be assumed to function as enzyme activators or as constituents of enzymes. The effects of suboptimal concentrations are manifested in various ways: reduced growth, decreased sporulation, increased or decreased concentrations of various enzymes in the cells, and increased or decreased synthesis of various metabolites. Many of the essential elements are toxic in too high a concentration.

A. Potassium

Spores and mycelium usually contain more potassium than any other metal. Rennerfelt (1934) found spores of *Aspergillus niger* to contain 4.9 times as much potassium as did mycelium. Rippel and Behr (1934) analyzed the mycelium of 5 isolates of *A. niger* and found the potassium con-

tent to depend on age of the culture, potassium content of the medium, and the isolate. Young mycelium contained more potassium than old mycelium. The highest potassium content found was 2.95%. Rosselet (1953) used *A. niger* to assay the available potassium content of soil.

Nakamigawa and Okuda (1960) found *Mucor mandschuricus* to accumulate pyruvic acid in a medium lacking thiamine, but containing potassium or rubidium ions. Pyruvic acid accumulated when the medium contained thiamine and rubidium, but not when both thiamine and potassium were present. If pantothenic acid and thiamine were added to a medium containing rubidium, pyruvic acid did not accumulate.

B. Magnesium

This element appears to be essential for all fungi. Nicholas and Fielding (1951) found the growth of *Aspergillus niger* to be proportional to the magnesium content of the medium; 20 mg/l was required for maximum production of mycelium. Vail and Lilly (1961) found *Phycomyces blakesleeanus* to tolerate magnesium concentrations of 820 mg/l without inhibition of growth, although carotene production was depressed at this concentration. *Allomyces arbuscula* required magnesium for growth; 9 mg/l sufficed for maximum growth and 200 mg/l was toxic (Ingraham and Emerson, 1954).

Rippel and Behr (1930) found the magnesium content of *Aspergillus niger* mycelium to be dependent on the age of the culture and on whether the medium contained ammonium or nitrate nitrogen. Three-day-old mycelium grown in the ammonium-nitrogen medium contained 0.13% magnesium, whereas comparable mycelium grown in the nitrate-nitrogen medium contained 0.265% magnesium. Rennerfelt (1934) found the spores of *A. niger* to contain 7.1 times as much magnesium as did mycelium.

Conway and Beary (1962) prepared a yeast containing 292 meq of magnesium per kilogram wet cells and found this "magnesium yeast" to grow slowly in the absence of potassium. The cells of the "magnesium yeast" were abnormal, being elongated and associated in chains.

VIII. MICROELEMENTS

Iron, zinc, manganese, copper, and molybdenum are variously called micro, minor, and trace elements because they are required in low concentrations. Calcium and perhaps other elements may be included in this group. Numerous reports in the literature state that this or that micro-

element is not required by a particular fungus. Judgment is required in evaluating negative results.

A. Iron

This element is essential for all fungi. As little as 0.1 μg of iron in 50 ml of medium may be detected by *Aspergillus niger* (Nicholas, 1952). The classical method of determining a requirement for iron is supported by the functional approach, since this element is a constituent of various enzymes including the cytochromes, cytochrome oxidase, catalase, and others.

Ferrous or ferric ions satisfy the iron requirements of most fungi. *Piloboolus* requires iron to be supplied as various chelated iron compounds (heme, coprogen, or ferrichrome) according to Page (1962). For a review of chelated iron compounds in nutrition, see Neilands (1957).

B. Zinc

This element, like iron, appears to be essential for all fungi. Zinc is a constituent, or activator, of a number of enzymes. Alcohol dehydrogenase contains 4 atoms of zinc per molecule (Vallee and Hoch, 1955). Zinc is essential for the functioning of this enzyme.

Grimm and Allen (1954) found a zinc concentration of 0.001 ppm to be sufficient for optimal growth of *Ustilago sphaerogena*. The cells were colorless when grown in media containing this concentration of zinc, but were pink when cultured in a sucrose-yeast extract medium. The pink cells contained as much as 1% cytochrome c. High yields of cytochrome c were obtained when the sporidia were cultured in a synthetic medium containing 1 ppm of zinc, 2 ppm of thiamine, and ammonium nitrogen. Thus, the concentration of zinc influenced the synthesis of cytochrome c, an iron-containing enzyme.

The reduced growth and sporulation of fungi cultured in media containing suboptimal concentrations of zinc (and other essential microelements) is readily apparent. Other effects may be detected by different techniques. Nason *et al.* (1951) cultured *Neurospora crassa* in a medium containing sufficient zinc for half-maximum growth, and also in the basal medium fortified with additional zinc. As expected, alcohol dehydrogenase was found in mycelium cultured in the medium containing added zinc; this zinc-containing enzyme was not detected in 4 out of 5 samples of mycelium grown in the low-zinc medium. The concentration of tryptophan synthetase was much lower in mycelium grown in the medium to which

zinc was not added than when adequate concentrations of this element were present. The DPNase content of the zinc-deficient mycelium was 10–20 times greater than in the control mycelium. Moderate zinc deficiency did not affect the concentration of fumarase.

C. Manganese

A requirement for manganese is difficult to establish for some fungi using classical methods. Nicholas (1952) found the mycelial weight of *Aspergillus niger* to be little affected by the omission of manganese from the medium, but very few spores formed in the absence of added manganese. Donald *et al.* (1952) reported a 50% reduction in growth of *A. niger* when manganese was omitted from media purified by certain techniques. These authors also showed that different isolates of *A. niger* varied in their requirements for manganese.

By using the functional method, Medina and Nicholas (1957) demonstrated that hydroxylamine reductase from *Neurospora crassa* requires manganese for activity. In addition, mycelium grown in media low in manganese contained 57% as much hydroxylamine reductase as "normal" mycelium. Mycelium of *N. crassa* grown in media low in manganese also contained reduced concentrations of nitrite and hyponitrite reductases.

D. Copper

The essential nature of copper has been demonstrated by classical methods. Steinberg (1950) found that the omission of copper from a highly purified medium resulted in a reduction of growth of *Sclerotium rolfsii, Cercospora nicotianae, Fusarium oxysporum, Pythium irregulare,* and *Thielaviopsis basicola.* Nicholas (1952) found the growth of *Aspergillus niger* to be proportional, within limits, to the copper concentration of the medium. As little as 0.05 μg/50 ml could be detected. Many investigators have noted that the spore color of *A. niger* changes from yellow to brown and then to black as the copper concentration is increased.

A number of enzymes, including tyrosinase and laccase, contain copper. Medina and Nicholas (1957) showed that copper is involved in the biosynthesis of nitrite and hyponitirite reductases.

E. Molybdenum

Steinberg (1936, 1937), using the classical method, found that *Aspergillus niger* required more molybdenum when cultured on a medium containing nitrate nitrogen than when ammonium nitrogen was used. Ex-

tremely low concentrations of molybdenum are detected by *A. niger*. Nicholas and Fielding (1950) found the addition of 0.1 mμg of molybdenum to 50 ml of purified medium resulted in an increase of 57 mg of mycelium. For other fungi that require molybdenum, see Steinberg (1950).

Molybdenum is known to be required for the formation and activation of nitrate reductase in fungi (and other organisms). Nicholas *et al.* (1953) cultured *Neurospora crassa* and *A. niger* in the absence and presence of added molybdenum. In all experiments, the omission of added molybdenum reduced the amount of nitrate reductase synthesized. Nicholas and Nason (1954) showed that *N. crassa* nitrate reductase is a flavoprotein containing molybdenum. Nicholas (1957) found the nitrate reductase content of mycelium of *N. crassa* grown in the absence of added molybdenum to be 10% of that found in mycelium grown in its presence. Growth in the absence of added molybdenum was 29% of the control. Nitrate reductase is an induced enzyme, little or none being synthesized by *A. niger* and *N. crassa* grown in media containing ammonium nitrogen. Molybdenum was not replaced by iron, zinc, manganese, nickel, silver, tungsten, chromium, vanadium, cobalt, or boron.

Medina and Nicholas (1957) summarized the steps in the reduction of nitrate to ammonia by *N. crassa* as follows:

$$\text{Nitrate} \xrightarrow{\text{Mo}} \text{nitrite} \xrightarrow{\text{Cu.Fe}} \text{hyponitrite} \xrightarrow{\text{Cu.Fe}} \text{hydroxylamine} \xrightarrow{\text{Mn}} \text{ammonia}$$

F. Calcium

Steinberg (1948) reinvestigated the calcium requirements of fungi and concluded that this element was not required by *Aspergillus niger* or *Fusarium oxysporum* var. *nicotianae*. *Alternaria solani* and other species made reduced growth in the absence of added calcium. For a review of the early literature, see Rippel and Stoess (1932).

Calcium does not appear to be required for the growth of *Chaetomium* sp., but it frequently increases the formation of perithecia (Basu, 1951). More investigation will be required to determine the role of calcium in fungi.

G. Scandium, Vanadium, and Gallium

Steinberg (1939a) found *Aspergillus niger* to make increased growth in a glycerol medium when scandium was added. Steinberg (1950) reported that scandium partially replaced calcium for *Alternaria solani*. Vanadium appeared to be essential for an isolate of *A. niger* (Bertrand, 1941). Omission of gallium from a highly purified medium led to a reduc-

tion in growth and sporulation by an isolate of *A. niger* (Steinberg, 1938). Under some conditions, growth of *Corticium solani* and *Pythium irregulare* was reduced when gallium was omitted from the medium (Steinberg, 1950).

H. Essential Elements Not Required by Fungi

Boron, fluorine, sodium, chlorine, iodine, sodium, cobalt, and vanadium are essential for various organisms, but none of them appear to be required by fungi.

I. Nonessential Metals

Often fungi are in the presence of, and may absorb, nonessential metals. Presumably, none of these metals are inert, and in sufficient concentration they may be toxic. The heavy metals as fungicides are treated in Chapter 20. A few examples of the effects of nonessential metals on particular fungi are noted below.

Lilly *et al.* (1962) found sodium and lithium chlorides to inhibit germination of unheated sporangiospores of *Phycomyces blakesleeanus*. Lithium chloride (4.3 mmoles/l) inhibited growth of *P. blakesleeanus*, whereas the same concentration of sodium chloride did not. Cobalt is not essential for any fungus, so far as is known, but Nicholas (1952) reported that *Aspergillus niger* synthesized vitamin B_{12} when cobalt was added to the medium. Mandels and Reese (1957) noted that the production of cellulase by *Trichoderma viride* increased when calcium (30 mg/l) was added to an unpurified medium when cobalt was present.

IX. SUMMARY

The elements essential for all fungi include hydrogen, carbon, nitrogen, oxygen, phosphorus, sulfur, potassium, magnesium, iron, zinc, manganese, copper, and molybdenum. Some fungi require calcium. The actual composition of fungi growing in nature, or on nonpurified media, may contain 2 to 3 times as many elements as are listed above. The following elements comprise the greater part of fungus cells: hydrogen, oxygen, carbon, nitrogen, phosphorus, sulfur, potassium, and magnesium. Iron, zinc, manganese, copper, and molybdenum occur in low concentrations in fungus cells and exert their physiological effects as components or activators of enzymes. The nonessential elements may be accumulated; in sufficient concentration many of them are toxic.

REFERENCES

Abelson, P. H., and E. Aldous. (1950). Ion antagonism in microorganisms: interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc, and manganese. *J. Bacteriol.* **60**:401–413.

Bajaj, V., S. P. Damle, and P. S. Krishnan. (1954). Phosphate metabolism of mold spores. 1. Phosphate uptake by the spores of *Aspergillus niger*. *Arch. Biochem. Biophys.* **50**:451–460.

Basu, S. N. (1951). Significance of calcium in the fruiting of *Chaetomium* species, particularly *Chaetomium globosum*. *J. Gen. Microbiol.* **5**:231–238.

Bertrand, D. (1941). Vanadium comme facteur de croissance pour l'*Aspergillus niger*. *Bull. Soc. Chim. Biol.* **23**:467–471.

Challenger, F., D. B. Lisle, and P. B. Dransfield. (1954). Biological methylation. 14. The formation of trimethylarsine and dimethylselenide in mould cultures from methyl sources containing C^{14}. *J. Chem. Soc.* pp. 1760–1771.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Conway, E. J., and M. E. Beary. (1962). A magnesium-yeast and its properties. *Biochem. J.* **84**:328–333.

Conway, E. J., and P. T. Moore. (1954). A sodium-yeast and some of its properties. *Biochem. J.* **57**:523–528.

Donald, C., B. I. Passey, and R. J. Swaby. (1952). A comparison of methods for removing trace metals from microbiological media. *J. Gen. Microbiol.* **7**:211–220.

Foster, J. W. (1939). The heavy metal nutrition of fungi. *Botan. Rev.* **5**:207–239.

Foster, J. W. (1949). "Chemical Activities of Fungi," 648 pp. Academic Press, New York.

Grant, C. L., and D. Pramer. (1962). Minor element composition of yeast extract. *J. Bacteriol.* **84**:869–870.

Grimm, P. W., and P. J. Allen. (1954). Promotion by zinc of the formation of cytochromes in *Ustilago sphaerogena*. *Plant Physiol.* **29**:369–377.

Hawker, L. E. (1950). "Physiology of Fungi," 360 pp. Univ. of London Press, London.

Hilpert, R. S., G. Friesen, and W. Rosseé. (1937). Der Einfluss das Nährbodens auf die chemische Zusammensetzung des *Aspergillus niger*. *Biochem. Z.* **289**:193–197.

Holzapfel, L., and W. Engel. (1954). Den Einfluss organischer Kieselsäure-Verbindungen auf das Wachstum von *Aspergillus niger* und *Triticum*. *Z. Naturforsch.* **9b**:602–606.

Ingraham, J. L., and R. Emerson. (1954). Studies of the nutrition and metabolism of the aquatic phycomycete, *Allomyces*. *Am. J. Botany* **41**:146–152.

Javillier, M. (1914). Une cause d'erreur dans l'étude de l'action biologique des éléments chimiques: la présence de traces de zinc dans la verre. *Compt. Rend.* **158**:140–143.

Lavollay, J. (1955). Les oligo-éléments dans la nutrition et la croissance des microorganismes. *Rev. Gen. Sci. Pure Appl. Bull. Assoc. Franc. Avan. Sci.* **62**:102–126.

Lilly, V. G., and H. L. Barnett. (1951). "Physiology of the Fungi," 464 pp. McGraw-Hill, New York.

Lilly, V. G., H. L. Barnett, and R. F. Krause. (1962). Effects of the alkali metal chlorides on spore germination, growth, and carotenogenesis of *Phycomyces blakesleeanus*. *Mycologia* **54**:235–248.

MacLeod, R. A., and E. S. Snell. (1951). Ion antagonism in bacteria as related to antimetabolites. *Ann. N.Y. Acad. Sci.* **52**:1249–1259.

Mandels, M., and E. T. Reese. (1957). Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. *J. Bacteriol.* **73**:269–278.

Medina, A., and D. J. D. Nicholas. (1957). Metallo-enzymes in the reduction of nitrite to ammonia in *Neurospora*. *Biochem. Biophys. Acta* **25**:138–141.

Nakamigawa, K., and A. Okuda. (1960). Physiological role of K. 4. K requirements for pyruvic acid fermentation by *Mucor*. *Nippon Dojo-Hiryogaku Zasshi* **31**:380–382. [*Chem. Abstr.* (1962) **57**:10331.]

Nason, A., N. O. Kaplan, and S. P. Colowick. (1951). Changes in enzymatic constitution in zinc-deficient *Neurospora*. *J. Biol. Chem.* **188**:397–406.

Neilands, J. B. (1957). Some aspects of microbial iron metabolism. *Bacteriol. Rev.* **21**:101–111.

Nicholas, D. J. D. (1952). The use of fungi for determining trace metals in biological materials. *Analyst* **77**:629–642.

Nicholas, D. J. D. (1957). The function of trace metals in the nitrogen metabolism of plants. *Ann. Botany (London)* [N.S.] **21**:587–598.

Nicholas, D. J. D. (1961). *Trans. 7th Intern. Congr. Soil Sci., Madison, Wisc., 1960* **4**:168–180.

Nicholas, D. J. D., and A. H. Fielding. (1950). Use of *Aspergillus niger* as a test organism for determining molybdenum in soils available to crop plants. *Nature* **166**:342–343.

Nicholas, D. J. D., and A. H. Fielding. (1951). The use of *Aspergillus niger* (M) for the determination of magnesium, zinc, copper, and molybdenum available in soils to crop plants. *J. Hort. Sci.* **26**:125–147.

Nicholas, D. J. D., and A. Nason. (1954). Mechanism of action of nitrate reductase from *Neurospora*. *J. Biol. Chem.* **211**:183–197.

Nicholas, D. J. D., A. Nason, and W. D. McElroy. (1953). The effect of molybdenum deficiency on nitrate reductase in cell-free extracts of *Neurospora* and *Aspergillus*. *Nature* **172**:34.

Nicholas, D. J. D., P. W. Wilson, W. Heinen, G. Palmer, and H. Beinert. (1962). The use of electron paramagnetic resonance spectroscopy in investigations of functional metal components in micro-organisms. *Nature* **196**:433–436.

Nielsen, N., and H. Lundin. (1955). The amount of some amino acids in protein-rich and protein-poor *Rhodotorula gracilis*. *Ann. Acad. Sci. Fennicae: Ser. A II* **60**:455–459.

Nielsen, N., and G. Schneider. (1957). On the production of vitamins by protein-rich and protein-poor *Rhodotorula gracilis* yeast. *Acta Chem. Scand.* **11**:685–689.

Page, R. M. (1962). Light and the asexual reproduction of *Pilobolus*. *Science* **138**: 1238–1245.

Perlman, D. (1949). Effects of minor elements on the physiology of fungi. *Botan. Rev.* **15**:195–220.

Raistrick, H., J. H. Birkinshaw, J. H. V. Charles, P. W. Clutterbuck, F. P. Coyne, A. C. Hetherson, C. H. Lilly, M. L. Rintoul, W. Rintoul, R. Robertson, J. A. R. Stoyle, C. Thom, and W. Young. (1931). Studies in the biochemistry of micro-organisms. *Phil. Trans. Roy. Soc.* **B220**:1–367.

Rennerfelt, E. (1934). Untersuchung über die Salzaufnahme bei *Aspergillus niger*. *Planta* **22**:221–239.

Richards, O. W., and M. C. Troutman. (1940). Spectroscopic analysis of the mineral content of yeast grown on synthetic and natural media. *J. Bacteriol.* **39**:739–746.

Rippel, A., and G. Behr. (1930). Über die Verteilung des Magnesiums im Pilzmycel. *Arch. Mikrobiol.* 1:271–276.

Rippel, A., and G. Behr. (1934). Über die Bedeutung des Kaliums im Stoffwechsel von *Aspergillus niger*. *Arch. Mikrobiol.* 5:561–577.

Rippel, A., and U. Stoess. (1932). Ist Calcium ein für Mikroorganismen notwendiges Element? *Arch. Mikrobiol.* 3:492–506.

Rosselet, F. (1953). *Aspergillus niger* as a test organism for measuring plant available potassium in lowveld soils. *S. African J. Sci.* 49:344–347.

Steinberg, R. A. (1935). Nutrient-solution purification for the removal of heavy metals in deficiency investigations with *Aspergillus niger*. *J. Agr. Res.* 52:413–424.

Steinberg, R. A. (1936). Relation of accessory growth substances to heavy metals, including molybdenum, in the nutrition of *Aspergillus niger*. *J. Agr. Res.* 52:439–448.

Steinberg, R. A. (1937). Role of molyhdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. *J. Agr. Res.* 55:891–902.

Steinberg, R. A. (1938). The essentiality of gallium to growth and reproduction of *Aspergillus niger*. *J. Agr. Res.* 57:569–574.

Steinberg, R. A. (1939a). Relating carbon nutrition to trace element and accessory requirements of *Aspergillus niger*. *J. Agr. Res.* 59:749–763.

Steinberg, R. A. (1939b). Growth of fungi in synthetic nutrient solutions. *Botan. Rev.* 5:327–350.

Steinberg, R. A. (1946). Specificity of potassium and magnesium for the growth of *Aspergillus niger*. *Am. J. Botany* 33:210–214.

Steinberg, R. A. (1948). Essentiality of calcium in the nutrition of fungi. *Science* 107:423.

Steinberg, R. A. (1950). Growth on synthetic nutrient solutions of some fungi pathogenic to tobacco. *Am. J. Botany* 37:711–714.

Todd, G. W., and J. Levitt. (1951). Bound water in *Aspergillus niger*. *Plant Physiol.* 26:331–336.

Vail, W. J., and V. G. Lilly. (1961). The effect of magnesium concentration on growth and carotene production of *Phycomyces blakesleeanus*. *Proc. West Va. Acad. Sci.* 33:11–14.

Vallee, B. L., and F. L. Hoch. (1955). Zinc, a component of yeast alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U.S.* 41:327–338.

Weissman, G. S., and S. F. Trelease. (1955). Influence of sulfur on the toxicity of selenium to *Aspergillus*. *Am. J. Botany* 42:489–495.

Whitaker, D. R. (1951). Studies in the biochemistry of cellulolytic microorganisms. 1. Carbon balances of wood-rotting fungi in surface culture. *Can. J. Botany* 29: 159–175.

Yarwood, C. E. (1950). Water content of fungus spores. *Am. J. Botany* 37:636–639.

CHAPTER 9

Chemical Constituents of the Fungal Cell

2. Special Chemical Products

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I. INTRODUCTION

Green plants, as autotrophic organisms, utilize the energy of sunlight to synthesize complex organic substances of high energy content, starting from such simple materials as carbon dioxide and water together with small amounts of inorganic salts to provide nitrogen and phosphorus. They are enabled to do this through their content of chlorophyll, which acts as catalyst of the photosynthetic processes.

The organic substances produced by plants become available for the nutrition of another class of organisms termed heterotrophs, which do not possess chlorophyll and are incapable of photosynthesis. In the course of their metabolism, the heterotrophs, by processes which include both breakdown and synthesis, adapt and modify the food materials into molecules suitable for their own cell structure and reproductive activities. These activities require energy, which is derived from oxidative processes, by which a part of the food is converted into carbon dioxide and water, any excess energy being dissipated as heat.

Both animals and fungi belong to the class of heterotrophs. The animals, however, require their nitrogen to be supplied in organic form, whereas the needs of many fungi are satisfied with purely inorganic sources of nitrogen such as nitrate or ammonium ions. The fungi produce—in addition to carbon dioxide, water, and all the complex materials required for cell structure and the vital processes—a large number of substances of varying com-

plexity, whose function is still obscure. These metabolites are considered in the present chapter.

Given the necessary organic food materials and inorganic salts, most of the fungi can be grown on relatively simple media and thus form excellent subjects for laboratory study. It is usually convenient to cultivate a pure strain of the organism to be examined on simple media such as Czapek-Dox or Raulin-Thom, which consist of aqueous solutions of sugar (usually glucose) and inorganic salts to supply nitrogen, phosphorus, and certain trace elements. In Czapek-Dox medium, glucose is the sole source of carbon; Raulin-Thom medium contains tartrate in addition. The fungi may be grown as a surface film on a static medium or submerged in an agitated medium; in either case a sufficient air supply must be ensured. Some of the higher fungi are more exacting in their food requirements, and the growth rate may be very slow. In such cases it may be more convenient to examine specimens collected in their natural habitats.

The products of the fungi, formed by a chain of reactions, each catalyzed by an enzyme, will normally be elaborated within the cell, where the whole process is organized and controlled, but, once formed, most of these products when not forming part of the cell substance or otherwise completely utilized, are able to escape from the cell. If they are soluble in water they will be found mainly in the aqueous medium, from which they may be obtained by processes of distillation, extraction with solvents, chromatography, and so on. Further purification by crystallization, sublimation, or preparation of derivatives is usually necessary to obtain pure materials. Relatively insoluble products may be found as deposits in the culture flasks or intermingled with the mycelium, where they may in some cases be seen as crystalline aggregates surrounding the hyphae. In the latter case extraction of the mycelium with organic solvents is usually applicable. If the product is an acid it will probably be present as a salt from which the free acid must be liberated before extraction with solvents. Examination of the metabolic products of many species and strains of fungi by the methods indicated has led to the isolation and characterization of hundreds of metabolites, for many of which the chemical structures have been determined.

These studies may be said to have been initiated by Carl Wehmer toward the end of the nineteenth century; the earlier literature contains only a few scattered references to fungal products. Wehmer's observations on the production from sugar of oxalic and citric acids in high yield through the agency of fungi stimulated interest in the commercial possibilities of mold fermentation and led to the industrial production of citric acid from sugar by *Aspergillus niger*.

Other workers, particularly Raistrick and his school, began to search for new metabolites in a systematic manner and discovered quite a range of

new and interesting products. A further stimulus to the search was provided by Fleming's discovery of penicillin. This focused attention on the possibility of finding other useful antibiotics and led to the intense screening of fungi and other microorganisms, notably actinomycetes, for substances of value for chemotherapy.

The beneficial activities of fungi in producing metabolites of value and commercial importance must be contrasted with their harmful and destructive effects. Their role as pathogens of animals and plants and in damaging and destroying all kinds of useful organic materials, such as foodstuffs and textiles, is well known. Attention has latterly been directed to the poisoning produced by groundnuts infected with *Aspergillus flavus.* The extremely potent toxin, even though present only in minute amount, has caused serious losses in turkeys fed on infected meal. The toxin has now been shown to be a mixture of four new closely related substances of known structure which have been named aflatoxins B_1, B_2, G_1, and G_2.

For the purpose of reducing the heterogeneous collection of fungal metabolites to some sort of order, it is convenient to classify them on the basis of chemical structure, even though difficulties arise because many products, owing to their polyfunctional nature, fall into more than one category. This classification often reveals similarities and minor differences that are of some value in suggesting possible pathways of biosynthesis, particularly when several chemically related products are formed by the same fungus and the order of their appearance can be determined.

We are here concerned more particularly with those products which are characteristic of the fungi. We shall therefore exclude from consideration the commoner amino acids, the proteins, the nucleic acids, and the sterols which are common to all forms of living matter. We shall also omit the polysaccharides and the products concerned in the Embden-Meyerhof pathway and the Krebs cycle since these are not specific for the fungi and are dealt with elsewhere.

In the chemical classification here adopted, it is not possible to consider more than a few examples of metabolic products in each category, but those chosen are intended to be fairly representative of the whole group. The selection has to some extent been influenced by the information available as to the pathway of biosynthesis.

II. METHODS OF BIOSYNTHESIS

The identification of fungal products was a necessary preliminary to studies on the mechanism of their biosynthesis, which have latterly been pursued with much vigor. Such studies have been greatly aided by two techniques that were first employed toward the middle of this century. One

was the use of mutant strains of fungi lacking some specific enzyme responsible for a particular link in the chain of reactions comprising the metabolic pathway. The other was the use of substrates labeled with radioactive isotopes, usually C^{14}; this, by the application of suitable methods of chemical breakdown to the metabolite, enables the fate of individual atoms in the substrate to be traced.

It appears that there are two main methods employed in the biosynthesis of fungal metabolites: (1) the shikimic acid pathway and (2) the acetate pathway.

A. The Shikimic Acid Pathway

This pathway was elucidated mainly by the work of Beadle, Bonner, Davis, Tatum, and their collaborators, reviewed by Davis (1955). The organisms chiefly employed were the mold *Neurospora crassa* and the bacteria *Escherichia coli* and *Aerobacter aerogenes*. Mutant strains of these organisms were obtained, in which the biosynthesis of aromatic amino acids was blocked at various points. Intermediates which accumulated at the points of blockage could then be isolated and examined. When such mutants were supplied with the critical substance whose synthesis was blocked, the microorganisms could then complete the chain of reactions leading to the amino acids. In this way the chain of reactions was found to be as represented in Fig. 1.

In this scheme phosphoenol pyruvate and D-erythrose 4-phosphate derived via sedoheptulose diphosphate from the pool of reactions associated with carbohydrate metabolism, are condensed to 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate. This by rearrangement and aldol-like condensation is transformed successively to 5-dehydroquinic acid, 5-dehydroshikimic acid, and shikimic acid. Shikimic acid condenses with a C_3 unit to form, through an intermediate Z_1, prephenic acid. Aromatization at this stage leads through phenylpyruvic and *p*-hydroxyphenylpyruvic acid to phenylalanine and tyrosine, respectively. Modifications may occur in the later stages of this sequence, giving rise to other benzenoid compounds such as vanillic, protocatechuic, 3,4-dihydroxyphenylacetic, and 2,5-dihydroxyphenylacetic acids.

B. The Acetate Pathway

The establishment of the acetate pathway as a common mechanism of biosynthesis of mold metabolites, particularly in the aromatic series, is mainly due to the work of Birch and his collaborators, although the possibility of some such method of biosynthesis was foreshadowed in the specu-

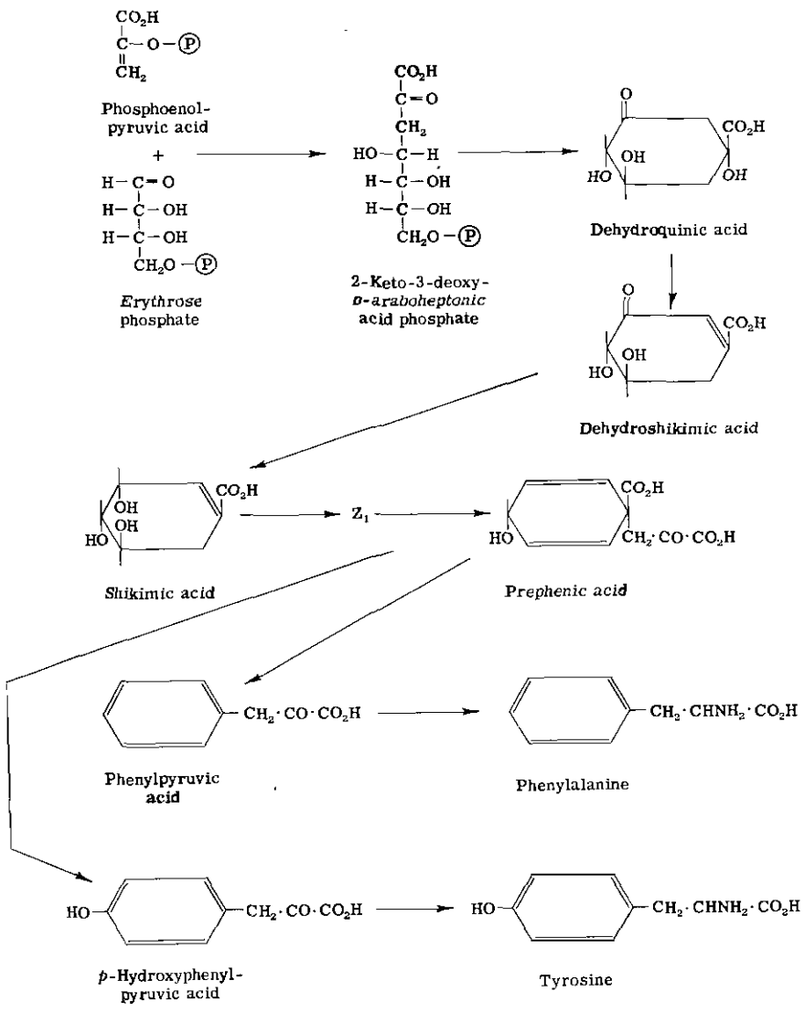


FIG. 1. Shikimic acid pathway. Ⓟ = $-PO(OH)_2$.

lations of Collie and the later suggestions of Sir Robert Robinson (1955). The latter pointed out that a chain of sixteen carbon atoms, built up from eight acetate units by head-to-tail linkage to form a polyketo acid which, by reduction would give palmitic acid, could, by abstraction of water molecules with appropriate ring closures, be folded into a form identical with the structure of the anthrone corresponding to endocrocin, a metabolite of

Aspergillus amstelodami (see Fig. 2). The anthrone is readily oxidized chemically, and hence presumably biochemically, to the anthraquinone.

Birch drew attention to the fact that a similar process could account for the observed structure of many other fungal metabolites and that the molec-

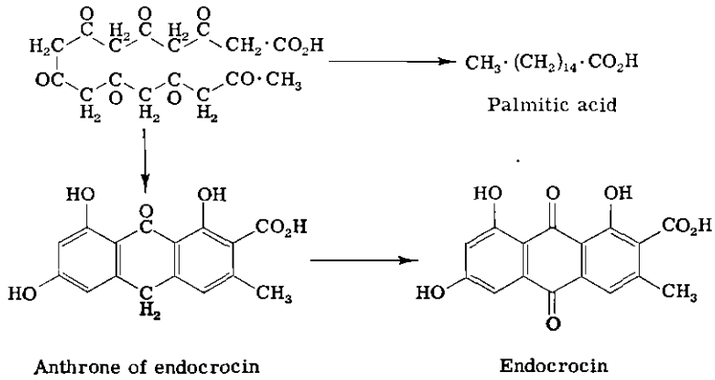


FIG. 2. Biosynthesis of endocrocin from acetate units.

ular arrangements so obtained would have, in the main, the oxygen functions attached to the correct carbon atoms, although in some cases further oxidations or reductions might be involved. These, however, could be accommodated within the framework of known biochemical modifications.

In a brilliant series of investigations Birch was able to establish beyond doubt the correctness of his main thesis. He employed acetate labeled in one of the two possible positions with C^{14} as precursor. This was fed to the mold and the labeled product was isolated and degraded by suitable chemical methods to establish the position and degree of labeling of the tagged carbon atoms.

Thus, in a classical experiment, Birch *et al.* (1955) grew *Penicillium griseofulvum* on a glucose medium supplemented with sodium [1-C^{14}]acetate. The labeled 6-methylsalicylic acid produced was degraded chemically by suitable methods to reveal the tagged atoms which are marked by asterisks in Fig. 3. The positions of these atoms in the molecule and their approximately equal degree of labeling are in accord with the hypothesis that the molecule of 6-methylsalicylic acid originates from a chain of four acetate molecules linked head-to-tail. Orsellinic acid has here been postulated as an intermediate since it is itself a fungal metabolic product, and has similar labeling when derived from carboxyl-labeled acetate. The conversion to 6-methylsalicylic acid requires a reduction step to eliminate the hydroxyl group para to the carboxyl.

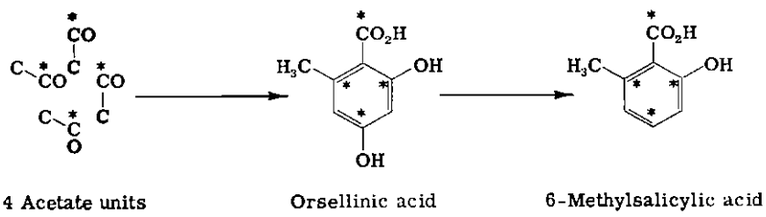


FIG. 3. Biosynthesis of 6-methylsalicylic acid from acetate units.

In some cases a methyl group attached to carbon to form a branched chain or to oxygen to form a methoxyl group may be inserted. These additions to the acetate-based molecule can be detected by employing a single-carbon labeled precursor such as formate ($HC^{14}O_2Na$). Examples of this will be noted later.

Further work has shown the necessity for amplification of the original acetate hypothesis of biosynthesis. Bu'Lock and Smalley (1961), using labeled ethyl malonate have now established that three of the four "acetate units" forming 6-methylsalicylic acid actually undergo condensation as malonate units (presumably in the form of malonyl coenzyme A); these three malonate units subsequently lose three molecules of carbon dioxide to give the metabolite, the total effect being the same as that which would be produced by linkage of four acetate units. When malonate is not supplied, it is synthesized by the fungus from acetyl coenzyme A and carbon dioxide. This participation of malonate has been confirmed by Bentley and Keil (1961) in the case of the production of penicillic acid from *Penicillium cyclopium*.

Bu'Lock and Smalley (1962) have further shown that a polyacetylene is similarly produced. Incubation of *Tricholoma grammopodium* cultures with diethyl α-C^{14} malonate afforded dehydromatricarianol, $H_3C—(C\equiv C)_3—CH—CH—CH_2OH$, in which 97% of the labeling was incorporated into C-1 to C-8 of the C_{10} chain, presumably in even-numbered carbon atoms since C-1 was inactive. This contrasts with the uniform distribution of labeling from acetate found in polyacetylenes. The participation of malonate in these fungal biosyntheses affords a close parallel with fatty acid synthesis in which malonate is also involved. Bu'Lock and Smalley represent the synthesis of the three types, polyacetylenes, fatty acids, and acetate-derived aromatics (polyketides), as occurring according to the scheme shown in Fig. 4.

The essential stages of this biosynthesis are (1) combination of acetyl thiolester and malonyl CoA with simultaneous or subsequent decarboxylation, and (2) reduction of β-ketoacetyl thiolester by hydride transfer to yield the fatty acids. Polyacetylene synthesis is visualized as arising in step

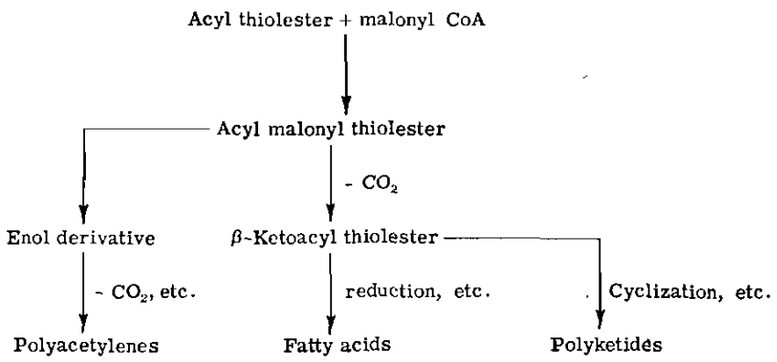


FIG. 4. Scheme of biosynthesis from acetate-malonate units.

(1) by failure to decarboxylate until after formation of an enolic derivative, which then undergoes decarboxylation and water elimination. The polyketides would arise by omission of reduction steps in stage (2) leading to β-diketone types which are able to undergo cyclization.

There is little direct evidence regarding the intermediate stages in these syntheses from acetate and malonate with one exception. Mevalonic acid, $HOCH_2—CH_2—C(CH_3)(OH)—CH_2—CO_2H$, a product first isolated from yeast, is known to be a building unit in the biosynthesis of terpenes and

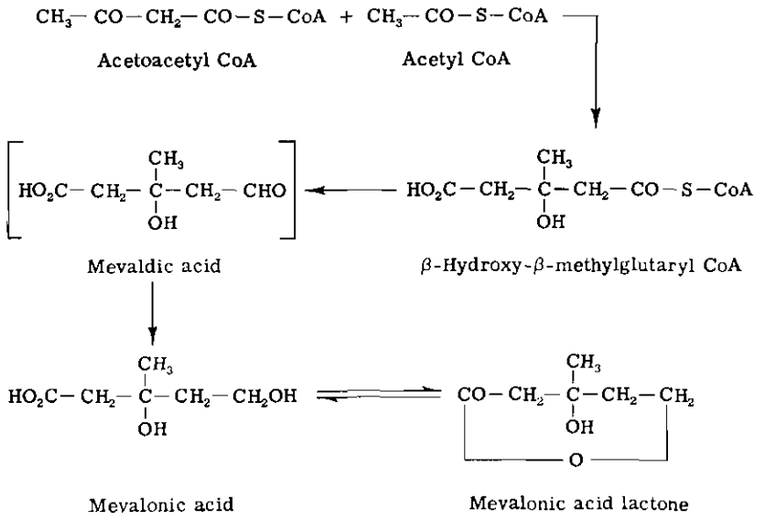


FIG. 5. Biosynthesis of mevalonic acid.

sterols. This acid, by decarboxylation, produces the well-known isoprene skeleton, which had long been recognized as a repetitive unit in sterol structure. Mevalonic acid is biosynthesized in effect from three acetic acid units according to the scheme shown in Fig. 5.

By the use of labeled mevalonic acid (or its lactone), it has been shown that this acid is an irreversible intermediate in the synthesis not only of the terpenes and sterols, but also of terpenoid side chains attached to aromatic nuclei as, for example, in mycophenolic acid (Fig. 17).

III. ALIPHATIC METABOLITES

A. Saturated Compounds

1. Simple Alcohols, Acids, Esters, and Fats

Many of the metabolic products in this group represent intermediates in the main track of the common metabolic pathways or in slight divergences therefrom, but, excluding these, there remain some products of interest which may be briefly considered.

Acetic acid, derivable by oxidation from the acetaldehyde produced in the Embden-Meyerhof pathway, and the starting point for an important biosynthetic route, has been isolated in small amount as a metabolic product of many fungi. In some cases it is present as simple esters, e.g., ethyl acetate, $CH_3CO_2C_2H_5$, derived from *Penicillium digitatum* (Birkinshaw *et al.* 1931) and isobutyl acetate, $CH_3CO_2CH_2CH(CH_3)CH_3$, from *Endoconidiophora coerulescens* (Birkinshaw and Morgan, 1950). Branched-chain acids of small molecular weight are obtained from some fungi; thus α-methylbutyric acid, $CH_3CH_2CH(CH_3)CO_2H$, is obtained from *Penicillium notatum* (Cram and Tishler, 1948), and dimethylpyruvic acid, $CH_3CH(CH_3)COCO_2H$, from *Aspergillus* species (Ramachandran and Radha, 1955).

Hydroxy acids are not uncommon; for example, itatartaric acid, $HO_2CCH_2C(OH)(CH_2OH)CO_2H$ was isolated from cultures of *Aspergillus terreus* (Stodola *et al.*, 1945). The acids α,β-dihydroxyisovaleric, $CH_3C(OH)(CH_3)CH(OH)CO_2H$, and α,β-dihydroxy-β-methylvaleric, $CH_3CH_2C(OH)(CH_3)CH(OH)CO_2H$, both isolated from a *Neurospora crassa* mutant by Sjölander *et al.* (1954), were shown to be precursors of valine and isoleucine, respectively. It is well known that γ- and δ-hydroxy acids undergo ready lactonization; hence the fungal hydroxy acids that have this structure are usually isolated in the lactone form.

Synthesis of fat is an important function of the fungi, and the usual saturated and unsaturated fatty acids (free or as glycerides) are obtained in high yield in many cases. Thus *Aspergillus nidulans* produces the

saturated acids myristic, palmitic, stearic, arachidic, behenic, and lignoceric acids (39%) together with even larger amounts of the unsaturated acids hexadecenoic, oleic, linoleic, linolenic, and C_{20} acids (61%). The predominating acids are palmitic (21%), stearic (16%), oleic (40%), and linoleic (17%) (Singh *et al.*, 1955). Other closely related products probably arise from the fatty acids by reduction of the carboxyl group. Cetyl alcohol, $CH_3(CH_2)_{14}CH_2OH$, related to palmitic acid, is a product of *Amanita phalloides* (H. Wieland and Coutelle, 1941), and stearyl alcohol, $CH_3(CH_2)_{16}CO_2H$, is a product of *Penicillium notatum* (Angeletti *et al.*, 1952). The long-chain saturated hydrocarbon octacosane, $CH_3(CH_2)_{26}CH_3$ has been obtained from *A. phalloides* (H. Wieland and Coutelle, 1941).

2. *Products Related to Citric Acid*

Certain hydroxylated tribasic acids are closely related to citric acid as regards the functional groups, but they contain in addition a saturated chain of ten to sixteen carbon atoms. Such are spiculisporic and minioluteic acids from *Penicillium*, agaricic acid from *Fomes officinalis*, and caperatic acid from the lichen *Parmelia caperata*. These structures are shown in Fig. 6 together with that of citric acid. Spiculisporic and minioluteic acids are actually the γ-lactones of the structures shown; they are represented in the hydrated form for easier comparison. Caperatic acid is a monomethyl ester

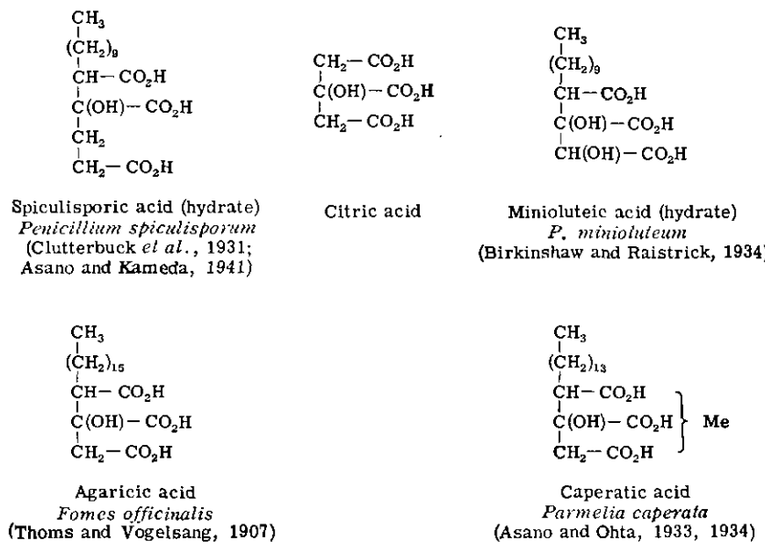


FIG. 6. Acids related to citric acid.

of tetradecylcitric acid; which of the three carboxyl groups is esterified has not been determined.

The final stage of the citric acid biosynthesis is known to be a condensation between an acetic acid unit (acetyl coenzyme A) and oxalacetic acid. It is surmised that a similar condensation in which a higher fatty acid replaces acetic acid may produce the acids in this group. Spiculisporic acid however would require the participation of a five-carbon acid, e.g. α-ketoglutaric acid in place of oxalacetic acid.

B. Unsaturated Compounds

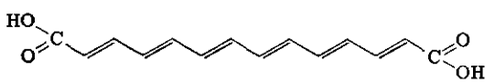
1. Ethylene and Polyenes

The ethylenic linkage, —CH CH—, is of very frequent occurrence in fungal metabolites. The double bond may occur in isolation as one element of an otherwise saturated chain of carbon atoms, or in conjugated form in a chain containing alternate single and double bonds. Only a few examples of these linkages are mentioned in this section; other cases will be encountered later, particularly in the substituent groups of cyclic compounds.

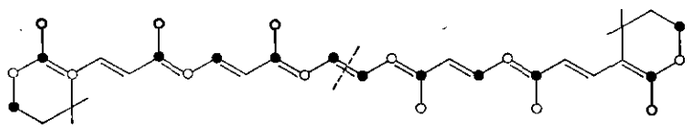
The simplest example of the isolated double bond is, of course, ethylene, $CH_2=CH_2$. It is evolved by respiring plant tissue and promotes the ripening of fruit. This property led to its detection as a product of *Penicillium digitatum* by Hall (1951), when he grew the fungus on glucose- or arabinose-agar medium. Ethylene is also produced by other fungi, e.g. the pathogenic *Blastomyces dermatitidis* (Nickerson, 1948). Another example of the isolated double bond is provided by 2-methyl-2-butene, $CH_3C(CH_3)$ $CHCH_3$, a product of *Puccinia graminis* f. sp. *tritici* (Forsyth, 1955).

Chains of conjugated double bonds are present in a number of fungal metabolites, many of which are colored—a characteristic effect of conjugation. A typical example is corticrocin, which forms yellow needles or prisms. The carbonyl groups present in the carboxyls at either end of the polyene chain here contribute to the conjugation (Fig. 7). The carotenoids, which are common constituents of fungi and other microorganisms, also contain long conjugated polyene chains. The many examples known represent minor variations of a characteristic pattern, of which β-carotene is an example. The subject has been well reviewed by Goodwin (1954) and others.

It will be noted that the β-carotene molecule is symmetrical about the dotted line, indicating that it is produced by joining two similar moieties tail-to-tail. It has been found that *Mucor hiemalis* uses acetate for the production of β-carotene; with C^{14}-labeled acetate the partial distribution is as shown in Fig. 7 (Grob and Bütler, 1956). Mevalonic acid is an



Corticrocin
Corticium croceum
(Erdtman, 1948; B. L. Shaw and Whiting, 1954)



β-Carotene
o Carbon atom from the methyl group of acetate
• Carbon atom from the carboxyl group of acetate

FIG. 7. Polyenes.

effective precursor of carotene for certain fungi (Braithwaite and Goodwin, 1957; Grob, 1957), as might be anticipated from the fact that the skeleton can be constructed from eight isoprene units linked in appropriate fashion.

2. Acetylenic Compounds, Polyynes, Polyenynes

The triple bond as present in acetylene occurs in a number of fungal products, particularly those derived from the higher fungi. This type of linkage is almost always conjugated with other triple bonds or with double bonds, forming long unbranched chains. When both types of bond occur in the same molecule however, the triple bonds do not alternate with double bonds in the conjugated chain, but are segregated. This will be seen from examples (Fig. 8).

These acetylenic compounds usually occur in low yield and as complex mixtures. Isolation is often difficult since they are frequently unstable, but they possess characteristic absorption spectra that aid in identification. The antibiotic activity that is a feature of many of these products first drew attention to their presence in the expressed juices of some of the higher fungi. The pure polyyne type represented by agrocybin occurs much less frequently than the mixed polyenyne type represented by diatretynes I and II.

With improvements in the techniques of isolation and identification of the polyacetylenic metabolites, the recording of new discoveries shows no signs of abatement. Thus Cambie *et al.* (1963) have recently reported the

$HOCH_2-C\equiv C-C\equiv C-C\equiv C-CONH_2$

Agrocybin
Agrocybe dura
(Anchel, 1952; Bu' Lock *et al.*, 1954)

$HO_2C-CH=CH-C\equiv C-C\equiv C-R$

Diatretyne I; R = $CONH_2$
Diatretyne II; R = CN
Clitocybe diatreta
(Anchel, 1953, 1955; Ashworth *et al.*, 1958)

I $CH_3-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH_2OH$
II $HC\equiv C-C\equiv C-C\equiv C-CH=CH-CH_2OH$
III $HOCH_2-CH_2-C\equiv C-C\equiv C-CH=CH-CH_2OH$
IV $HOCH_2-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH_2OH$
V $HC\equiv C-C\equiv C-C\equiv C-CH=CH-CO_2H$
VI $HC\equiv C-C\equiv C-C\equiv C-CH=CH-CO-NH-CH(CO_2H)-CH(CH_3)_2$
VII $HO_2C-C\equiv C-C\equiv C-C\equiv C-CH_2-CH=CH-CH_2-CH_2-CH_2-CO_2H$
VIII $HO_2C-C\equiv C-C\equiv C-C\equiv C-CH=CH-CO_2H$
IX $HOCH_2-C\equiv C-C\equiv C-C\equiv C-CH_2-CH(OH)-CO_2H$
X $HOCH_2-C\equiv C-C\equiv C-CH=CH-CH_2-CH(OH)-CO_2H$

Polyacetylenic metabolites of *Poria sinuosa*
(Cambie *et. al.*, 1963)

FIG. 8. Polyacetylenes.

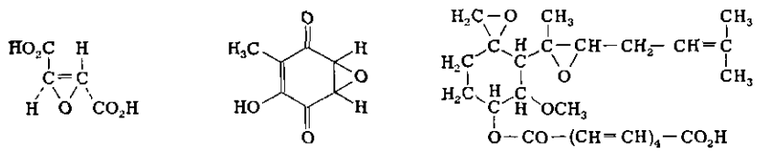
solation and characterization of ten polyacetylenic compounds from *Poria sinuosa*. The structures (I–X) (Fig. 8) illustrate the variety and range of polyacetylenes derivable from a single species. All these with the exception of (I), (II), and (IV) are new. The alcohol compound (I) had previously been obtained from *Pleurotus ulmarius, Tricholoma grammopodium, Clitocybe obbata,* and *C. candida*. Alcohols (II) and (IV) had been isolated from *Coprinus quadrifidus*. The acid (V) is also a product of *Psilocybe sarcocephala*.

The diacid (VII) is of special interest because it contains the longest carbon chain (C_{14}) yet found in fungal polyacetylenes. It is also unusual because it shows unsaturation separated from (i.e. not conjugated with) the main triyne chromophoric group. Product (VI) is the first example of a fungal polyacetylenic acid linked with an amino acid (L-valine).

C. Oxygen Heterocycles

1. Epoxides

Only a few examples of the epoxide group are known in fungal metabolic products (see Fig. 9). The simplest case, *l-trans*-oxidoethylene-α,β-dicarboxylic acid (epoxysuccinic acid) has been obtained from the culture filtrates of *Monilia formosa, Penicillium viniferum,* and *Aspergillus fumigatus*. Terreic acid, from *A. terreus* is closely related to 3,6-dihydroxy-2,5-toluquinone, into which it may readily be converted. A still more complex example of this group is fumagillin, an antiphage agent obtained from



Epoxysuccinic acid
Aspergillus fumigatus
(Sakaguchi *et al.*, 1939; Birkinshaw *et al.*, 1945)

Terreic acid
A. terreus
(Florey *et al.*, 1949; Sheehan *et al.*, 1958)

Fumagillin
A. fumigatus
(Eble and Hanson, 1951; Tarbell *et al.*, 1960)

FIG. 9. Epoxides.

Aspergillus fumigatus. It contains two epoxide rings; it is also an ester of decatetraenedioic acid—a further example of a conjugated double-bond system.

Several polyacetylenic epoxides have been obtained from plants (Compositae). The first fungal example has now appeared. Jones *et al.* (1963) have allocated the structure *trans*-2,3-epoxynona-4,6,8-triyn-1-ol to a product obtained in traces from *Coprinus quadrifidus*, and identical with biformyne 1 (originally known as biformin) isolated by Anchel and Cohen (1954) from *Polyporus biformis*. Jones *et al.* consider it likely that epoxidation of the ethylenic bonds in polyenynes is a standard metabolic process. Perhaps the epoxide groups in other types of natural structures arise by a similar process.

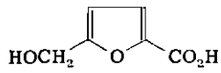
2. *Furan Rings*

The uncomplicated furan ring is present in 2-hydroxymethylfuran-5-carboxylic acid, a product of various aspergilli (Fig. 10). This five-membered ring also occurs fused to other ring systems as in sterigmatocystin, a xanthone derivative obtained from *Aspergillus versicolor*, which is readily transformed by alkali into isosterigmatocystin, a true furan derivative. The furan skeleton is also present in γ-lactones, which, as already mentioned, are of frequent occurrence as fungal metabolites.

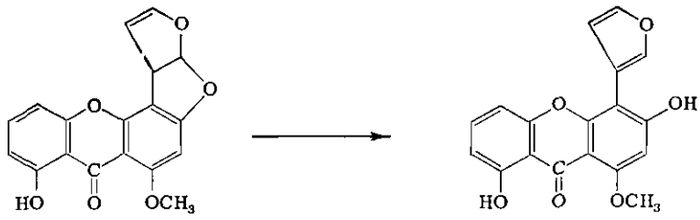
3. *Tetronic Acids*

The tetronic acids, a number of which have been identified as fungal metabolites, are in fact further examples of the furanoid structure. Since the special type of γ-lactone arrangement present confers upon them distinctive properties, they are here considered as a separate group.

As their name implies, the tetronic acids are acidic in nature, even when not possessing a free carboxyl group, as is ascorbic acid of similar structure. The simplest fungal product in this class, γ-methyltetronic acid, is



2-Hydroxymethylfuran-
5-carboxylic acid
Aspergillus niger, etc.
(Sumiki, 1931)



Sterigmatocystin
A. versicolor
(Hatsuda and Kuyama,
1954; Bullock *et al.*, 1962)

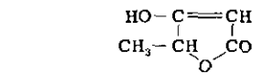
Isosterigmatocystin

FIG. 10. Furan rings.

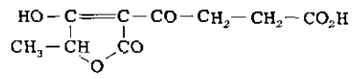
obtained from *Penicillium charlesii*, which also yields several other tetronic acids. Further examples are derived from other fungal sources. From an examination of the structures (Fig. 11) it is evident that substitution occurs at two points in the tetronic acid ring, the α-position which may have acyl substituents and the γ-position which is variously substituted by $—CH_3$, $—CH_2—CO_2H$, and $=CH_2$. In certain of these acids where the acyl group in the α-position is hydroxylated in the 4-position, as in carolic, terrestric, carlic, and dehydrocarolic acids, ether linkage has occurred with the β-hydroxyl group to form a second ring in the molecule.

Lybing and Reio (1958) studied the incorporation of 1-C^{14}-acetate into carolic and carlosic acids. They found that with carlosic acid, for example, there was high activity in carbons 1, 5, and 7, but only low activity in carbons 3, 4, 9, and 10, and they suggested that the acid was derived from a C_6 moiety containing three acetyl units and a C_4 dicarboxylic acid from the citric acid cycle.

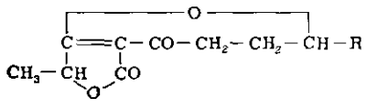
Ascorbic and penicillic acids, although structurally similar to the tetronic acids, are biosynthesized by other pathways. Ascorbic acid arises from D-glucuronic acid through L-gulonolactone and 2-keto-L-gulonolactone (Sastry and Sarma, 1957), whereas penicillic acid is formed from orsellinic acid by cleavage of the aromatic ring as shown in Fig. 12 (Mosbach, 1960;



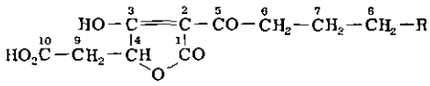
l-γ-Methyltetronic acid
Penicillium charlesii
(Clutterbuck *et al.*, 1935c)



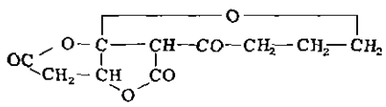
Carolinic acid
P. charlesii
(Clutterbuck *et al.*, 1935a)



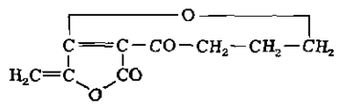
Carolic acid, R = H
Terrestric acid, R = C_2H_5
P. charlesii, P. terrestre
(Clutterbuck *et al.*, 1935a;
Birkinshaw and Raistrick, 1936)



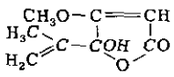
Carlosic acid, R = H
Viridicatic acid, R = C_2H_5
P. charlesii, P. cinerascens, P. viridicatum
(Clutterbuck *et al.*, 1935b; Bracken and
Raistrick, 1947; Birkinshaw and Samant, 1960)



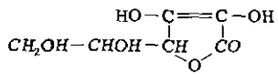
Carlic acid
P. charlesii
(Clutterbuck *et al.*, 1935b)



Dehydrocarolic acid
P. cinerascens
(Bracken and Raistrick, 1947)

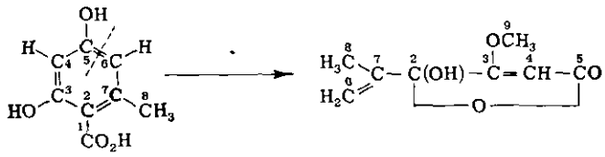


Penicillic acid
P. puberulum, P. cyclopium
(Alsberg and Black, 1913;
Birkinshaw *et al.*, 1936)



Ascorbic acid
Aspergillus niger
(Geiger-Huber and Galli, 1945)

FIG. 11. Tetronic acids.



Orsellinic acid — Penicillic acid

FIG. 12. *Biosynthesis of penicillic acid.*

Bentley and Keil, 1961). The orsellinic acid molecule, derived from one acetate and three malonate units, is cleaved between carbon atoms 5 and 6, carbon atom 1 being lost as CO_2. The additional carbon atom 9 of the methoxyl group is introduced from a C_1 unit.

4. Pyrones

The α-pyrone ring is representative of the δ-lactones, which have already been mentioned. The γ-pyrone ring is exemplified by kojic acid, a product formed in high yield by the *Aspergillus flavus-A. oryzae* group. A related product is patulin, which contains an unsaturated γ-lactone ring. Kojic acid appears to be synthesized directly from glucose by oxidation and elimination of water without cleavage of the 6-carbon chain (Arnstein and Bentley, 1953); whereas patulin is probably produced by fission of an aromatic precursor such as gentisaldehyde, which is derived via the shikimic acid pathway (Fig. 13).

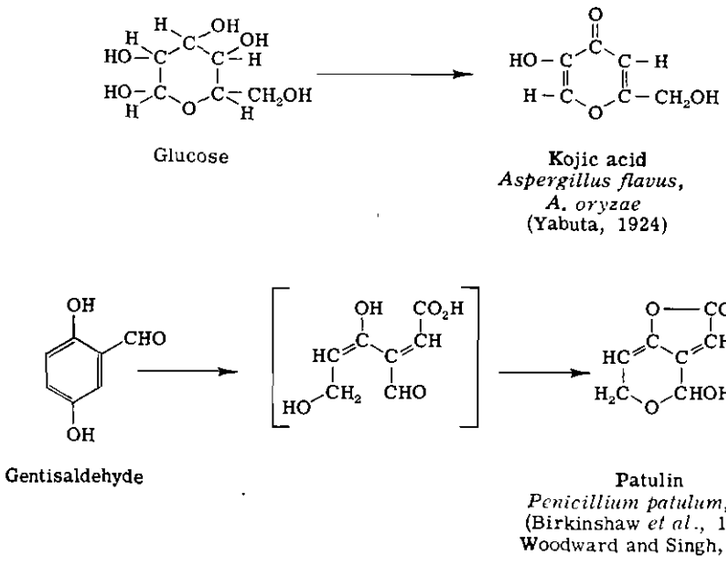


FIG. 13. Biosynthesis of kojic acid and patulin.

IV. AROMATIC METABOLITES

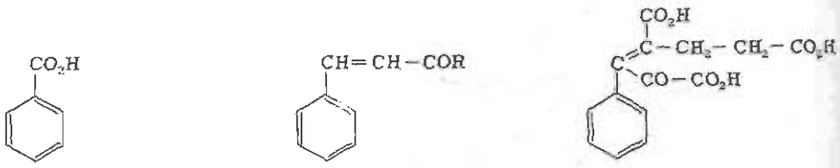
Since nearly all the aromatic products of the fungi contain phenolic groups, the number and arrangement of these groups (whether methylated or not) has been adopted as a means of classification in this category. This may aid in predicting the mode of biosynthesis since hydroxyls *meta*-disposed, as in resorcinol, are an indication of biosynthesis from acetate units, whereas *ortho*- and *para*-hydroxylation (to hydroxyl) is found more often in shikimic acid-derived products. This, however, is merely a rough

indication, not always borne out in practice, since the fungi are able to introduce or remove phenolic hydroxyl groups. A frequent substituent of the phenols is the methyl group, which occurs as such or in oxidized forms represented by hydroxymethyl, formyl, or carboxyl groups.

A. Monocyclic Structures

1. No Nuclear Hydroxyl Groups

A few aromatic products of fungi contain no nuclear hydroxyl groups. Such are benzoic acid and cinnamic acid and its derivatives. A product, $C_{14}H_{10}O_7$, from *Chaetomium indicum* is also of this nature (Fig. 14). The



Benzoic acid
Penicillium roseopurpureum
(Posternak, 1940)

Cinnamaldehyde; R = H
Cinnamic acid; R = OH
Stereum subpileatum
(Birkinshaw *et al.*, 1957)
Methyl cinnamate; R = OCH_3
Lentinus lepideus
(Birkinshaw and Findlay, 1940)

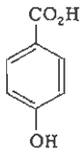
C_{14} Tribasic acid
Chaetomium indicum
(Johnson *et al.*, 1953)

FIG. 14. Benzene derivatives.

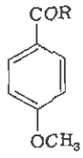
cinnamic acid derivatives have the C_6–C_3 structure which relates them to the shikimic acid pathway through which they are formed. Benzoic acid may be a product of further oxidation of cinnamic acid.

2. Phenol Derivatives

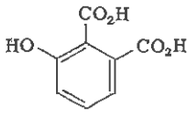
A single phenolic hydroxyl (or methoxyl) is present in various products shown in Fig. 15. *p*-Hydroxybenzoic acid, anisaldehyde, and anisic acid are obviously closely related. Their structure suggests that they are derived via the shikimic acid pathway. 6-Methylsalicylic acid (Fig. 3), already mentioned as derived via the acetate pathway, is an example of the elimination of a hydroxyl group at some stage during synthesis. 3-Hydroxyphthalic acid could be formed by a similar process with oxidation of the methyl group. In ochracein a second ring due to lactonization is present. Gladiolic and dihydrogladiolic acids are capable of undergoing a similar ring closure between carboxyl and (hydrated) aldehyde group.



p-Hydroxybenzoic acid
Penicillium patulum
(Bassett and
Tanenbaum, 1958)

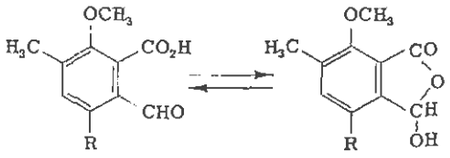


Anisaldehyde; R = H
Daedalea juniperina
(Birkinshaw and
Chaplen, 1955)

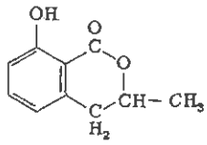


3-Hydroxyphthalic acid
Penicillium islandicum,
P. patulum
(Gatenbeck, 1957)

Methyl anisate; R = OCH_3
Lentinus lepideus
(Birkinshaw and Findlay, 1940)



Gladiolic acid; R = CHO
Dihydrogladiolic acid, R = CH_2OH
Penicillium gladioli
(Grove, 1952; Raistrick and Ross, 1952;
Brown and Newbold, 1954;
Duncanson *et al.*, 1953)

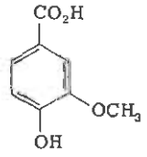


Ochracein (Mellein)
Aspergillus ochraceus
A. melleus
(Yabuta and Sumiki, 1933, 1934;
Blair and Newbold, 1955)

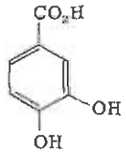
FIG. 15. Phenol derivatives.

3. *Pyrocatechol Derivatives*

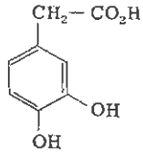
Only a few examples of this arrangement are known (Fig. 16). Vanillic and protocatechuic acid were obtained from a mutant of *Neurospora crassa* having strict nutritional requirements. Protocatechuic acid is produced also



Vanillic acid



Protocatechuic acid
Phycomyces blakesleeanus
(Schröter, 1956)



Homoprotocatechuic acid
Polyporus lumulosus
(Ralph and
Robertson, 1950)

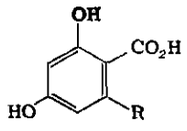
Neurospora crassa
(Metzenberg and Mitchell, 1958)

FIG. 16. Pyrocatechol derivatives.

by *Phycomyces blakesleeanus,* homoprotocatechuic acid by *Polyporus tumulosus.*

4. Resorcinol Derivatives

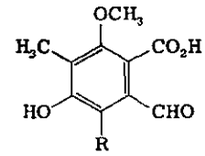
Many fungal metabolites have the resorcinol (1,3) arrangement of the phenolic groups, usually with carboxyl in position 4, since this orientation is readily attained through the acetate pathway. Orsellinic acid, already mentioned, is a case in point. 3,5-Dihydroxyphthalic acid could be derived by oxidation of the methyl group of orsellinic acid. This and other examples are illustrated in Fig. 17.



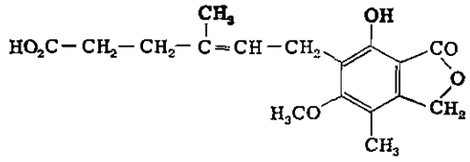
3, 5-Dihydroxyphthalic acid; R = CO_2H

Three C_{10} acids; R = $CH_2-CO-CH_3$; R = $CH(OH)-CO-CH_3$; R = $CO-CO-CH_3$

Penicillium brevi-compactum
(Oxford and Raistrick, 1932, 1933)



Cyclopolic acid; R = CH_2OH
Cyclopaldic acid; R = CHO
P. cyclopium
(Birkinshaw *et al.*, 1952b; Duncanson *et al.*, 1953)

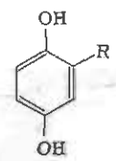


Mycophenolic acid
P. brevi-compactum
(Alsberg and Black, 1913; Birkinshaw *et al.*, 1952a)

FIG. 17. Resorcinol derivatives.

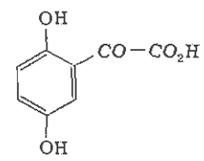
5. Quinol Derivatives

Gentisyl alcohol, gentisic acid, and 2,5-dihydroxyphenylglyoxylic acid are simple representatives of this group (Fig. 18). Auroglaucin and flavoglaucin, both derived from *Aspergillus glaucus,* have extended side chains; the seven-membered straight chain suggests an origin from acetate, the five-membered branched chain is of isoprenoid nature, probably arising from mevalonic acid. 5-Methoxycoumarone which is included here is also a representative of the furan group (Fig. 10).

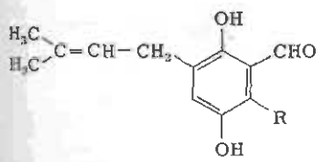


Gentisyl alcohol; R = CH_2OH
Penicillium patulum
(Birkinshaw *et al.*, 1943b)

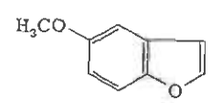
Gentisic acid; R = CO_2H
P. griseofulvum
(Raistrick and Simonart, 1933)



2, 5-Dihydroxyphenylglyoxylic acid
Polyporus tumulosus
(Moir and Ralph, 1954)



Auroglaucin; R = $(CH{=}CH)_3{-}CH_3$
Flavoglaucin; R = $(CH_2)_6{-}CH_3$
Aspergillus glaucus
(Raistrick *et al.*, 1937;
Quilico *et al.*, 1953a, b)

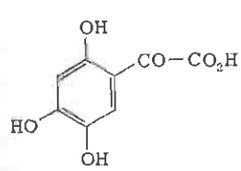


5-Methoxycoumarone
Stereum subpileatum
(Birkinshaw *et al.*, 1957)

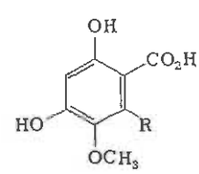
FIG. 18. Quinol derivatives.

6. Hydroxyquinol Derivatives

2,4,5-Trihydroxyphenylglyoxylic acid, which falls into this group, was obtained, together with homoprotocatechuic acid (Fig. 16), from *Polyporus tumulosus* cultured in the laboratory. Other examples (Fig. 19) of



2, 4, 5-Trihydroxyphenylglyoxylic acid
Polyporus tumulosus
(Ralph and Robertson, 1950)



Ustic acid; R = $CH(OH){-}CO{-}CH_3$
Aspergillus ustus
(Raistrick and Stickings, 1951)

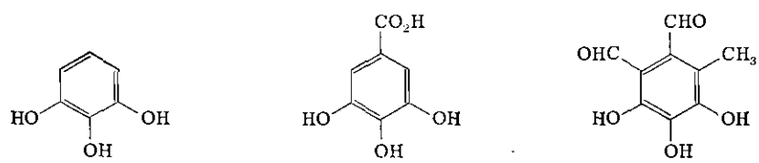
4,6-Dihydroxy-3-methoxyphthalic acid; R = CO_2H
Paecilomyces victoriae
(Vora, 1954)

FIG. 19. Hydroxyquinol derivatives.

this 1,2,4-arrangement of hydroxyl groups are ustic acid and 4,6-dihydroxy-3-methoxyphthalic acid. These products have the structure of the acid $C_{10}H_{10}O_6$ and of 3,5-dihydroxyphthalic acid, respectively (Fig. 17), with an additional methoxyl group.

7. Pyrogallol Derivatives

Pyrogallol, gallic acid, and flavipin (Fig. 20) are examples of this structure.



Pyrogallol
Penicillium patulum
(Bassett and Tanenbaum, 1958)

Gallic acid
Phycomyces blakesleeanus
(Bernhard and Albrecht, 1947)

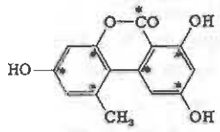
Flavipin
Aspergillus flavipes
(Raistrick and Rudman, 1956)

FIG. 20. Pyrogallol derivatives.

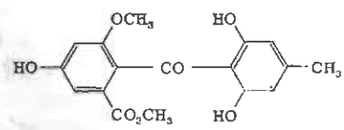
B. Polycyclic Structures

Examples of this group are shown in Fig. 21. The biosynthesis of alternariol was studied by feeding *Alternaria tenuis* with sodium 1-C^{14}-acetate (Thomas, 1961a). The alternariol produced was labeled on the carbon atoms marked with an asterisk. It is immediately evident that the labeling is on alternate carbon atoms and that only labeled atoms have oxygen attached. This is consistent with biosynthesis from an unbranched folded chain of seven acetate units. Although the activity of each labeled atom was broadly the same, the difference in labeling between the carbon adjacent to the methyl group and the carbonyl carbon of the lactone ring was considered to be outside the limits of experimental error. These atoms represent the carboxyls of the initial and terminal acetate units of the C_{14} chain. This difference is readily explicable if the chain is built up from one acetyl (represented by the methyl group and its partner) and six malonyl units.

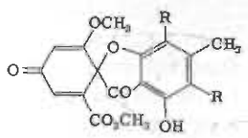
Sulochrin, bisdechlorogeodin, and asterric acid occur together as metabolites of at least two fungi, *Oospora sulphurea-ochracea* and *Penicillium frequentans* (Stickings and Mahmoodian, 1962). R. F. Curtis *et al.* (1960) have shown that sulochrin can readily be converted *in vitro* through bisdechlorogeodin into asterric acid, and they have suggested that the same route is probably followed in biosynthesis. It is assumed that the chlorine atoms are introduced in a secondary reaction into bisdechlorogeodin to



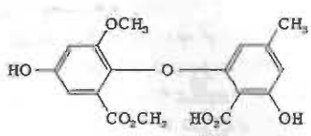
Alternariol
Alternaria tenuis
(Raistrick *et al.*, 1953)



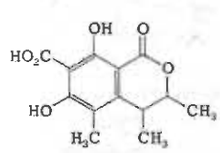
Sulochrin
Oospora sulphurea-ochracea
(Nishikawa, 1936)



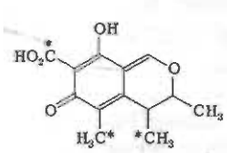
Bisdechlorogeodin; R = H
Geodin; R = Cl
Aspergillus terreus
(Raistrick and Smith, 1936;
Barton and Scott, 1958;
R. F. Curtis *et al.*, 1961)



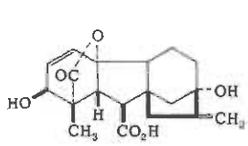
Asterric acid
A. terreus
(R. F. Curtis *et al.*, 1960)



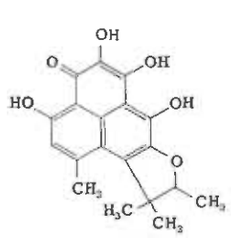
Isocoumarin derivative
A. *terreus* (mutant)
(Hassall and Jones, 1962)



Citrinin
Penicillium citrinum, A. *terreus*
(Hetherington and Raistrick, 1931;
Johnson *et al.*, 1950)



Gibberellic acid
Gibberella fujikuroi
(P. J. Curtis and Cross, 1954;
Aldridge *et al.*, 1963)



Atrovenetin
P. atrovenetum
(Neill and Raistrick, 1957;
Barton *et al.*, 1959)

FIG. 21. Polycyclic metabolites.

form geodin. This is supported by studies of Rhodes *et al.* (1962) which indicate that in *Aspergillus terreus* bisdechlorogeodin is a common intermediate in the biosynthesis of both geodin and asterric acid.

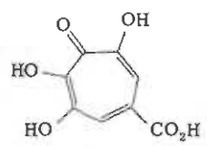
The close relationship between citrinin, formed by *A. terreus* wild type, and the isocoumarin derivative produced by a mutant, suggests a similar origin for these two products. Further, Birch *et al.* (1958a) found that the carbon atoms marked with an asterisk in the formula shown differ from others in citrinin in being derived from a C_1 precursor (formic acid). Hassall and Jones (1962) therefore have suggested (1) that citrinin may be derived from the acetonyl-β-resorcylic acid derivative $C_{10}H_{10}O_5$ (Fig. 17) formed by *P. brevi-compactum,* through a series of steps involving methylation, oxidation, and reduction, and (2), since the *A. terreus* mutant no longer produces citrinin, that the reduction of the carboxyl function of the β-resorcylic acid derivative occurs at a late stage in the biosynthesis of citrinin.

The biosynthesis of the atrovenetin structure has been examined by Thomas (1961b) for the related products herqueinone and norherqueinone. Deoxynorherqueinone is identical with atrovenetin. Sodium 1-C^{14}-acetate and DL-2-C^{14}-mevalonic lactone were incorporated by *P. herquei* into norherqueinone in a manner consistent with the derivation of the perinaphthenone nucleus from acetate and the C_5 side chain from acetate or mevalonate.

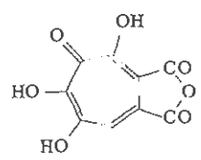
Gibberellic acid is a representative of a group of related substances known as gibberellins, which are produced by *Gibberella fujikuroi.* Although strictly out of place among the polycyclic structures of Fig. 21, which are partly or mainly aromatic in nature, gibberellic acid is nevertheless included because of its interesting hormonal characteristics. It stimulates shoot, but not root, growth, reverses genetic dwarfing, and induces the formation of the flowering hormones of many growing plants. The gibberellins are present in higher plants, particularly in immature seeds, and also in ferns and algae. Gibberellic acid has potential uses in agriculture and malting.

C. Tropolones

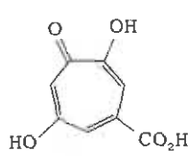
Several fungal metabolites contain the tropolone nucleus, which consists of a seven-membered carbocyclic structure, to which the special system of conjugated double bonds imparts aromatic properties. Some of these structures are illustrated in Fig. 22. The actual method of biosynthesis of the tropolone ring has not yet been clearly established although two possible mechanisms for the expansion of a six-membered aromatic ring have been suggested. Further examples of the seven-membered tropane ring are seen in the condensed ring system of lactarazulene and lactaroviolin.



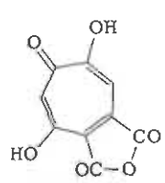
Puberulic acid
Penicillium puberulum, etc.
(Birkinshaw and Raistrick, 1932;
Corbett *et al.*, 1950a, b)



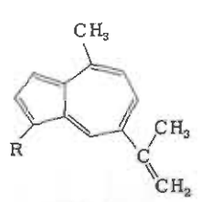
Puberulonic acid
P. puberulum, etc.
(Corbett *et al.*, 1950a, b;
Aulin-Erdtman, 1951)



Stipitatic acid
P. stipitatum
(Birkinshaw *et al.*, 1942c;
Corbett *et al.*, 1950c)



Stipitatonic acid
P. stipitatum
(Segal, 1957, 1959)

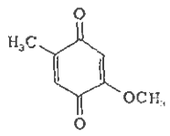


Lactarazulene; R = CH_3
Lactaroviolin; R = CHO
Lactarius deliciosus
(Willstaedt and Zetterberg, 1946;
Sorm *et al.*, 1953; Heilbronner and Schmid, 1954)

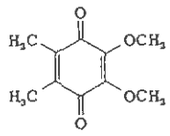
FIG. 22. Tropolones.

V. QUINONES AND QUINONOID PRODUCTS

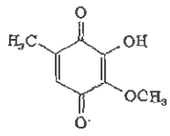
The quinones are closely related to the quinols, which have already been discussed as belonging to the aromatic series. The quinols are readily convertible by atmospheric oxidation to the quinones and the reverse change can readily be accomplished by reducing agents. The fungi are capable of inducing these changes, so that a quinol and its corresponding quinone may in some cases be isolated from the same fungal culture. Thus gentisyl



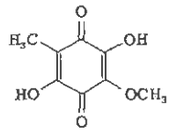
2-Methoxy-5-methylbenzoquinone
Coprinus similis
(Anchel *et al.*, 1948)



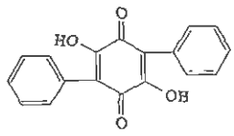
Aurantiogliocladin
Gliocladium sp.
(Vischer, 1953)



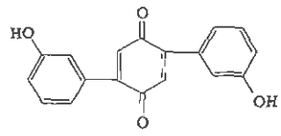
Fumigatin
Aspergillus fumigatus
(Anslow and Raistrick, 1938;
Baker and Raistrick, 1941)



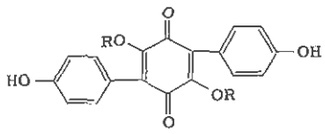
Spinulosin
Penicillium spinulosum
(Birkinshaw and Raistrick, 1931)



Polyporic acid
Polyporus nidulans
(Kögl, 1926)

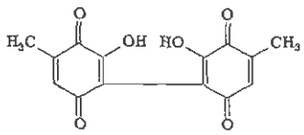


Volucrisporin
Volucrispora aurantiaca
(Divekar *et al.*, 1959)

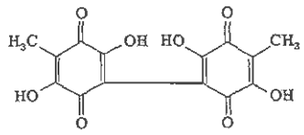


Atromentin; R = H
Polyporus nidulans
(Kögl and Becker, 1928)

Aurantiacin; R = C_6H_5CO
Hydnum aurantiacum
(Gripenberg, 1956)



Phoenicin
Penicillium phoenicium
(Posternak, 1938)



Oosporein
Oospora colorans
(Kögl and van Wessem, 1944)

FIG. 23. Benzoquinones.

alcohol (Fig. 18) and its quinone have been obtained from *Penicillium patulum* as a deep violet-colored complex (Engel and Brzeski, 1947).

The substituents of the quinone nucleus are in general therefore similar to those found in the phenolic derivatives and include methyl, hydroxyl, and methoxyl groups. The carboxyl group is of infrequent occurrence. It cannot be a substituent of the quinone ring, but may be found on an aromatic nucleus fused with the quinone ring as in the anthraquinone series. All the known fungal quinones are *p*-quinones. More complex structures (diquinones) in this series are represented by direct carbon-to-carbon linkage of two similar quinone molecules.

A. Benzoquinones

Various examples of fungal benzoquinones are shown in Fig. 23. It is to be noted that in some cases symmetrically placed phenyl groups (which may or may not be hydroxylated) are present as substituents. Where there is a high degree of symmetry in a fairly complex structure it is tempting to conclude that the molecule is biosynthesized from two similar moieties. In tracer studies Birch *et al.* (1958b) found that aurantiogliocladin is synthesized from four acetate units with loss of a molecule of CO_2 and the introduction of one nuclear methyl and two *O*-methyl groups from C_1 units (formic acid). The relationship of terreic acid (Fig. 9) to a quinone has already been noted.

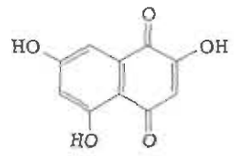
B. Naphthoquinones

The fungal naphthoquinones, examples of which are shown in Fig. 24, probably arise by the acetate pathway. Birch and Donovan (1954), on the assumption that this pathway was in operation for flaviolin, were able to predict the correct structure before the position of the hydroxyl groups had been completely determined. The structure was later confirmed by synthesis.

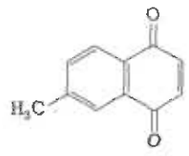
C. Anthraquinones

Anthraquinone pigments are found as products of a number of fungal species and are often responsible for bright colors in the mycelium. They are usually present as mixtures and may be formed in surprisingly high yield. As much as 30% of the dried mycelium of *Helminthosporium*

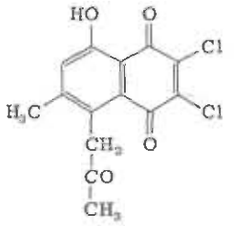
gramineum was found to consist of a mixture of polyhydroxyanthraquinones. The fungal anthraquinones, as illustrated in Fig. 25, contain from one to five hydroxyl groups, one of which may be methylated. A β-methyl group is almost always present as such or in one of its oxidized forms (CH_2OH, CHO, or CO_2H). The possible derivation of endocrocin from a C_{16} polyketo acid has already been mentioned (p. 183). Endocrocin on decarboxylation gives emodin. Tracer studies have in fact shown that helminthosporin and emodin arise from acetate; it may be inferred that



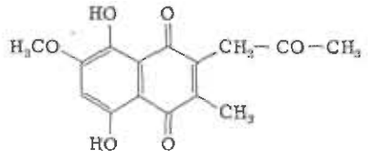
Flaviolin
Aspergillus citricus
(Davies *et al.*, 1955)



6-Methyl-1, 4-naphthoquinone
Marasmius gramineum
(Bendz, 1948)



Mollisin
Mollisia caesia
(Van der Kerk and Overeem, 1957)

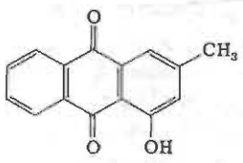


Javanicin
Fusarium javanicum
(Arnstein and Cook, 1947;
Ruelius and Gauhe, 1950)

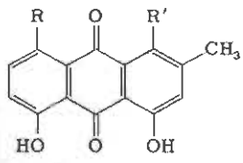
FIG. 24. Naphthoquinones.

this derivation is generally applicable to the fungal anthraquinones. Emodin may be regarded as the type structure from which many others may be derived by insertion or removal of hydroxyl groups, *O*-methylation, and methyl oxidation.

Several dianthraquinones are known. These are formed by carbon-to-carbon linkage of two similar anthraquinone molecules in the 8-position. Two examples of this type of structure are shown in Fig. 26. Skyrin is presumably formed by linkage of two emodin molecules, iridoskyrin is a diislandicin.

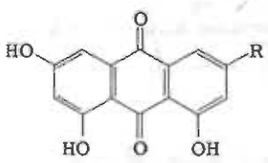


Pachybasin
Pachybasium candidum
(Shibata and Takido, 1955)



Chrysophanol; R = R' = H
Islandicin; R = H, R' = OH
Penicillium islandicum
(Howard and Raistrick, 1949, 1950)

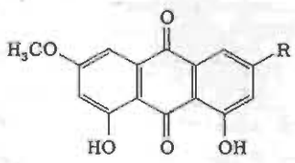
Helminthosporin; R = OH, R' = H
Helminthosporium gramineum
(Charles *et al.*, 1933)



Emodin; R = CH_3
Cortinarius sanguineus
(Kögl and Postowsky, 1925)

ω-Hydroxyemodin; R = CH_2OH

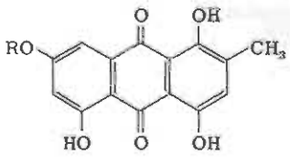
Emodic acid; R = CO_2H
Penicillium cyclopium
(Anslow *et al.*, 1940)



Physcion; R = CH_3
Aspergillus glaucus
(Ashley *et al.*, 1939)

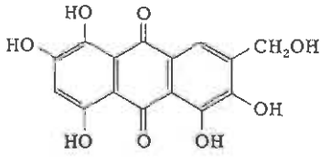
Teloschistin; R = CH_2OH
Teloschistes flavicans
(Seshadri and Subramanian, 1949)

Fallacinal; R = CHO
Xanthoria fallax
(Murakami, 1956)



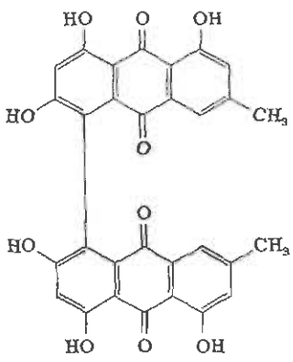
Catenarin; R = H
Helminthosporium catenarium
(Anslow and Raistrick, 1940)

Erythroglaucin; R = CH_3
Aspergillus glaucus
(Ashley *et al.*, 1939)

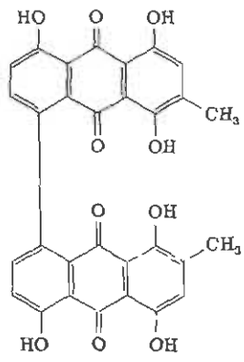


Asperthecin
Aspergillus quadrilineatus
(Howard and Raistrick, 1955;
Birkinshaw and Gourlay, 1961)

FIG. 25. Anthraquinones.



Skyrin
Penicillium islandicum, etc.
(Shibata *et al.*, 1955)

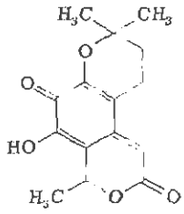


Iridoskyrin
P. islandicum
(Howard and Raistrick, 1954)

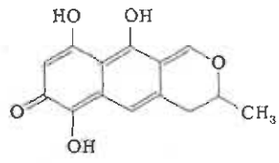
FIG. 26. Dianthraquinones.

D. Hemiquinones

Some of the fungal metabolites possess a hemiquinonoid (or methylenequinone) structure, in which one of the doubly linked oxygen atoms of the quinone nucleus is replaced by a doubly linked carbon atom. Citrinin (Fig. 21) is an example of this type. Others are shown in Fig. 27.



Fuscin
Oidiodendron fuscum
(Michael, 1948; Barton and Hendrickson, 1955)



Purpurogenone
Penicillium purpurogenum
(Roberts and Warren, 1955)

FIG. 27. Hemiquinones.

VI. METABOLITES CONTAINING NITROGEN

A. Acyclic Nitrogen Compounds

1. Amines

Ammonia and the simpler amines are of frequent occurrence, particularly in the higher fungi. Von Kamienski (1958) investigated the amine

content of 105 species representing 18 families of the higher fungi. Ammonia was universally present; methylamine, CH_3NH_2, occurred in 22 species; dimethylamine, $(CH_3)_2NH$, in 10; trimethylamine, $(CH_3)_3N$, in 8; isoamylamine, $(CH_3)_2CH—CH_2—CH_2NH_2$, in 19; and β-phenylethylamine, $C_6H_5—CH_2—CH_2NH_2$, in 4 species. In many cases these amines appear to arise by decarboxylation of the corresponding amino acids. Thus List and Hetzel (1960) obtained from *Coprinus micaceus* eight volatile amines, for which the amino acids were also detected. *Polyporus sulfureus* (List and Menssen, 1959) afforded methylamine, dimethylamine, ethylamine, *n*-propylamine, isoamylamine, colamine, and phenylethylamine. The amino acids corresponding with these bases were also present with the exception of sarcosine, which would yield dimethylamine. In *Coprinus atramentarius,* on the other hand, List and Reith (1960) found only a few bases that could be regarded as simple decarboxylation products of the numerous amino acids present, and it was concluded that the specific decarboxylases presumably present in the other fungi were lacking in this case.

Hydroxylated amines and their derivatives are present in fungi. Ethanolamine, $H_2NCH_2—CH_2OH$, is a constituent of phosphatides; the mono- and di-*N*-methyl derivatives have been obtained from *Neurospora crassa.* Further examples of the hydroxyamines are to be found in the phytosphingosines, which contain three hydroxyl groups, and the amino group is acylated, usually with a long-chain fatty acid. The fungal cerebrins are amides of the base with hydroxy acids, e.g., $CH_3(CH_2)_xCHOH—CO—NH—CH(CH_2OH)—CHOH—CHOH(CH_2)_nCH_3$, where $x = 21$ or 23, $n = 13$ or 15.

2. *Quaternary Ammonium Compounds*

Quaternization of ethanolamine gives choline, a constituent of phosphatides; other derivatives of choline are acetylcholine, present in ergot (*Claviceps purpurea*), and choline sulfate obtained from *Aspergillus sydowii* mycelium. The bases muscarine and muscaridine, derived from the fly agaric, *Amanita muscaria,* are further examples of this type (Fig. 28).

3. *Unusual Nitrogen Groups*

The groups illustrated in Fig. 29 are of infrequent occurrence in natural products although well known in organic chemistry. The nitro group is present in β-nitropropionic acid, which is formed by *Aspergillus flavus, A. oryzae,* and *Penicillium atrovenetum.* From the last-named species, when grown on Raulin-Thom medium, which supplies nitrogen only as ammonium ion, it is obtained in considerable yield. It appears to be biosyn-

thesized from aspartic acid by monodecarboxylation and conversion of the amino to the nitro group (Birch *et al.*, 1960).

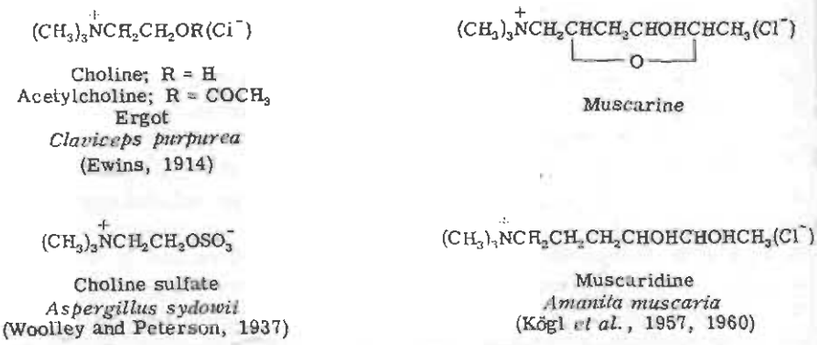


FIG. 28. Quaternary ammonium compounds.

The nitroso group occurs as *p*-methylnitrosamine benzaldehyde, a metabolite of *Clitocybe suaveolens*.

In certain of the polyacetylenes the more usual terminal carboxyl group is modified and contains nitrogen, present as the amide group in agrocybin and diatretyne I and as nitrile in diatretyne II (see Fig. 8).

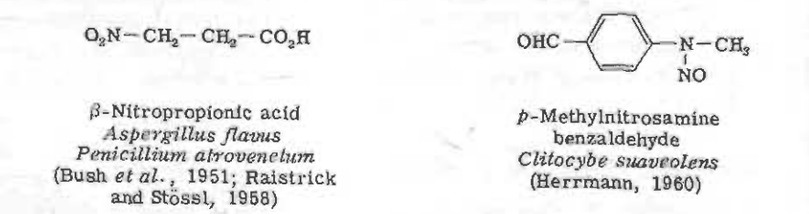


FIG. 29. Unusual nitrogen groups.

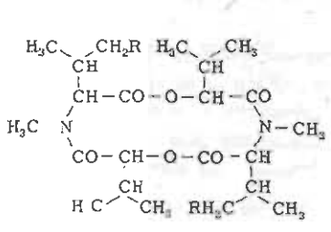
B. Oligopeptides

Proteins, macromolecules containing chains of amino acids, are characteristic of all forms of life. Many of the fungi produce in addition oligopeptides containing only a few structural units. These often have marked physiological action on other organisms. The term oligopeptide is here used to include chains or large rings having as building units not only amino acids, some of which may be unusual in type, but also nitrogen-free organic acids. Two types of linkage are involved: (1) the true peptide (—CONH—) linkage between a carboxyl and an amino group, and (2) the ester linkage between a carboxyl and a hydroxyl group. The term

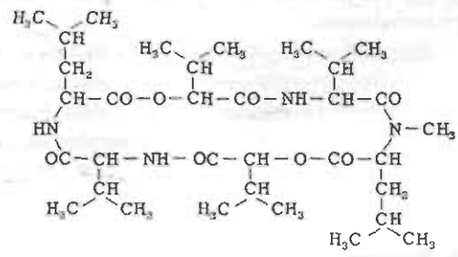
depsipeptide has been introduced to denote a product where both types of linkage occur in the same molecule.

The simplest fungal oligopeptide, containing only two building units, is DL-fumarylalanine, $HO_2C—CH=CH—CO—NH—CH(CH_3)—CO_2H$, obtained from *Penicillium resticulosum* (Birkinshaw *et al.*, 1942b). It is evidently formed by peptide linkage between DL-alanine and one of the carboxyl groups of fumaric acid. Its occurrence as the racemic form is unexpected, but Winterstein *et al.* (1913) have recorded the presence of DL-alanine in an aqueous extract from *Boletus edulis*.

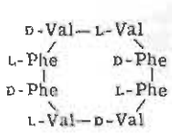
The genus *Fusarium* produces several oligopeptides. Some of these, e.g., lycomarasmin and culmomarasmin, were isolated from culture filtrates of species which produced a strong wilting action on plants such as tomatoes. Although the individual amino acid or other components of these products



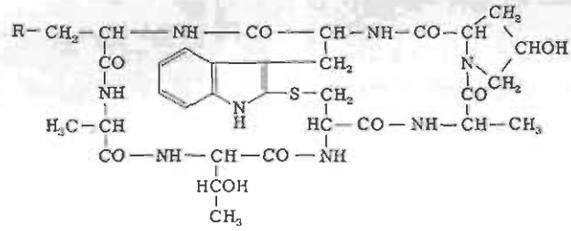
Enniatin A; R = CH_3
Enniatin B; R = H
Fusarium sp.
(Plattner *et al.*, 1948)

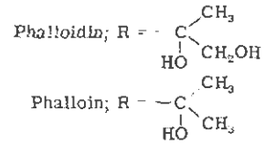


Sporidesmolide I
Pithomyces chartarum
(Russell, 1960)

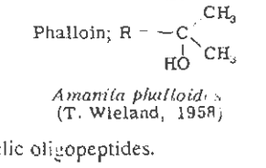


Fungisporin
(Val, valine;
Phe, phenylalanine)
Fungal spores
(Miyao, 1960)





Phalloidin; R =



Phalloin; R =

Amanita phalloides
(T. Wieland, 1958)

FIG. 30. Cyclic oligopeptides.

have been identified, no satisfactory structural formula can yet be advanced.

Greater success has been attained in the examination of the enniatins, also products of species or strains of fusaria. Enniatin A, $C_{24}H_{38}O_6N_2$, and enniatin B, $C_{22}H_{38}O_6N_2$, both show antibiotic action against *Mycobacterium tuberculosis*. From the structures shown in Fig. 30 it can be seen that these differ only by two methyl groups; both peptide and ether linkages are present. Other peptide antibiotics of *Fusarium* species investigated by Cook *et al.* (1949) appear to be similar in structure to the enniatins.

Sporidesmolides I and II were obtained from the pasture fungus *Sporidesmium bakeri* (=*Pithomyces chartarum*), which is associated with a facial eczema of ruminants. The structures suggested are similar to those of the enniatins.

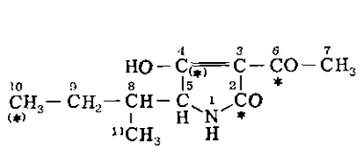
Fungisporin, a sublimable component of the spores of some fungi, is a cyclic polypeptide containing equal numbers of molecules of valine and phenylalanine.

The toxins of *Amanita phalloides* have aroused much interest owing to the extremely poisonous nature of the fungus. They have been subjected to intensive investigation by Wieland and co-workers, who have completely unraveled the structure of phalloidin and phalloin.

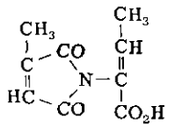
C. Heterocyclic Nitrogen Compounds

1. Pyrrole Derivatives

Proline (pyrrolidinecarboxylic acid) and its derivatives are constituents of some fungal peptides such as phalloidin and of some of the ergot alkaloids. A relatively simple example of this type of five-membered ring is tenuazonic acid, which has a structure similar to that of the tetronic acids, but with NH replacing the ring oxygen (Fig. 31). Degradative studies by Stickings and Townsend (1961) of the product obtained by feeding



Tenuazonic acid
* Main labeling from acetate-1-C^{14}
(*) Minor labeling from acetate-1-C^{14}
Alternaria tenuis
(Rosett *et al.*, 1957; Stickings, 1959)



Pencolide
Penicillium multicolor
(Birkinshaw *et al.*, 1963

FIG. 31. Pyrrole derivatives.

1-C^{14}-acetate to *Alternaria tenuis* showed that the primary labeling was on carbon atoms 2 and 6, with secondary labeling on carbons 4 and 10. Taking into account the similar labeling produced in corresponding atoms of isoleucine by *Torulopsis* [*candida*] *utilis* grown on 1-C^{14}-acetate, it seems probable that tenuazonic acid is synthesized from two molecules of acetate and one of isoleucine.

Whereas the tenuazonic acid ring structure may be considered to be derived from a γ-lactone by substitution of NH for oxygen, a similar substitution in an acid anhydride ring gives the imide form, which is the basis of the structure allocated to penicolide. This metabolite of *Penicillium multicolor* is easily hydrolyzed to citraconic acid, α-oxobutyric acid, and ammonia; the most probable structure is therefore 2-citraconimidobut-2-enoic acid.

2. *Indole Derivatives*

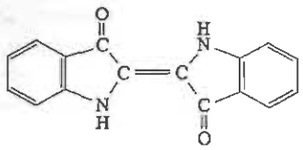
Only relatively simple indole derivatives are mentioned in this section; more complex representatives are considered in other groups.

The amino acid tryptophan is a common constituent of proteins. It is produced by condensation of indole with serine. The enzyme responsible for this synthesis has been extracted from wild-type *Neurospora* (Umbreit *et al.*, 1946). Indigo has been obtained from a mutant culture of *Schizophyllum commune* grown on a synthetic medium containing thiamine, with an ammonium salt as source of nitrogen. Indigo is probably produced by condensation of two molecules of indole, since certain bacteria are known to be capable of converting indole to indigo (Fig. 32).

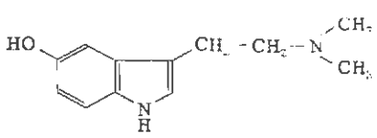
Bufotenin (5-hydroxy-*N*,*N*-dimethyltryptamine) is a constituent of the skin secretion of toads. It has been obtained by Wieland by extraction of *Amanita mappa* [A. citrina] and has also been detected in other *Amanita* species.

The Maya Indians have for centuries used the "magic-fungus" Teonanácatl to produce illusions and hallucinations in certain tribal ceremonies. The fungus responsible was found to be a *Psilocybe*. From *P. mexicana* and from *Stropharia cubensis*, Hofmann (1960) was able to isolate two active principles which produced the psychotomimetic effects of the Mexican magic-fungus. The pure products, named psilocin and psilocybin, were found to be 4-hydroxy-ω-*N*,*N*-dimethyltryptamine and the corresponding phosphoryl ester.

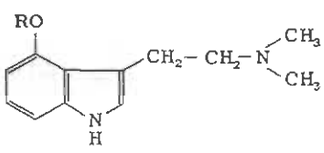
The indole nucleus is also present in the large group of ergot alkaloids derived from *Claviceps purpurea*. The natural ergot alkaloids and those obtainable by laboratory culture of the fungus are all related to lysergic acid. Some are peptides, others differ in substituents and in configuration. Other fungi, e.g., *Aspergillus fumigatus*, also produce good yields of indole



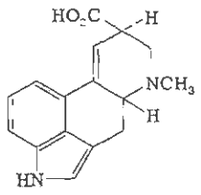
Indigo
Schizophyllum commune
(Miles *et al.*, 1956)



Bufotenin
Amanita citrina
(T. Wieland *et al.*, 1953)



Psilocybin; R = $-PO_3H_2$
Psilocin; R = H
Psilocybe mexicana
(Hofmann *et al.*, 1959)



Lysergic acid
Claviceps purpurea

FIG. 32. Indole derivatives.

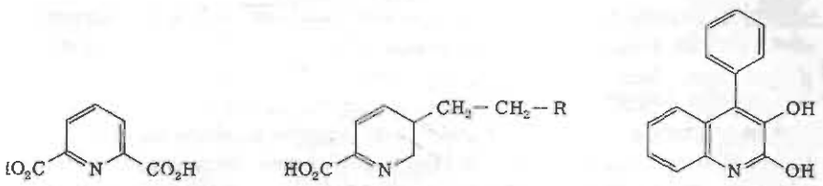
alkaloids. It has been found that tryptophan can act as precursor of lysergic acid (Mothes *et al.*, 1958), and the remainder of the molecule appears to arise by incorporation of mevalonate.

3. Pyridine Derivatives

The pyridine derivatives nicotinic acid and pyridoxine are vitamins. They are of widespread occurrence in living cells since they function as coenzymes and act as prosthetic groups in a variety of enzymatic reactions.

Other examples of metabolites more specific for the fungi are dipicolinic acid (originally obtained, however, from a bacterial food inoculant), fusaric acid, and dehydrofusaric acid (Fig. 33). Fusaric acid represents yet another wilting agent derived from *Fusarium* species. It causes wilting by destroying the differential permeability of leaf cells.

An example of a quinoline metabolite is viridicatin, $C_{15}H_{11}O_2N$, derived from *Penicillium viridicatum* and *P. cyclopium*. It can also be obtained by mild acid hydrolysis of the more complex structure cyclopenin, $C_{17}H_{14}O_4N_2$, also produced by *P. cyclopium*. The degradation products of cyclopenin, in



Dipicolinic acid
Penicillium sp.
)oyama *et al.*, 1960)

Fusaric acid; R = $-C_2H_5$
Dehydrofusaric acid;
R = $-CH=CH_2$
Fusarium sp.
Gibberella fujikuroi
(Yabuta *et al.*, 1934;
Plattner *et al.*, 1954)

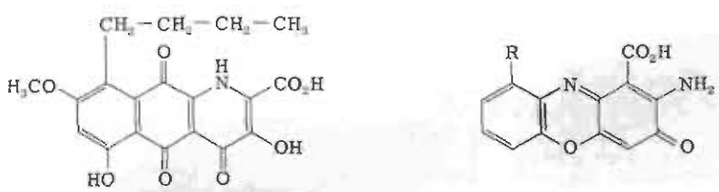
Viridicatin
Penicillium viridicatum,
P. cyclopium
(Cunningham and
Freeman, 1953;
Bracken *et al.*, 1954)

FIG. 33. Pyridine and quinoline derivatives.

addition to viridicatin are methylamine and carbon dioxide. The structure of cyclopenin is still under investigation.

4. *Azanthracene and Phenoxazone Derivatives*

One example of each of these two types of tricyclic structures is illustrated (Fig. 34). The azanthracene derivative phomazarin was obtained



Phomazarin
Phoma terrestris
(Kögl and Quackenbush, 1944;
Birch *et al.*, 1964)

Cinnabarin; R = CH_2OH
Cinnabarinic acid; R = CO_2H
Polystictus sanguineus
(Cavill *et al.*, 1959;
Gripenberg, 1958)

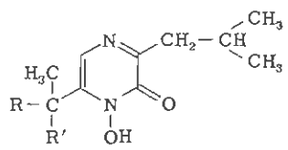
FIG. 34. Tricyclic nitrogen derivatives.

from the mycelium of *Phoma terrestris*. The general form of the molecule is that of an anthraquinone with one of the α-carbon atoms replaced by nitrogen. Work with labeled acetate as precursor indicates that phomazarin is biosynthesized from eight acetate units, possibly with addition of glycine and carbon dioxide. Cinnabarin (polystictin) is derived from *Coriolus* [*Polystictus*] *sanguineus*. In cinnabarinic acid from the same source, the hydroxymethyl group is replaced by carboxyl. Cinnabarin is structurally

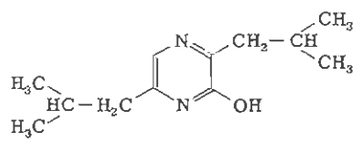
related to the insect pigments known as ommochromes and to the actinomycins obtained from *Streptomyces* species.

5. Pyrazine Derivatives

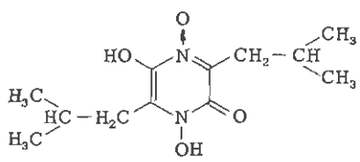
Aspergillic acid and hydroxyaspergillic acid are products of *Aspergillus flavus*. The first indication of the presence of aspergillic acid in the culture filtrate was given by its antibiotic activity. Muta-aspergillic acid and flavacol have also been obtained from the same mold. The four structures (Fig. 35)



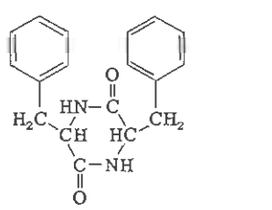
Aspergillic acid; $\begin{cases} R = C_2H_5 \\ R' = H \end{cases}$
Hydroxyaspergillic acid; $\begin{cases} R = C_2H_5 \\ R' = OH \end{cases}$
Muta-aspergillic acid; $\begin{cases} R = CH_3 \\ R' = OH \end{cases}$
Aspergillus flavus
(Glister, 1941; Dutcher, 1958; Nakamura, 1960, 1961)



Flavacol
Aspergillus flavus
(Dunn *et al.*, 1949)



Pulcherriminic acid
Candida pulcherrima
(Kluyver *et al.*, 1953; Cook and Slater, 1956)



L-Phenylalanine anhydride
Penicillium nigricans
(Birkinshaw and Mohammed, 1962)

FIG. 35. Pyrazine derivatives.

are closely related; flavacol was shown to be identical with 3-hydroxy-2,5-diisobutylpyrazine prepared by dehydration of DL-leucine anhydride with phosphoryl chloride. There is definite evidence that aspergillic acid is synthesized from leucine and isoleucine. Pulcherrimin, obtained from a yeast in a medium containing a ferric salt, is a deep maroon powder containing 12.7% of iron. Removal of the iron with aqueous sodium hydroxide affords pulcherriminic acid, which has two identical side chains.

An even more symmetrical example of this type of ring structure is furnished by L-phenylalanine anhydride (*cis*-L-3,6-dibenzyl-2,5-dioxopiperazine), which is obtained from the mycelium of *Penicillium nigricans*. It was synthesized by warming L-phenylalanine ethyl ester, which suggests the method of biosynthesis. Other more complex fungal metabolites in this group such as echinulin and mycelianamide are known. It is highly probable that in all cases the biosynthesis follows the same course, the pyrazine ring being first produced by double peptide linkage between two similar or dissimilar α-amino acids. Further modifications may then be introduced by oxidation or other processes.

VII. METABOLITES CONTAINING SULFUR

A. No Nitrogen Present

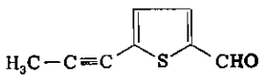
Sulfur is only rarely encountered as a constituent of fungal metabolites unless the molecule also contains nitrogen. However *Schizophyllum commune*, a wood-rotting fungus, was found to convert inorganic sulfate into the methylated derivatives of sulfur listed in Fig. 36. Traces of hydrogen

CH_3SH **Methyl mercaptan**

CH_3S-SCH_3 **Dimethyl disulfide**

$(CH_3)_2S$ **Dimethyl sulfide**

Schizophyllum commune
(Birkinshaw *et al.*, 1942a;
Challenger and Charlton, 1947)



Junipal
Daedalea juniperina
(Birkinshaw and Chaplen, 1955)

$(CH_3)_2SO_2$ **Dimethyl sulfone**

Cladonia deformis
(Bruun and Sorensen, 1954)

FIG. 36. Metabolites containing sulfur, but no nitrogen.

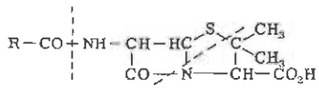
sulfide were also detected. From the lichen *Cladonia deformis* dimethyl sulfone has been obtained.

A further example of a metabolite containing sulfur is the acetylenic thiophen derivative junipal. This is the only thiophen derivative to be recorded so far from fungi although α-terthienyl has been obtained from the Indian marigold (Zechmeister and Sease, 1947). It seems probable that the thiophen ring in such natural products arises by interaction of hydrogen sulfide with a straight-chain compound containing an acetylenic-olefinic system.

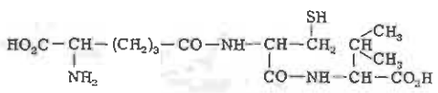
B. Nitrogen Present

A number of sulfur-containing fungal metabolites also contain nitrogen. One reason for this is the fact that cysteine, which contains both nitrogen and sulfur, can act as building unit for more complex products of polypeptide nature. Examples of oligopeptides containing sulfur, namely phalloidin and phalloin (Fig. 30), have already been mentioned.

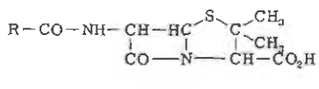
An important example of this type is the antibiotic penicillin, which has proved to be an extremely valuable chemotherapeutic agent for the treatment of many bacterial infections. The biogenesis of penicillin has been studied [for reviews see Arnstein (1957) and Demain (1959)]. The thiazolidine-β-lactam structure is derived from L-cystine and L-valine. The side-chain precursor is the appropriate substituted acetic acid; in benzylpenicillin this is phenylacetic acid. Arnstein *et al.* (1960) obtained from



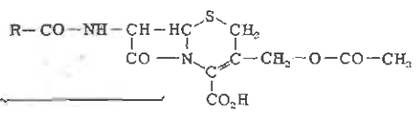
The Penicillins
(Benzylpenicillin; R = $CH_2-C_6H_5$)
Penicillium chrysogenum, etc.



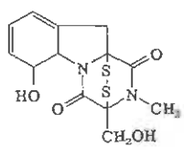
δ-(α-Aminoadipoyl) cysteinylvaline
P. chrysogenum
(Arnstein *et al.*, 1960)



Cephalosporin N
Cephalosporium sp.
(Newton and Abraham, 1954)



Cephalosporin C
Cephalosporium sp.
(Abraham and Newton, 1961)



Gliotoxin
Gliocladium fimbriatum, etc.
(Weindling, 1932; Bell *et al.*, 1958; Winstead and Suhadolnik, 1960)

FIG. 37. Metabolites containing sulfur and nitrogen.

cillium chrysogenum, after brief incubation with C^{14}-valine, a tripeptide
ıg the structure δ-(α-aminoadipoyl)cyst(e)inylvaline (Fig. 37).
ninoadipic acid was also obtained chiefly in the L-form. The possible
enetic relationship with cephalosporin N, another antibiotic, is im-
ately evident. The pathway for penicillin biosynthesis proposed by
tein and Morris (1960) involves the conversion of the tripeptide to
alosporin N by appropriate ring closures. The final stage requires ex-
ge of the α-aminoadipoyl side chain of cephalosporin N for a phenacyl
p.

nother antibiotic from a *Cephalosporium* species, cephalosporin C,
a structure which could also arise from the tripeptide δ-(α-amino-
oyl)cysteinylvaline by suitable oxidation of the *gem*-dimethyl group
a different type of ring closure to form a six-membered dihydrothia-
ring in place of the five-membered thiazolidine ring of penicillin.

further member of this group, gliotoxin, derived from several fungal
ies, has bacteriostatic and fungicidal properties. Radioactive tracer
es have shown that gliotoxin is biosynthesized by *Trichoderma viride*
one molecule each of phenylalanine and serine, which combine to
an anhydropeptide ring, with addition of two sulfur atoms to form a
fide bridge. Since *m*-tyrosine can act as precursor, it appears that
oxylation can occur before cyclization of the aliphatic side chain of
ıylalanine.

VIII. METABOLITES CONTAINING CHLORINE

hlorine is a constituent of a number of fungal metabolites, some of
h have already been mentioned, e.g., geodin (Fig. 21) and mollisin
. 24). Organically bound chlorine is not a necessary precursor in their
ynthesis since these products are readily formed in synthetic media in
h the chlorine is present as chloride ion. When the fungus is deprived
ıloride, it may produce the dechloro analog of the metabolite. In most
s the chlorine is attached to a carbon atom of a carbocyclic ring of
ıatic type. Exceptionally, caldariomycin, a dihydroxycyclopentane de-
ive, carries two chlorine atoms attached to the same carbon atom.

ı studying the synthesis of caldariomycin, P. D. Shaw *et al.* (1959)
found that the formation of the carbon-chlorine bond is catalyzed by
nzyme which is present in *Caldariomyces fumago*. The enzyme pro-
:s the conversion of β-ketoadipic acid and chloride ion into δ-chloro-
linic acid. The latter, when labeled with Cl^{36}, is converted by the mold
labeled caldariomycin. The reactions are presumed to follow the
me shown in Fig. 38.

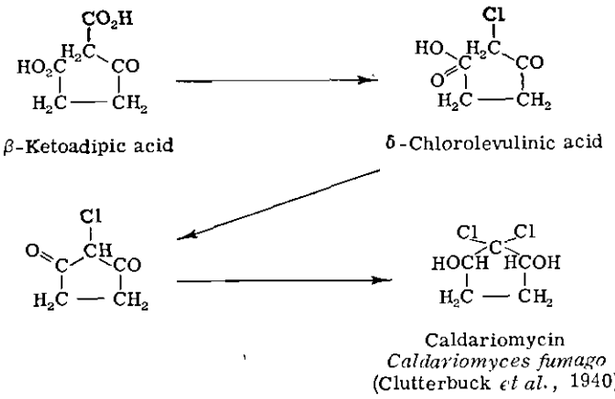


FIG. 38. Biosynthesis of caldariomycin.

Further examples of chlorine substitution in aromatic rings are shown in Fig. 39.

Griseofulvin is of considerable commercial importance since it is a valuable chemotherapeutic agent. It was obtained independently in two labora-

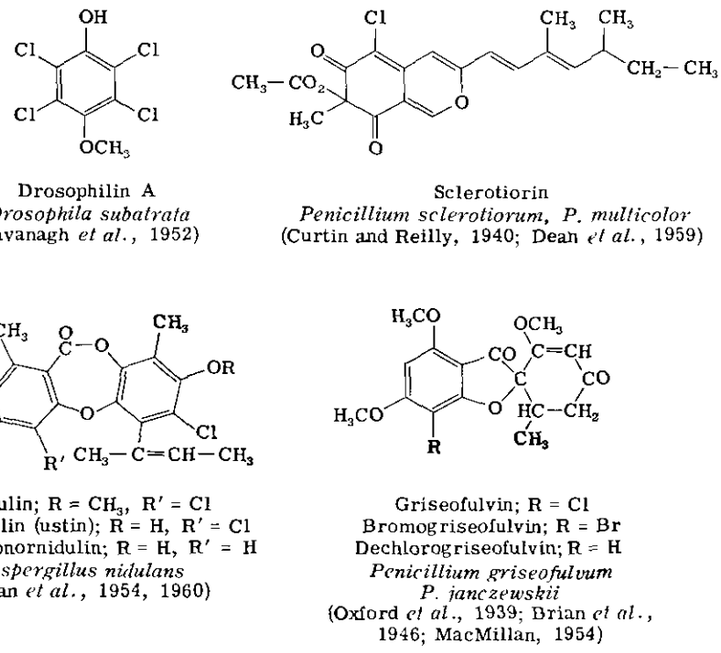


FIG. 39. Metabolites containing chlorine.

tories. Oxford *et al.* (1939) isolated it from *Penicillium griseofulvum* cultures and determined its chemical properties and empirical formula. Brian *et al.* (1949) obtained it from *P. janczewskii* and, noting its peculiar stunting and distorting effect on the germ tubes and hyphae of *Botrytis allii* and other fungi, named it "curling factor." The two products were found to be identical. It was found to act systemically in plants, being taken up by the roots and protecting seedlings against mildew and tomato plants against *Botrytis*. Soil application was not very profitable, however, since griseofulvin was readily destroyed in the ground. It was not readily taken up by the leaves of plants. The main application of griseofulvin is now in the cure of dermatoses in man; it is manufactured for this purpose from molds of the *P. griseofulvum-P. urticae* group since from this source the metabolite can be obtained by the deep-culture method. It is the agent of choice in many mycoses of skin, hair, and nails.

The structure of griseofulvin is reminiscent of that of geodin; it is probably biosynthesized by a similar route. When grown on a medium devoid of chlorine, the fungi yielding griseofulvin produce the chlorine-free analog dechlorogriseofulvin. The bromo analog has also been obtained by substituting bromide for chloride in the culture medium.

Drosophilin A is of interest in containing no less than four chlorine atoms although it is a relatively simple molecule.

Nidulin has a depsidone type of structure with unsaturated side chain. Nornidulin (with OH replacing OCH_3) and dechloronornidulin (in which one of the three chlorine atoms is missing) are variants of the same basic structure.

Sclerotiorin is the only chlorine-containing member of a group of products of similar basic form which have been termed azaphilones. They are so named because they react avidly with ammonia, the cyclic oxygen atom being replaced by NH.

IX. METABOLITES CONTAINING ARSENIC, ETC.

A number of cases of arsenical poisoning have been ascribed to the use of domestic wallpapers colored with pigments containing arsenic (usually as copper hydrogen arsenite). The toxic compound evolved as a result of the growth of molds on the damp wallpaper has been shown to be trimethylarsine, $(CH_3)_3As$. Challenger *et al.* (1933) obtained this gas by growth of *Scopulariopsis brevicaulis* (*Penicillium brevicaule*) on sterile bread crumbs moistened with solutions of various arsenic compounds such as arsenious oxide, As_2O_3, sodium methyl arsonate, $CH_3AsO(ONa)_2$, or sodium cacodylate, $(CH_3)_2AsOONa$. Sodium ethyl arsonate and other alkylarsonic acids gave rise to mixed methylated arsines.

Similar results were obtained by the use of inorganic selenium and tellurium compounds as substrates, dimethyl selenide, $(CH_3)_2Se$, and dimethyl telluride, $(CH_3)_2Te$ being evolved. These results are comparable with the production of methyl sulfides by the action of *Schizophyllum commune* on inorganic sulfate; they are all examples of biological methylation (see review by Challenger, 1959).

X. FUNCTION

So far as present knowledge extends, most of the metabolic products here discussed appear to be off the main pathways of biosynthesis required for building cell structure, enzymes, genes, etc., or for the production of energy (e.g., the Krebs cycle). Further research may show that some of these metabolites function in processes as yet undiscovered. Various roles have been suggested, such as respiratory factors (e.g., quinones), photosensitive pigments involved in phototropic response mechanisms (gallic acid), food storage (fats and carbohydrates), and defense mechanisms (antibiotics).

Even when these possibilities are taken into account, however, there still appears to remain a solid core of products to which no useful purpose can be assigned. These have been termed overflow or shunt metabolites and would represent "errors in metabolism." Shunt metabolites may accumulate as a result of changes in genetic factors (mutations) characterized either by loss of an enzyme required for a fundamental pathway or by acquisition of a new enzyme specific for a particular reaction.

Alternatively, environmental factors may in some cases be responsible. The few well-established laboratory media regularly employed for the cultivation of the fungi have been evolved mainly on the basis of convenience, reproducibility, and ability to support growth of a large number of species. They may lack one or more nutritional factors required for specific organisms or may present an incorrect balance of components, producing pH changes or osmotic effects inimical to the "normal" metabolism.

Shunt metabolites produced owing to the operation of any or all of these causes may be regarded as pathological in nature, but are obviously without marked detrimental effect on the survival of the organisms concerned in their production.

REFERENCES

Abraham, E. P., and G. G. F. Newton. (1961). *Biochem. J.* 79:377.

Aldridge, D. C., J. F. Grove, R. N. Speake, B. K. Tidd, and W. Klyne. (1963). *J. Chem. Soc.* p. 143.

Alsberg, C. L., and O. F. Black. (1913). *Bull. U.S. Bur. Plant Ind.* **270.**
Anchel, M. (1952). *J. Am. Chem. Soc.* **74**:1588.
Anchel, M. (1953). *J. Am. Chem. Soc.* **75**:4621.
Anchel, M. (1955). *Science* **121**:607.
Anchel, M., and M. P. Cohen. (1954). *J. Biol. Chem.* **208**:319.
Anchel, M., A. Hervey, F. Kavanagh, J. Polatnick, and W. J. Robbins. (1948). *Proc. Natl. Acad. Sci. U.S.* **34**:498.
Angeletti, A., G. Tappi, G. Biglino. (1952). *Ann. Chim. (Rome)* **42**:502.
Anslow, W. K., and H. Raistrick. (1938). *Biochem. J.* **32**:687.
Anslow, W. K., and H. Raistrick. (1940). *Biochem. J.* **34**:1124.
Anslow, W. K., J. Breen, and H. Raistrick. (1940). *Biochem. J.* **34**:159.
Arnstein, H. R. V. (1957). *Ann. Rept. Progr. Chem. (Chem. Soc. London)* **54**:339.
Arnstein, H. R. V., and R. Bentley. (1953). *Biochem. J.* **54**:493.
Arnstein, H. R. V., and A. H. Cook. (1947). *J. Chem. Soc.* p. 1021.
Arnstein, H. R. V., and D. Morris. (1960). *Biochem. J.* **76**:323.
Arnstein, H. R. V., M. Artman, D. Morris, and E. J. Toms. (1960). *Biochem. J.* **76**:353.
Asano, M., and Y. Kameda. (1941). *J. Pharm. Soc. Japan* **61**:57, 80.
Asano, M., and Z. Ohta. (1933). *Ber. Deut. Chem. Ges.* **66**:1020.
Asano, M., and Z. Ohta. (1934). *Ber. Deut. Chem. Ges.* **67**:1842.
Ashley, J. N., H. Raistrick, and T. Richards. (1939). *Biochem. J.* **33**:1291.
Ashworth, P. J., E. R. H. Jones, G. H. Mansfield, K. Schlögl, J. M. Thompson, and M. C. Whiting. (1958). *J. Chem. Soc.* p. 950.
Aulin-Erdtman, G. (1951). *Chem. & Ind. (London)* p. 28.
Baker, W., and H. Raistrick. (1941). *J. Chem. Soc.* p. 670.
Barton, D. H. R., and J. B. Hendrickson. (1955). *Chem. & Ind. (London)* p. 682.
Barton, D. H. R., and A. I. Scott. (1958). *J. Chem. Soc.* p. 1767.
Barton, D. H. R., P. de Mayo, G. A. Morrison, and H. Raistrick. (1959). *Tetrahedron* **6**:48.
Bassett, E. W., and S. W. Tanenbaum. (1958). *Biochim. Biophys. Acta* **28**:247.
Bell, M. R., J. R. Johnson, B. S. Wildi, and R. B. Woodward. (1958). *J. Am. Chem. Soc.* **80**:1001.
Bendz, G. (1948). *Acta Chem. Scand.* **2**:192.
Bentley, R., and J. G. Keil. (1961). *Proc. Chem. Soc.* p. 209.
Bernhard, K., and H. Albrecht. (1947). *Helv. Chim. Acta* **30**:627.
Birch, A. J., and F. W. Donovan. (1954). *Chem. & Ind. (London)* p. 1047.
Birch, A. J., R. A. Massy-Westropp, and C. J. Moye. (1955). *Australian J. Chem.* **8**:539.
Birch, A. J., P. Fitton, E. Pride, A. J. Ryan, H. Smith, and W. B. Whalley. (1958a). *J. Chem. Soc.* p. 4576.
Birch, A. J., R. I. Fryer, and H. Smith. (1958b). *Proc. Chem. Soc.* p. 343.
Birch, A. J., B. J. McLoughlin, H. Smith, and J. Winter. (1960). *Chem. & Ind. (London)* p. 840.
Birch, A. J., D. N. Butler, and R. W. Rickards. (1964). *Tetrahedron Letters* p. 1853.
Birkinshaw, J. H., and P. Chaplen. (1955). *Biochem. J.* **60**:255.
Birkinshaw, J. H., and W. P. K. Findlay. (1940). *Biochem. J.* **66**:188.
Birkinshaw, J. H., and R. Gourlay. (1961). *Biochem. J.* **81**:618.
Birkinshaw, J. H., and Y. S. Mohammed. (1962). *Biochem. J.* **85**:523.
Birkinshaw, J. H., and E. N. Morgan. (1950). *Biochem. J.* **47**:55.

Birkinshaw, J. H., and H. Raistrick. (1931). *Phil. Trans. Roy. Soc. London* **B220**:245.
Birkinshaw, J. H., and H. Raistrick. (1932). *Biochem. J.* **26**:441.
Birkinshaw, J. H., and H. Raistrick. (1934). *Biochem. J.* **28**:828.
Birkinshaw, J. H., and H. Raistrick. (1936). *Biochem. J.* **30**:2194.
Birkinshaw, J. H., and M. S. Samant. (1960). *Biochem. J.* **74**:369.
Birkinshaw, J. H., J. H. V. Charles, and H. Raistrick. (1931). *Phil. Trans. Roy. Soc. London* **B220**:355.
Birkinshaw, J. H., A. E. Oxford, and H. Raistrick. (1936). *Biochem. J.* **30**:394.
Birkinshaw, J. H., W. P. K. Findlay, and R. A. Webb. (1942a). *Biochem. J.* **36**:526.
Birkinshaw, J. H., H. Raistrick, and G. Smith. (1942b). *Biochem. J.* **36**:829.
Birkinshaw, J. H., A. R. Chambers, and H. Raistrick. (1942c). *Biochem. J.* **36**:242.
Birkinshaw, J. H., A. Bracken, M. Greenwood, W. E. Gye, W. A. Hopkins, S. E. Michael, and H. Raistrick. (1943a). *Lancet* **II**:625.
Birkinshaw, J. H., A. Bracken, and H. Raistrick. (1943b). *Biochem. J.* **37**:726.
Birkinshaw, J. H., A. Bracken, and H. Raistrick. (1945). *Biochem. J.* **39**:70.
Birkinshaw, J. H., H. Raistrick, and D. J. Ross. (1952a). *Biochem. J.* **50**:630.
Birkinshaw, J. H., H. Raistrick, D. J. Ross, and C. E. Stickings. (1952b). *Biochem. J.* **50**:610.
Birkinshaw, J. H., P. Chaplen, and W. P. K. Findlay. (1957). *Biochem. J.* **66**:188.
Birkinshaw, J. H., M. G. Kalyanpur, and C. E. Stickings. (1963). *Biochem. J.* **86**:237.
Blair, J., and G. T. Newbold. (1955). *Chem. & Ind. (London)* p. 93.
Bracken, A., and H. Raistrick. (1947). *Biochem. J.* **41**:569.
Bracken, A., A. Pocker, and H. Raistrick. (1954). *Biochem. J.* **57**:587.
Braithwaite, G. D., and T. W. Goodwin. (1957). *Biochem. J.* **66**:31P.
Brian, P. W., P. J. Curtis, and H. G. Hemming. (1946). *Brit. Mycol. Soc. Trans.* **29**:173.
Brian, P. W., P. J. Curtis, and H. G. Hemming. (1949). *Brit. Mycol. Soc. Trans.* **32**:30.
Brown, J. J., and G. T. Newbold. (1954). *J. Chem. Soc.* p. 1076.
Bruun, T., and N. A. Sorensen. (1954). *Acta Chem. Scand.* **8**:703.
Bullock, E., J. C. Roberts, and J. G. Underwood. (1962). *J. Chem. Soc.* p. 4179.
Bu'Lock, J. D., and H. M. Smalley. (1961). *Proc. Chem. Soc.* p. 209.
Bu'Lock, J. D., and H. M. Smalley. (1962). *J. Chem. Soc.* p. 4662.
Bu'Lock, J. D., E. R. H. Jones, G. H. Mansfield, J. W. Thompson, and M. C. Whiting. (1954). *Chem. & Ind. (London)* p. 990.
Bush, M. T., O. Touster, and J. E. Brockman. (1951). *J. Biol. Chem.* **188**:685.
Cambie, R. C., J. N. Gardner, E. R. H. Jones, G. Lowe, and G. Read. (1963). *J. Chem. Soc.* p. 2056.
Cavill, G. W. K., P. S. Clezy, J. R. Tetaz, and R. L. Werner. (1959). *Tetrahedron* **5**:275.
Challenger, F. (1959). "Aspects of the Organic Chemistry of Sulphur." Butterworths, London.
Challenger, F., and P. T. Charlton. (1947). *J. Chem. Soc.* p. 424.
Challenger, F., C. Higginbottom, and L. Ellis. (1933). *J. Chem. Soc.* p. 95.
Charles, J. H. V., H. Raistrick, R. Robinson, and A. R. Todd. (1933). *Biochem. J.* **27**:499.
Clutterbuck, P. W., H. Raistrick, and M. L. Rintoul. (1931). *Phil. Trans. Roy. Soc. London* **B220**:301.

Clutterbuck, P. W., H. Raistrick, and F. Reuter. (1935a). *Biochem. J.* **29**:300.
Clutterbuck, P. W., H. Raistrick, and F. Reuter. (1935b). *Biochem. J.* **29**:871.
Clutterbuck, P. W., H. Raistrick, and F. Reuter. (1935c). *Biochem. J.* **29**:1300.
Clutterbuck, P. W., S. L. Mukhopadhyay, A. E. Oxford, and H. Raistrick. (1940). *Biochem. J.* **34**:664.
Cook, A. H., and C. A. Slater. (1956). *J. Chem. Soc.* p. 4133.
Cook, A. H., S. F. Cox, and T. H. Farmer. (1949). *J. Chem. Soc.* p. 1022.
Corbett, R. E., C. H. Hassall, A. W. Johnson, and A. R. Todd. (1950a). *J. Chem. Soc.* p. 1.
Corbett, R. E., A. W. Johnson, and A. R. Todd. (1950b). *J. Chem. Soc.* p. 6.
Corbett, R. E., A. W. Johnson, and A. R. Todd. (1950c). *J. Chem. Soc.* p. 147.
Cram, D. J., and M. Tishler. (1948). *J. Am. Chem. Soc.* **70**:4238.
Cunningham, K. G., and G. G. Freeman. (1953). *Biochem. J.* **53**:328.
Curtin, T. P., and J. Reilly. (1940). *Biochem. J.* **34**:1419.
Curtis, P. J., and B. E. Cross. (1954). *Chem. & Ind. (London)* p. 1066.
Curtis, R. F., C. H. Hassall, D. W. Jones, and T. W. Williams. (1960). *J. Chem. Soc.* p. 4838.
Curtis, R. F., C. H. Hassall, S. Natori, and H. Nishikawa. (1961). *Chem. & Ind. (London)* p. 1360.
Davies, J. E., F. E. King, and J. C. Roberts. (1955). *J. Chem. Soc.* p. 2782.
Davis, B. D. (1954–1955). *Harvey Lectures* **50**:230.
Dean, F. M., J. C. Roberts, and A. Robertson. (1954). *J. Chem. Soc.* p. 1432.
Dean, F. M., J. Staunton, and W. B. Whalley. (1959). *J. Chem. Soc.* p. 3004.
Dean, F. M., D. S. Deorha, A. D. T. Erni, D. W. Hughes, and J. C. Roberts. (1960). *J. Chem. Soc.* p. 4829.
Demain, A. L. (1959). *Advan. Appl. Microbiol.* **1**:23.
Divekar, P. V., G. Read, and L. C. Vining. (1959). *Chem. & Ind. (London)* p. 731.
Duncanson, L. A., J. F. Grove, and J. Zealley. (1953). *J. Chem. Soc.* p. 3637.
Dunn, G., G. T. Newbold, and F. S. Spring. (1949). *J. Chem. Soc.* p. 2586.
Dutcher, J. D. (1958). *J. Biol. Chem.* **232**:785
Eble, T. E., and F. R. Hanson. (1951). *Antibiot. & Chemotherapy* **1**:54.
Engel, B. G., and W. Brzeski. (1947). *Helv. Chim. Acta* **30**:1472.
Erdtman, H. (1948). *Acta Chem. Scand.* **2**:209.
Ewins, A. J. (1914). *Biochem. J.* **8**:44.
Florey, H. M., E. B. Chain, N. G. Heatley, M. A. Jennings, A. G. Sanders, E. P. Abraham, and M. E. Florey. (1949). "Antibiotics," Vol. I, p. 388. Oxford Univ. Press, London and New York.
Forsyth, F. R. (1955). *Can. J. Botany* **33**:363.
Gatenbeck, S. (1957). *Acta Chem. Scand.* **11**:555.
Geiger-Huber, M., and H. Galli. (1945). *Helv. Chim. Acta* **28**:248.
Glister, A. (1941). *Nature* **148**:470.
Goodwin, T. W. (1954). "Comparative Biochemistry of Carotenoids." Chem. Publ. Co., New York.
Gripenberg, J. (1956). *Acta Chem. Scand.* **10**:1111.
Gripenberg, J. (1958). *Acta Chem. Scand.* **12**:603.
Grob, E. C. (1957). *Chimia (Aarau)* **11**:338.
Grob, E. C., and R. Bütler. (1956). *Helv. Chim. Acta* **39**:1975.
Grove, J. F. (1952). *Biochem. J.* **50**:648.
Hall, W. C. (1951). *Botan. Gaz.* **113**:55.
Hassall, C. H., and D. W. Jones. (1962). *J. Chem. Soc.* p. 4189.

Hatsuda, Y., and S. Kuyama. (1954). *J. Agr. Chem. Soc. Japan* **28**:989.
Heilbronner, E., and R. W. Schmid. (1954). *Helv. Chim. Acta* **37**:2018.
Herrmann, H. (1960). *Naturwissenschaften* **47**:162.
Hetherington, A. C., and H. Raistrick. (1931). *Phil. Trans. Roy. Soc. London* **B220**:269.
Hofmann, A. (1960). *Verhandl. Naturforsch. Ges. Basel* **71**:239.
Hofmann, A., R. Heim, A. Brack, H. Kobel, A. Frey, H. Ott, T. H. Petrzilka, and F. Troxler. (1959). *Helv. Chim. Acta* **42**:1557.
Howard, B. H., and H. Raistrick. (1949). *Biochem. J.* **44**:227.
Howard, B. H., and H. Raistrick. (1950). *Biochem. J.* **46**:49.
Howard, B. H., and H. Raistrick. (1954). *Biochem. J.* **57**:212.
Howard, B. H., and H. Raistrick. (1955). *Biochem. J.* **59**:475.
Johnson, D. H., A. Robertson, and W. B. Whalley. (1950). *J. Chem. Soc.* p. 2971.
Johnson, D. H., A. Robertson, and W. B. Whalley. (1953). *J. Chem. Soc.* p. 2429.
Jones, E. R. H., J. S. Stephenson, W. B. Turner, and M. C. Whiting. (1963). *J. Chem. Soc.* p. 2048.
Kavanagh, F., A. Hervey, and W. J. Robbins. (1952). *Proc. Natl. Acad. Sci. U.S.* **38**:555.
Kluyver, A. J., J. P. van der Walt, and A. J. van Triet. (1953). *Proc. Natl. Acad. Sci. U.S.* **39**:583.
Kögl, F. (1926). *Ann. Chem.* **447**:78.
Kögl, F., and H. Becker. (1928). *Ann. Chem.* **465**:211, 243.
Kögl, F., and J. J. Postowsky. (1925). *Ann. Chem.* **444**:1.
Kögl, F., and F. W. Quackenbush. (1944). *Rec. Trav. Chim.* **63**:251.
Kögl, F., and G. C. van Wessem. (1944). *Rec. Trav. Chim.* **63**:5.
Kögl, F., C. A. Salemink, H. Schouten, and F. Jellinek. (1957). *Rec. Trav. Chim.* **76**:109.
Kögl, F., C. A. Salemink, and P. L. Schuller. (1960). *Rec. Trav. Chim.* **79**:278.
List, P. H., and H. Hetzel. (1960). *Planta Med.* **8**:105.
List, P. H., and H. G. Menssen. (1959). *Arch. Pharm.* **292**:21.
List, P. H., and H. Reith. (1960). *Z. Physiol. Chem.* **319**:17.
Lybing, S., and L. Reio. (1958). *Acta Chem. Scand.* **12**:1575.
MacMillan, J. (1954). *J. Chem. Soc.* p. 2585.
Metzenberg, R. L., and H. K. Mitchell. (1958). *Biochem. J.* **68**:168.
Michael, S. E. (1948). *Biochem. J.* **43**:528.
Miles, P. G., H. Lund, and J. R. Raper. (1956). *Arch. Biochem. Biophys.* **62**:1.
Miyao, K. (1960). *Bull. Agr. Chem. Soc. Japan* **24**:23.
Moir, G. F. J., and B. J. Ralph. (1954). *Chem. & Ind. (London)* p. 1143.
Mosbach, K. (1960). *Acta Chem. Scand.* **14**:457.
Mothes, K., F. Weygand, D. Gröger, and H. Grisebach. (1958). *Z. Naturforsch.* **13b**:41.
Murakami, T. (1956). *Pharm. Bull. (Tokyo)* **4**:298.
Nakamura, S. (1960). *Bull. Agr. Chem. Soc. Japan* **24**:629.
Nakamura, S. (1961). *Agr. Biol. Chem. (Tokyo)* **25**:74.
Neill, K. G., and H. Raistrick. (1957). *Biochem. J.* **65**:166.
Newton, G. G. F., and E. P. Abraham. (1954). *Biochem. J.* **58**:103.
Nickerson, W. J. (1948). *Arch. Biochem.* **17**:225.
Nishikawa, H. (1936). *Bull. Agr. Chem. Soc. Japan* **12**:47.
Ooyama, J., N. Nakamura, and O. Tanabe. (1960). *Bull. Agr. Chem. Soc. Japan* **24**:743.

Oxford, A. E., and H. Raistrick. (1932). *Biochem. J.* **26**:1902.
Oxford, A. E., and H. Raistrick. (1933). *Biochem. J.* **27**:634.
Oxford, A. E., H. Raistrick, and P. Simonart. (1939). *Biochem. J.* **33**:240.
Plattner, P. A., U. Nager, and A. Boller. (1948). *Helv. Chim. Acta* **31**:594.
Plattner, P. A., W. Keller, and A. Boller. (1954). *Helv. Chim. Acta* **37**:1379.
Posternak, T. (1938). *Helv. Chim. Acta* **21**:1326.
Posternak, T. (1940). *Helv. Chim. Acta* **23**:1046.
Quilico, A., C. Cardani, and L. Panizzi. (1953a). *Atti Accad. Naz. Lincei, Rend., Classe Sci. Fis., Mat. Nat.* **14**:358.
Quilico, A., C. Cardani, and G. Stagno d'Alcontres. (1953b). *Gazz. Chim. Ital.* **83**:754.
Raistrick, H., and D. J. Ross. (1952). *Biochem. J.* **50**:635.
Raistrick, H., and P. Rudman. (1956). *Biochem. J.* **63**:395.
Raistrick, H., and P. Simonart. (1933). *Biochem. J.* **27**:628.
Raistrick, H., and G. Smith. (1936). *Biochem. J.* **30**:1315.
Raistrick, H., and C. E. Stickings. (1951). *Biochem. J.* **48**:53.
Raistrick, H., and A. Stössl. (1958). *Biochem. J.* **68**:647.
Raistrick, H., R. Robinson, and A. R. Todd. (1937). *J. Chem. Soc.* p. 80.
Raistrick, H., C. E. Stickings, and R. Thomas. (1953). *Biochem. J.* **55**:421.
Ralph, B. J., and A. Robertson. (1950). *J. Chem. Soc.* p. 3380.
Ramachandran, K., and V. Radha. (1955). *Current Sci. (India)* **24**:50.
Rhodes, A., M. P. McGonagle, and G. A. Somerfield. (1962). *Chem. & Ind. (London)* p. 611.
Roberts, J. C., and C. W. H. Warren. (1955). *J. Chem. Soc.* p. 2992.
Robinson, Sir R. (1955). "Weizmann Memorial Lectures." Dec. 1st, 1953. Oxford Univ. Press (Clarendon), London and New York.
Rosett, T., R. H. Sankhala, C. E. Stickings, M. E. U. Taylor, and R. Thomas. (1957). *Biochem. J.* **67**:390.
Ruelius, H. W., and A. Gauhe. (1950). *Ann. Chem.* **569**:38.
Russell, D. W. (1960). *Biochim. Biophys. Acta* **45**:411.
Sakaguchi, K., T. Inoue, and S. Tada. (1939). *Zentr. Bakteriol., Parasitenk., Abt. II* **100**:302.
Sastry, K. S., and P. S. Sarma. (1957). *Nature* **179**:44.
Schröter, H. B. (1956). *Angew. Chem.* **68**:158.
Segal, W. (1957). *Chem. & Ind. (London)* p. 1040.
Segal, W. (1959). *J. Chem. Soc.* p. 2847.
Seshadri, T. R., and S. S. Subramanian. (1949). *Proc. Indian Acad. Sci.* **A30**:67.
Shaw, B. L., and M. C. Whiting. (1954). *J. Chem. Soc.* p. 3217.
Shaw, P. D., J. R. Beckwith, and L. P. Hager. (1959). *J. Biol. Chem.* **234**:2560.
Sheehan, J. C., W. B. Lawson, and R. J. Gaul. (1958). *J. Am. Chem. Soc.* **80**:5536.
Shibata, S., and M. Takido. (1955). *Pharm. Bull. (Tokyo)* **3**:156.
Shibata, S., T. Murakami, O. Tanaka, G. Chihara, I. Kitagawa, M. Sumimoto, and C. Kaneko. (1955). *Pharm. Bull. (Tokyo)* **3**:160.
Singh, J., T. K. Walker, and M. L. Meara. (1955). *Biochem. J.* **61**:85.
Sjölander, J. R., K. Folkers, E. A. Adelberg, and E. L. Tatum. (1954). *J. Am. Chem. Soc.* **76**:1085.
Sorm, F., V. Benesova, and V. Herout. (1953). *Chem. Listy* **47**:1856.
Stickings, C. E. (1959). *Biochem. J.* **72**:332.
Stickings, C. E., and A. Mahmoodian. (1962). *Chem. & Ind. (London)* p. 1718.
Stickings, C. E., and R. J. Townsend. (1961). *Biochem. J.* **78**:412.

Stodola, F. H., M. Friedkin, A. J. Moyer, and R. D. Coghill. (1945). *J. Biol. Chem.* **161**:739.
Sumiki, Y. (1931). *J. Agr. Chem. Soc. Japan* 7:819.
Tarbell, D. S., R. M. Carman, D. D. Chapman, K. R. Huffman, and N. J. McCorkindale. (1960). *J. Am. Chem. Soc.* **82**:1005.
Thomas, R. (1961a). *Biochem. J.* **78**:748.
Thomas, R. (1961b). *Biochem. J.* **78**:807.
Thoms, H., and J. Vogelsang. (1907). *Ann. Chem.* **357**:145.
Umbreit, W. W., W. A. Wood, and I. C. Gunsalus. (1946). *J. Biol. Chem.* **165**:731.
Van der Kerk, G. J. M., and J. C. Overeem. (1957). *Angew. Chem.* **69**:623.
Vischer, E. B. (1953). *J. Chem. Soc.* p. 815.
von Kamienski, F. S. (1958). *Planta* **50**:331.
Vora, V. C. (1954). *J. Sci. Ind. Res. (India)* **13B**:842.
Weindling, R. (1932). *Phytopathology* **22**:837.
Wieland, H., and G. Coutelle. (1941). *Ann. Chem.* **548**:270.
Wieland, T. (1958). *Ann. Chem.* **617**:152.
Wieland, T., W. Motzel, and H. Merz. (1953). *Ann. Chem.* **581**:10.
Willstaedt, H., and B. Zetterberg. (1946). *Svensk Kem. Tidskr.* **58**:306.
Winstead, J. A., and R. J. Suhadolnik. (1960). *J. Am. Chem. Soc.* **82**:1645.
Winterstein, E., C. Reuter, and R. Korolew. (1913). *Landwirtsch. Vers.-Sta.* **79–80**:541.
Woodward, R. B., and G. Singh. (1949). *J. Am. Chem. Soc.* **71**:758.
Woolley, D. W., and W. H. Peterson. (1937). *J. Biol. Chem.* **122**:213.
Yabuta, T. (1924). *J. Chem. Soc.* **125**:575.
Yabuta, T., and Y. Sumiki. (1933). *J. Agr. Chem. Soc. Japan* 9:1264.
Yabuta, T., and Y. Sumiki. (1934). *J. Agr. Chem. Soc. Japan* 10:703.
Yabuta, T., K. Kambe, and T. Hayashi. (1934). *J. Agr. Chem. Soc. Japan* **10**:1059.
Zechmeister, L., and J. W. Sease. (1947). *J. Am. Chem. Soc.* **69**:273.

CHAPTER 10

Carbohydrate Metabolism

1. Glycolysis

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I. INTRODUCTION

Glycolysis is the physiological conversion of glucose to pyruvate or lactate *without* regard to mechanism. The glycolytic pathways in fungi, as in all cells, serve to furnish energy, precursors, or components for synthesis and for oxidation or reduction reactions necessary for converting these precursors to the appropriate intermediates or end products.

It has been a little more than a century since Pasteur published his memoirs on the alcoholic fermentation of sugar. He also studied certain fungi extensively from the standpoint of corroborating and generalizing his concepts of fermentation already conceived with ordinary brewers' yeast (*Saccharomyces cerevisiae*) (cf. Foster, 1949). However, it was in the three to four decades following the demonstration in 1897 by Büchner of glycolysis in extracts of yeast that studies with isolated en-

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[2] The following abbreviations are used throughout this paper: ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide, or diphosphopyridine nucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate, or triphosphopyridine nucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EM, Embden-Meyerhof pathway; HMP, hexose monophosphate pathway; ED, Entner-Doudoroff pathway; TCA, tricarboxylic acid cycle; -P, -phosphate; P_i, inorganic orthophosphate; RSA, relative specific activity.

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zymes resulted in the recognition of the pathway now known as the Embden-Meyerhof (EM) pathway.

The remarkable successes in elucidating this pathway in yeast, mammalian, and bacterial cells resulted in the general acceptance, until about fifteen years ago, of the EM pathway as the *sole* one for glycolysis in living cells. Whether this was true in filamentous fungi was less certain, primarily because of technical difficulties. This situation led, in fact, to a school of thought, championed by Nord, which suggested that phosphorylation played little part in glycolysis by molds such as *Fusarium*. Foster summarized the situation in 1949: "It is a surprising fact, though typical of molds, that the occurrence of phosphorylated intermediary products of carbohydrate metabolism has not yet been conclusively demonstrated. The elimination of this very real stumbling block to the elucidation of dissimilation mechanisms of molds doubtless can be achieved, to a large extent at least, by the development of methods for obtaining active cell-free juices of mold mycelium, so that the study of them becomes one of isolated enzyme action rather than cellular action."

Almost at the time Foster was writing these words, Lynen and Hoffmann-Walbeck (1948) were able to demonstrate the presence of aldolase and triose-P dehydrogenase in *Penicillium notatum* extracts. Within five years it became apparent that the EM enzymes could be demonstrated in many fungal extracts. During this same interval, studies by Horecker, Racker, Cohen, and others extended the earlier studies of Warburg, Lipmann, and Dickens so that another glycolytic pathway, the hexose monophosphate (HMP) pathway, was recognized in yeast (cf. Dickens, 1958). This time, the existence of HMP enzymes in filamentous fungi was demonstrated without delay. Thus it was possible to establish that the Kluyverian concept of the "unity of biochemistry" did hold for fungal glycolysis in that they possessed the enzymatic potential for EM and HMP pathways.

During the past decade, with the availability of differently C^{14}-labeled glucoses, procedures were devised that made it possible to demonstrate that intact fungal and other cells not only had the potential for the EM and HMP pathways, but that these pathways actually operated, usually simultaneously. With the demonstration that intact *Fusarium* mycelium utilized a pathway involving phosphorylated EM intermediates, the chief objection of the proponents of a nonphosphorylated pathway for glucose catabolism in filamentous fungi was overcome. This objection was that studies with cell-free extracts involved pathological disorganization and did not reflect what occurs in normal cells (Foster, 1949; Cochrane, 1958).

The notion of the "unity of biochemistry" has been advanced in an over simplified form that sometimes leads to concentrated effort on similarities while that which is novel and different is overlooked or discounted (Cohen,

963). Lest anyone should assume that pathways involving phosphorylated termediates are the only ones, it may be noted that three pathways for arbohydrate catabolism that do not involve phosphorylated sugars have ready been discovered in bacteria. These are the conversion of glucose to -ketoglutarate by *Acetobacter melanogenum* and the conversion of L-arabinose to α-ketoglutarate and of D-arabinose to glycolate and pyruvate by *seudomonas saccharophila* (cf. Hollmann, 1964). The chances are very reat that there are still undiscovered pathways for carbohydrate metabosm in fungi. Furthermore, "Whereas the tell-tale evidence of existence of ew metabolic pathways in the great majority of other organisms cannot e foretold without laborious studies, . . . it seems to be uniquely charcteristic of fungi that they virtually advertise the presence of new metabolic athways by the piling up of compounds representing one or more stages n a pathway" (Foster, 1958).

II. METHODOLOGY

The classical procedures for the investigation of carbohydrate catabolism ave not been very successful when applied to fungi. The many advances n our understanding of fungal glycolytic processes during the past decade ave resulted primarily from studies both with isolated enzymes and with adioisotopic techniques applied to intact cells. Consequently, emphasis ill be placed on the recent and important isotopic techniques and only a superficial summary of other techniques will be presented. A concise synopsis of some general procedures of value in microbial metabolic studies an be found in Lamanna and Mallette (1959), and more extensive discussions of techniques particularly suited to fungal metabolism in Foster (1949) and Cochrane (1958).

There are many levels of organization which an investigator may select for studying fungal metabolism. For example, he may study fungi in their normal environment, in mixed cultures with complex media, in pure cultures with complex or chemically defined media, in resting cell suspensions or crude cell extracts, or in systems employing highly purified enzymes. At each successive level there is a gain in the form of more control of variables and greater simplicity. With these advantages, however, there usually is a concomitant loss due to a greater degree of disorganization. If at all possible, one should attempt to verify conclusions at more than one level.

A. Carbon and Oxidation-Reduction Balances

Measurements of the stoichiometry of substrate conversion to products from anaerobic fermentations, a technique that has been described briefly

by W. A. Wood (1961), was an early and valuable tool for microbiologists. Cochrane (1958) has discussed the use of this technique with fungi, and Foster (1949) has described the use of respirometers in fungal carbon-balance studies. Although the measurement of carbon and oxidation-reduction balances by itself has little use in determining glycolytic pathways, it is a valuable method in ascertaining whether or not all the products, and the appropriate ones in terms of degree of oxidation, have been recovered. The carbon balance itself often is used in studies with C^{14}-sugars (Wang and Krackov, 1962).

B. Growth: Sources of Energy

The ability of fungi to use certain sugars as sole sources of carbon and energy, as well as the efficiency of conversion of these compounds into cell mass (cf. Perlman, Chapter 18), may sometimes provide valuable clues to the glycolytic pathways available to a cell. For example, a cell that can utilize gluconate or 2-ketogluconate very likely possesses a modified HMP pathway (Fig. 3). The fact that xylose or gluconate is a better carbon source than glucose may mean that the HMP pathway is more active than the EM pathway although other explanations, such as limitations due to specific transport mechanisms (permeases), are possible. However, if good enzymatic and isotopic techniques are available, problems such as this are amenable to experimentation. The biochemical basis of nutritional capabilities in many fungi has been little studied and may well repay investigation.

C. Manometry

Although manometric techniques have been used successfully with many microorganisms, the results of quantitative studies with fungi generally have been less successful. Among the filamentous fungi the rate of endogenous respiration usually is bigh compared to the total respiration in the presence of an exogenous substrate. Methods have now been devised that aid in the decision whether or not the endogenous respiration continues in the presence of a utilizable substrate (cf. Section V). The determination of whether or not a substrate is oxidized at a rate higher than the endogenous rate is still very useful and often is applied in attempts to identify possible metabolic intermediates. A recent example of such a use was in studies on the glucose catabolism of *Caldariomyces fumago* (Ramachandran and Gottlieb, 1963). In this study, both resting cell suspensions (i.e., nongrowing cells) and cell-free extracts of the organism were found to be capable of oxidizing glucose, gluconolactone, 6-phosphogluconate, and 2-ketogluco-

ıate aerobically but not anacrobically; glucuronic acid, glucose-6-P or ructose-1,6-di-P were not oxidized. These results, as well as supplementary nanometric studies employing metabolic poisons and isotopic glucose, led o the conclusion that this fungus employs a modified ED pathway. Negaive data obtained by this technique, or any other, must be interpreted :arefully. Many compounds, particularly the phosphorylated sugars in-:olved in the EM, HMP, or ED pathways, are usually impermeable to :ells. In addition, phosphatases often are located on the cell surface (cf. Rothstein, Chapter 15) and can hydrolyze the phosphate ester so that it s the free sugar that actually is being metabolized. This problem can be-:ome even more complicated if the phosphatase has some degree of speci-ficity, as in the case of a highly purified acid phosphomonoesterase from *Neurospora crassa* which hydrolyzes phosphorylated sugars at different ·ates (Kuo and Blumenthal, 1961). These facts suggest that manometry, while a valuable supplementary technique, should not be used alone.

Induced enzyme synthesis (cf. Zalokar, Chapter 14) often can be meas-ured employing manometric techniques. In the case of *C. fumago* which was referred to above, suggestive evidence was obtained that an induced enzyme was involved in the oxidation of 2-ketogluconate. The same situa-tion for 2-ketogluconate oxidation probably exists in a number of other fungi (DeLey and Vandamme, 1955). These data do not establish whether enzymes which facilitate entry, or oxidation, are involved.

D. Inhibitors

The results of studies with metabolic inhibitors such as fluoride and iodoacetate played an important role in the definition of the EM pathway. Inasmuch as cells usually possess more than one glycolytic pathway, and many enzymes are common to all three such pathways known to be present in fungi (EM, HMP, and ED pathways; Table I), it is unlikely that poisons will play such an important role again. Unfortunately, inhibitors specific for the enzymes which are unique to one pathway, such as phosphofructo-kinase of the EM pathway, are as yet unknown. Inhibitors are still very useful in metabolic studies although the interpretation of results obtained with them, especially when using intact cells, is difficult.

The inhibition of glycolysis by fluoride, with the concomitant accumula-tion of phosphoglycerate, formerly was considered sufficient evidence to indicate that the EM pathway was *the* glycolytic pathway present. This was due to the fact that Mg^{++}, required in relatively high concentrations by enolase, was removed as the insoluble magnesium fluorophosphate. Therefore, if an organism was insensitive to fluoride, it was thought to be utilizing the HMP pathway or other non-EM means of glycolysis.

TABLE I

REACTIONS AND ENZYMES INVOLVED IN THE EMBDEN-MEYERHOF (EM), HEXOSE MONOPHOSPHATE (HMP), AND ENTNER-DOUDOROFF (ED) PATHWAYS FOR GLUCOSE CATABOLISM

Substrate(s)	Product(s)	Enzyme		Pathway(s) involved
		Trivial name(s)	Systematic name	
Glucose + ATP	Glucose-6-P + ADP	Hexokinase	ATP: D-Hexose 6-phospho-transferase	EM, HMP, ED
Glucose-6-P	Fructose-6-P	Glucosephosphate isomerase	D-Glucose-6-P ketol isomerase	EM, HMP
Fructose-6-P + ATP	Fructose-1, 6-di-P + ADP	Phosphofructokinase	ATP: D-Fructose-6-P 1-phosphotransferase	EM
Fructose-1,6-di-P	D-Glyceraldehyde-3-P + dihydroxyacetone-P	Aldolase; fructose-1,6-di-P aldolase	Fructose-1,6-di-P aldehyde lyase	EM, HMP
D-Glyceraldehyde-3-P	Dihydroxyacetone-P	Triosephosphate isomerase	D-Glyceraldehyde-3-P ketol isomerase	EM, HMP, ED
D-Glyceraldehyde-3-P + NAD + P_i	1,3-Diphosphoglycerate + NADH	Glyceraldehyde phosphate dehydrogenase; triose phosphate dehydrogenase	D-Glyceraldehyde-3-P: NAD oxidoreductase (phosphorylating)	EM, HMP, ED
1,3-Diphosphoglycerate + ADP	3-Phosphoglycerate + ATP	Phosphoglycerate kinase	ATP: D-3-phosphoglycerate 1-phosphotransferase	EM, HMP, ED
3-Phosphoglycerate	2-Phosphoglycerate	Phosphoglycerate mutase	D-Phosphoglycerate-2, 3-phosphomutase	EM, HMP, ED
2-Phosphoglycerate	Phosphoenolpyruvate	Enolase; phosphopyruvate hydratase	D-2-Phosphoglycerate hydrolyase	EM, HMP, ED
Phosphoenolpyruvate + ADP	Pyruvate + ATP	Pyruvate kinase	ATP: pyruvate phosphotransferase	EM, HMP, ED
Glucose-6-P + NADP	6-phosphoglucono-δ-lactone + NADPH	Glucose-6-P dehydrogenase	D-Glucose-6-P: NADP oxidoreductase	HMP, ED
6-Phosphoglucono-δ-lactone + H_2O	6-Phosphogluconate	6-Phosphogluconolactonase	D-Glucono-δ-lactone 6-phosphate hydrolase	HMP, ED

6-Phosphogluconate + NADP	D-Ribulose-5-P + CO_2 + NADPH	Phosphogluconate dehydrogenase (decarboxylating)	6-Phospho-D-gluconate: NADP oxidoreductase (decarboxylating)	HMP
D-Ribulose-5-P	D-Ribose-5-P	Ribose-P isomerase	D-Ribose-5-P ketol isomerase	HMP
D-Ribulose-5-P	D-Xylulose-5-P	Ribulose-P epimerase	D-Ribulose-5-P 3-epimerase	HMP
D-Xylulose-5-P + D-ribose-5-P	D-Sedoheptulose-7-P + D-Glyceraldehyde-3-P	Transketolase; glyceraldehyde transferase	D-Sedoheptulose-7-P: D-glyceraldehyde-3-P glycolaldehyde transferase	HMP
D-Sedoheptulose-7-P + D-glyceraldehyde-3-P	D-Fructose-6-P + D-Erythrose-4-P	Transaldolase; dihydroxyacetone transferase	D-Sedoheptulose-7-P: D-glyceraldehyde-3-P dihydroxyacetone transferase	HMP
6-Phosphogluconate	2-Keto-3-deoxy-6-phospho-D-gluconate + H_2O	6-Phosphogluconate dehydratase (or dehydrase)	D-6-Phosphogluconate hydrolyase	ED
2-Keto-3-deoxy-6-phospho-D-gluconate	Pyruvate + D-glyceraldehyde 3-P	2-Keto-3-deoxy-6-phosphogluconate aldolase	2-Keto-3-deoxy-D-gluconate 6-phosphate aldehyde lyase	ED

Such reasoning has been shown to be fallacious in *Bacillus subtilis* (Goldman and Blumenthal, 1963). Thus, cells were insensitive to fluoride when grown in a medium with an inorganic nitrogen source, but not when grown in a medium with a complex organic source of nitrogen. When this problem was investigated using isotopic techniques, it was clearly shown that the EM pathway was the major glycolytic pathway in both types of cells (Table II), even when the determinations were made in the presence

TABLE II

COMPARISON OF ESTIMATIONS OF THE AEROBIC GLUCOSE CATABOLIC PATHWAYS IN *Bacillus subtilis* RESTING CELL SUSPENSION USING TWO METHODS SIMULTANEOUSLY

Cells grown in medium with complex or inorganic nitrogen source	Method of estimation	Equation	Glucose utilized via HMP pathway (%)
Complex	CO_2	(3)	36
Complex	Intermediates (acetate)	(9)	41
Inorganic	CO_2	(3)	26
Inorganic	Intermediates (acetate)	(9)	35

of fluoride. The reasons for the differences in the effectiveness of fluoride are not known although there is likely to be some difference in the penetrability of fluoride to the sensitive sites in the two types of cells. Consequently, insensitivity of an intact cell to an agent such as fluoride does not necessarily imply the operation of an alternate pathway, or innate resistance to the agent by a particular enzyme or group of enzymes.

Another important point to consider when using metabolic poisons such as fluoride is that the poison may act at more than one locus and, depending upon the concentrations employed, the so-called secondary site may be the one that is being blocked. Such a case was recently described in *Aspergillus terreus* (Lal and Bhargava, 1962). In this mold, which uses the EM pathway as the major glycolytic pathway, fluoride inhibits a step in the conversion of pyruvate to itaconate at concentrations that inhibit pyruvate formation from glucose only slightly. Furthermore, this slight inhibition of glycolytic rate is completely reversed by the addition of pyruvate (through an unknown mechanism) although itaconate formation remains blocked. Consequently, a step in the conversion of pyruvate to itaconate is most susceptible to fluoride.

Arsenite often is employed when the accumulation of α-keto acids such as pyruvate or α-ketoglutarate is desired. Although such accumulation alone does not give information as to which glycolytic pathway is being used, it can do so when used in conjunction with the determination of the distribution of C^{14} in pyruvate after utilization of glucose-1-C^{14}. This is one type

evidence used by Ramachandran and Gottlieb (1963) to determine that ıcose was catabolized by the ED pathway in *Caldariomyces fumago*.

Cycloheximide (Actidione) is another agent that can be used to cause ə accumulation of α-keto acids in fungi. The action of the cycloheximide n be reversed in *Neurospora sitophila* by the addition of thiamine (Sey-ıux and Turian, 1962).

Useful information may sometimes be secured by means of studies on the echanism of resistance to a poison. For example, in a study on the sistance of *Aspergillus niger* to the fungistatic action of *m*-dinitrobenzene, iggins and Chambers (1963) reported that some important changes in rbohydrate metabolism had accompanied the acquisition of resistance. ıe resistant strain could metabolize glucose anaerobically whereas the .rent strain could not, and the former could no longer oxidize gluconate.

In addition to studies of the types just described, poisons are used to use the accumulation of intermediates which can then be identified by rious chromatographic techniques (cf. Godin, 1955). It is not always ·cessary to add poisons, however, to induce the accumulation of inter-ədiates. Duff and Webley (1959) were able to accomplish this with HMP :thway components from soil nocardias simply by keeping the pH near ·utrality or by using gluconate as the carbon source rather than glucose.

The compounds found in the medium will usually be dephosphorylated ' cell phosphatases, so in order to obtain phosphate esters it is necessary extract the mycelium. Thus, Moses (1959) used this technique to study e effect of azide on the intracellular intermediates formed during glucose ətabolism in *Zygorhynchus moelleri*.

The effect of respiratory poisons, such as cyanide, on fungi has been ınsidered by Lindenmayer in Chapter 12. In addition, Hochster and uastel (1963) have written a comprehensive treatment of metabolic in-bitors.

E. Mutants

Although *Neurospora* mutants have been used for the determination of rtain biochemical pathways, particularly those involving amino acids, they ıve not been employed for the investigation of glycolytic mechanisms. rauss and Pierog (1954) did, however, report an apparent increase in e utilization of the HMP pathway as a result of a mutation in *N. crassa* at nearly completely blocked its pyruvate metabolism.

Mutations affecting glycolytic enzymes were apparently unknown before e recent report of a mutant of *Salmonella typhimurium* deficient in ucosephosphate isomerase (Fraenkel *et al.*, 1963). Because of this de-ct, glucose, but not fructose, must be glycolyzed in this bacterium by eans other than the EM pathway.

Studies have been made of the enzymes of glucose catabolic pathways of a high oxytetracycline-producing strain of *Streptomyces rimosus* and of a mutant producing much smaller amounts of the antibiotic (Horváth and Szentirmai, 1962). Accompanying the loss in antibiotic productivity, the 6-phosphogluconate dehydrogenase content of the mycelium diminished, while the content of EM enzymes increased leading to a high pyruvate content in the medium. The authors concluded from these data, and others, that the low yield of antibiotic was due to a block in the utilization of pyruvate for antibiotic synthesis rather than to a direct effect on the glycolytic pathways. The addition of high concentrations of P_i to the medium, which also inhibits the HMP pathway and stimulates the EM pathway, resulted in increased production of pyruvate, which was promptly used to produce greater quantities of mycelium but not antibiotic.

Bacterial mutants are very useful for identifying intermediate steps in the conversion of D-galactose to D-glucose and in the catabolism of L-arabinose (cf. Hollmann, 1964). It is hoped that the use of fungal mutants lacking specific glycolytic enzymes may prove to be as valuable to the fungal physiologist.

F. Enzymes

The presence of a glycolytic enzyme in a cell extract indicates that the cell has the potential to use it. Three glycolytic pathways are known in fungi, the EM, HMP, and ED pathways, the components of which are listed in Table I. The first ten enzymes in the list are those ordinarily associated with the EM pathway. Note that, of these ten enzymes, all except phosphofructokinase also are associated with the HMP pathway and seven of the ten also are part of the ED pathway. There is good evidence that aldolase is involved in the resynthesis of hexoses via the HMP pathway by catalyzing the condensation of erythrose-4-P and triose-P to yield sedoheptulose-1,7-di-P. The latter compound can then be cleaved by a specific phosphatase to yield sedoheptulose-7-P, an intermediate in the HMP cycle (Horecker, 1962a).

It is possible that the presence of both NADPH oxidase and transhydrogenase activity in cell extracts may point to the predominant utilization of the HMP and/or ED pathways. This correlation has been observed in microorganisms (Eagon, 1963).

The significance of multiple forms of enzymes, the "hybrid" enzymes or isozymes, is not completely understood by enzymologists although the fungi also have them. Thus, *Neurospora crassa* has three glucose-6-P dehydrogenases and two 6-phosphogluconate dehydrogenases that are separable by gel electrophoresis. The same number of isozymes appear in the

mycelium of eight *N. crassa* strains after growth in three different media (Tsao, 1962). It is possible that the isozymes are representatives of glycolytic sequences from different cellular locations (cf. Zalokar, Chapter 14).

The successes associated with the demonstration of glycolytic enzymes in molds have paralleled the general rapid rise in enzyme technology. A list of the EM enzymes and some HMP enzymes that have been found in representative fungi is presented in Table III. The phosphorolytic cleavage of fructose-6-P or xylulose-5-P that is catalyzed by the enzyme phosphoketolase in certain bacteria (Horecker, 1962a) has not been reported in fungi.

The preparation of active cell-free extracts has been discussed by Cochrane (1958). Very often protective materials, such as reducing agents and/or chelating agents, are required to protect against heavy metal inactivation of the enzymes. As a general rule, the crude extracts must be kept cold and fractionated rapidly, at least during the primary purification stages, in order to remove many of the very active proteinases present in fungal extracts. Indeed, it is likely that the inability for many years to demonstrate fungal glycolytic enzymes was due in part to the very high concentration of such proteinases in fungi, as compared with other cells. A procedure for the 200-fold purification of *Aspergillus parasiticus* hexokinase (Davidson, 1960) is typical of enzymatic methods now in common use.

Even when a fungal enzyme cannot be stabilized, techniques are now available that will allow some rapid purification procedures. Thus, it was possible to remove the hexosephosphate isomerase from a labile *N. crassa* enzyme preparation, using a rapid fractionation technique employing calcium phosphate gel, to determine that fructose-6-P, not glucose-6-P, was the true substrate for the transamidase reaction yielding glucosamine-6-P (Ghosh *et al.*, 1960).

Column chromatographic techniques employing DEAE-cellulose or other materials have been of immense value in enzyme purification. For an example of the use of this technique in preparing a 1400-fold purified *Neurospora* acid phosphomonoesterase, see Kuo and Blumenthal (1961); for an example of its use in fractionating crude extracts from *Blastocladiella emersonii*, see Goldstein and Cantino (1962).

A "constant-proportion" group of five enzymes has been found in a wide variety of cells, including bakers' yeast. This group includes triose-P isomerase and dehydrogenase, phosphoglycerate kinase and mutase, and enolase. For example, cells that contained large amounts of one enzyme also had large amounts of the other four, and the proportion was similar in many different cells (Pette *et al.*, 1962). Relationships such as this may prove to be useful when any of these enzymes must be determined in cell extracts.

TABLE III

PRESENCE OF EMBDEN-MEYERHOF AND SOME HEXOSE MONOPHOSPHATE ENZYMES IN FUNGAL EXTRACTS

Microorganism	Hexokinase	Glucose-P isomerase	Phosphofructokinase	Aldolase	Triose-P isomerase	Triose-P dehydrogenase	Phosphoglycerate kinase	Phosphoglycerate mutase	Enolase	Pyruvate kinase	Glucose-6-P dehydrogenase	6-Phosphogluconate dehydrogenase	References
Aspergillus niger	+	+	+	+		+		+	+	+	+	+	Jagannathan and Singh (1953)
Caldariomyces fumago	+							+	+	+			Ramachandran and Gottlieb (1963)
Claviceps purpurea	+	+	+	+							+	+	McDonald *et al.* (1960)
Fusarium lini				+		+	+	+	+	+			Cochrane (1956)
Microsporum canis	+	+	−	+		+		+	+	+	+	+	Chattaway *et al.* (1960)
Penicillium chrysogenum	+	+	−	+		+		+	+	+	+	+	Sih and Knight (1956), Sih *et al.* (1957)
Rhizopus MX	+	+		+		+	+	+	+	+	+		Margulies and Vishniac (1961)
Streptomyces coelicolor	+	+	+	+	+	+	+	+	+	+	+	+	Cochrane *et al.* (1953), Cochrane (1955)
Streptomyces rimosus	+	+	+	+	+	+		+	+	+	+	+	Horváth and Szentirmai (1962)
Tilletia caries	+	+		+							+	+	Newburgh *et al.* (1955)
Ustilago maydis, mycelium				+		+	+				+		McKinsey (1959)
Ustilago maydis, spores	+	+	+	+	+	+	+	+	+	+	+[a]	+[a]	Gottlieb and Caltrider (1963)

[a] Absent until 12 hours after germination.

The enzymatic constitution of fungal cells is not constant and is affected by many cultural and physiological conditions. Damodaran *et al.* (1955) observed the variation in glycolytic enzyme activity of *Aspergillus niger* mycelium during a citric acid fermentation. Maruyama and Alexander (1962) found that the location and activity of aldolase in spores and mycelium of *Fusarium oxysporum* depended on the growth conditions. Zinc appears to be a particularly important trace element for *A. niger* enzymes of the EM and HMP pathways (Bertrand and deWolf, 1959; Geser, 1962). Franke *et al.* (1963) observed that the culture medium, conditions of aeration, age, and glucose concentration all affected the relative activities of glucose oxidase and EM enzymes in *A. niger* mycelium. Conditions that increased glucose oxidase activity decreased the EM enzymes, and vice versa. It should be noted, however, that many metabolic sequences are limited by the low concentrations of intermediates, not by enzyme activity.

The use of synchronized cultures (cf. Campbell, Chapter 28) allows certain changes in enzymatic constitution to become more apparent. In germinating *Ustilago zeae* [*U. maydis*] spores glucose-6-P and 6-phosphogluconate dehydrogenases, transketolase, transaldolase, and certain TCA-cycle enzymes do not appear until 12 hours after germination although all EM enzymes are present at all times in the spores (Gottlieb and Caltrider, 1963). Changes in the enzymatic activity and content of bacterial spores as they develop synchronously into vegetative cells have also been observed (Goldman and Blumenthal, 1964a), and the same situation may hold in *Neurospora ascospores* (Sussman, 1961). Improved methods for the large-scale growth of synchronized single generations of *Blastocladiella emersonii* have enabled the study of the stimulatory effect of white light on the synthesis of protein. Light caused a sharp decrease in the total glucose-6-P dehydrogenase activity per cell during the last stages of growth when light-induced formation of soluble polysaccharide per cell was most pronounced (Goldstein and Cantino, 1962). Other aspects of the enzymatic changes occurring during the development of phycomycetes are reviewed by Cantino and Turian (1959).

Another major reason for the increased success of studies on glycolytic enzymes from fungi is the use of assays of greater sensitivity and specificity. Spectrophotometric assays for many glycolytic enzymes have replaced Thunberg tubes and less sensitive chemical analyses. Also, the utilization of enzymes with defined specificities has enabled the activity of other enzymes to be measured which could not have been previously.

G. Isotopes

With the ready availability of differently labeled C^{14}-glucoses it became possible to devise quantitative procedures for the analysis of the pathways glucose traverses in the intact cell. The methods most often used for these estimations can be classified as (1) methods based on yields of $C^{14}O_2$; (2) methods based on specific activities of C^{14}-labeled intermediates.

It will not be possible to consider here the details and all the possible limitations of the techniques that have been devised. Details of much of the methodology have been reviewed by the workers most active in this field, including the Cleveland group (H. G. Wood, 1955; Katz and Wood, 1960, 1963; H. G. Wood *et al.*, 1963) and the Oregon group (Wang *et al.*, 1958a; Cheldelin, 1961; Cheldelin *et al.*, 1962; Wang and Krackov, 1962). An attempt will be made, however, to consider the major aspects of those techniques which seem to offer, at this time, the best chance for successfully estimating the glucose catabolic pathways in fungi and other microorganisms. An abbreviated discussion of the two main isotopic techniques is not available elsewhere, and this information is necessary for an adequate understanding of the results.

It should be emphasized at this point that, regardless of the methods used, the values are *approximate*. As will be discussed later, some variation in the pathways utilized can be expected in a given organism under different growth conditions, and factors such as oxygen tension and the use of growing or resting cells may also influence the pathways utilized. Finally, there is some uncertainty due to theoretical considerations.

1. Methods Based on Yields of $C^{14}O_2$

When glucose-1-C^{14} is metabolized via the HMP pathway, there is an early loss of glucose C-1 as $C^{14}O_2$ whereas glucose-6-C^{14} does not yield $C^{14}O_2$ directly through this pathway. On the other hand, when glucose-1- or -6-C^{14} is utilized via the EM pathway there is a symmetrical cleavage of the intermediate fructose-1,6-di-P yielding two triose-P molecules equilibrated by triose-P isomerase, hence making equivalent glucose C-1 and -6, C-2 and -5, and C-3 and -4. This is shown in Fig. 1 with the triose-P in the form of pyruvate. The subsequent catabolism of the two pyruvate molecules by decarboxylation and then by passage through the TCA cycle would yield glucose C-3 and -4 first as CO_2 followed next by C-2 and -5, and finally C-1 and -6 would be converted to $C^{14}O_2$. Thus, when equal numbers of cells are allowed to metabolize equal amounts of glucose-1- and -6-C^{14}, the yield of $C^{14}O_2$ from glucose-1-C^{14} would be expected to be

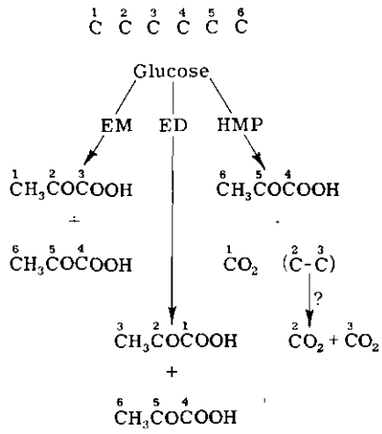

FIG. 1. Distribution of the carbon atoms of glucose in pyruvic acid formed by the Embden-Meyerhof (EM), Entner-Doudoroff (ED), and hexose monophosphate (HMP) pathways of glucose catabolism.

greater if the glucose were metabolized by the HMP pathway. Bloom and Stetten (1953) suggested that the ratio

$$\frac{\%\text{ radiochemical yield from glucose-6-C}^{14}}{\%\text{ radiochemical yield from glucose-1-C}^{14}}$$

the so-called C-6:C-1 ratio, might be used to give directly the maximal fraction of the glucose respired by the EM pathway. This technique overestimated the extent of the HMP pathway because the amount of C^{14} converted to CO_2 from the 1-position of glucose through the HMP pathway after only a few enzymatic steps, was compared to that coming from the 1-position of glucose that had to go through the entire EM sequence followed by several passages through the TCA cycle. If there is a block in the TCA cycle, as in instances where large amounts of lactate or ethanol accumulate, the C-1 via the EM pathway would not yield CO_2 whereas it would via the HMP. Attempts have been made to correct this method, but the correction factors are often of too great magnitude. Consequently, the ratio methods are no longer considered adequate (H. G. Wood, 1955; Katz and Wood, 1960).

A modification of the $C^{14}O_2$ method was introduced by Wang *et al.* (1956), who have since extended and modified the technique which they now refer to as radiorespirometry (Wang *et al.*, 1958a). The main change from previous methods, as far as the estimation is concerned, is that the cumulative amount of $C^{14}O_2$ released is measured at the time when all the

glucose has been utilized. Consequently, a specific yield of $C^{14}O_2$ is measured from a known amount of the glucose carbons used. A modified Warburg respirometer with a system of manifolds and sampling devices is used so that the $C^{14}O_2$ from a large number of flasks can be handled conveniently. However, this is just a convenience, and one can use ordinary Erlenmeyer flasks with alkali traps suspended in them. The flasks can be mounted on a shaker and the alkali traps changed at hourly intervals (Goldman and Blumenthal, 1963). The procedure consists of carrying out simultaneously, incubations of the microbe in question in a growth medium (or buffer, if resting cells are used) where glucose labeled with C^{14} at C-1, C-2, C-3,4, or C-6 is the sole carbon source. The hourly $C^{14}O_2$ yields from each labeled glucose, expressed as the percentage of the radioactivity of the administered glucose, is measured and plotted versus the time interval in hours or relative time units (RTU). An RTU is the time required for disappearance of the added glucose and is judged from the shapes of the curves (Wang *et al.*, 1958a) or from analyses of the medium (Wang *et al.*, 1956; Goldman and Blumenthal, 1963). The results from such time-course plots with four representative microorganisms, *Saccharomyces cerevisiae, Bacillus subtilis, Aspergillus niger,* and *Streptomyces griseus,* are shown in Fig. 2 (Wang *et al.*, 1958a).

The steep decline in the rate of $C^{14}O_2$ liberation from glucose-3,4-C^{14} marks the completion of the primary catabolic processes and is followed by a period when secondary reactions are resulting in enhanced production of $C^{14}O_2$ from the other carbon atoms. These patterns are useful for recognizing the types of glycolytic pathways, i.e., EM-TCA, HMP, or ED. However, only data from glucose-1- and -6-C^{14} are actually used to estimate the EM and HMP pathways quantitatively. The original equations for this calculation (Wang *et al.*, 1958a), with everything expressed as a fraction of unity, are

$$G_p = \frac{G_1 - G_6}{G_T - G'_T} \tag{1}$$

and

$$G_e = 1 - G_p \tag{2}$$

where G_p is the fraction of the administered glucose engaged in catabolism that has been routed into the HMP pathway; G_e is the fraction of the administered glucose engaged in catabolism that has been routed into the EM pathway; G_1 and G_6 are the $C^{14}O_2$ yields observed at time t after the metabolism of equal amounts (including radioactivity) of glucose labeled with C^{14} at C-1 or C-6, respectively; G_T is the total amount of each labeled substrate administered, expressed on the percentage basis as unity, i.e., $G_T = 1.00$; and G'_T is the fraction of the labeled substrate administered that was engaged in anabolic processes.

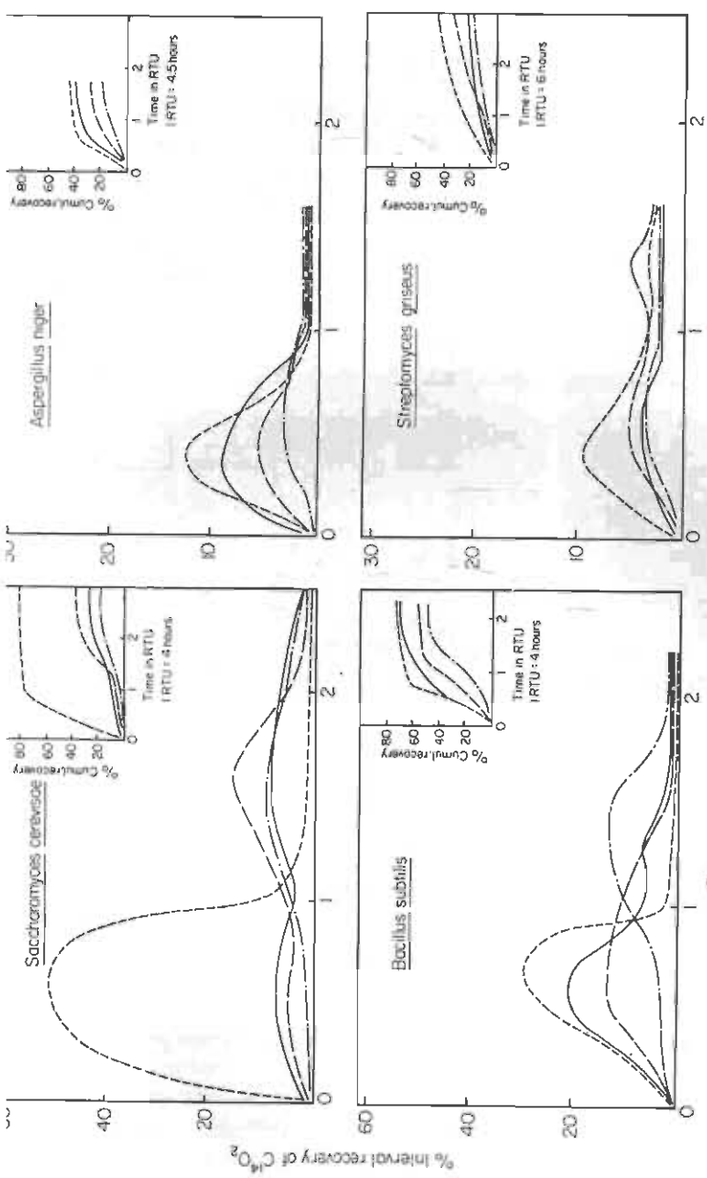

FIG. 2. Time-course plots of interval radiochemical recoveries from proliferating microorganisms metabolizing specifically labeled glucose. Legend: Glucose 1-C^{14} ———, 2-C^{14} — — —, 3,4-C^{14} - - - - - -, 6-C^{14} —·—·—. The insets show the cumulative recoveries of $C^{14}O_2$.

When the magnitude of G'_T is small, as it is usually assumed to be, Eq. (1) becomes

$$G_p = G_1 - G_6 \tag{3}$$

If necessary, the magnitude of the term G'_T can be estimated by determining the amount of C^{11} in cellular constituents using glucose-U-C^{11} or glucose-3,4-C^{11}. However, unless relatively large amounts of glucose are assimilated without prior catabolism this effect is not particularly great. If, for example, the value for the HMP pathway is 30% without any assimilation, even with 25% of the glucose being assimilated the apparent value for the HMP pathway would be raised only to 40%.

Similar equations can be derived for estimating the simultaneous operation of the HMP and ED pathways. In the ED pathway, one would expect the metabolic equivalence of C-1 with C-4, C-2 with C-5, and C-3 with C-6 (Fig. 1).

One of the assumptions made in the derivation of Eqs. (1–3), was that the pentose-P formed from glucose via the HMP pathway does not participate in CO_2 formation. Since there is information that some of the pentose-P can be converted to CO_2, Eqs. (1) and (3) represent *minimal* estimates of the HMP (see also Casselton and Syrett, 1962). A method to correct for pentose-P conversion to CO_2 through the use of gluconate-6-C^{11} along with the glucose-1- and -6-C^{11} has recently been reported (Wang and Krackov, 1962). If A_6 is equal to the $C^{11}O_2$ yield observed at time t following the metabolism of a given amount of gluconate-6-C^{11}, then Eq. (1) becomes

$$G_p = \frac{G_1 - (G_1 - G_p \cdot A_6)}{G_T - G'_T} \tag{4}$$

and Eq. (3) becomes

$$G_p = \frac{G_1 - G_6}{1 - A_6} \tag{5}$$

HMP pathway estimates of 19 and 12% employing Eq. (3) increased to values of 34 and 21%, respectively, when Eq. (5) was used for *B. subtilis* growing in two different media (Wang and Krackov, 1962).

At least one microbe, *Pseudomonas natriegens*, has been reported to use glucose by the EM and HMP pathways, although gluconate apparently is catabolized via the ED and HMP pathways (Eagon and Wang, 1962). Thus, the use of Eqs. (4) and (5) might not be entirely proper with an organism such as this.

The fact that C-6 of glucose traversing the EM or other glycolytic pathways does not appear as CO_2 until it has passed through an operative TCA cycle has led also to the use of the release of $C^{11}O_2$ from glucose-6-C^{11} to estimate the relative extent of operation of the TCA cycle (Goldman and Blumenthal, 1964b).

Some information on the comparison of different methods for the estimaon of pathways will be discussed in the next section. However, a new ıethod, based on the release of CO_2^{γ} from glucose-1- and -6-O^{γ}, has apeared (Rittenberg and Ponticorvo, 1962). By means of this technique, it ‹as estimated that *Escherichia coli* utilized between 10 and 30% of the lucose via the HMP pathway. This compares with a minimal value of 8% obtained by Wang *et al.* (1958a). Since there are some major differnces in the assumptions on which the two different CO_2 methods are based, is encouraging to see relatively good agreement in the estimates. Evidence ›r a similar concurrence of pathway estimates based on the isolation of ›¹⁴-intermediates and the $C^{14}O_2$ yields in the same experiment gives still ıore confidence in these techniques.

. Methods Based on the Specific Activities of C^{14}-Labeled Intermediates

A procedure has been devised for the estimation of the relative extents of ›articipation of the EM and HMP pathways based on the following asumptions: (a) that these pathways are the only ones involved; and (b) hat trioses, or 3-carbon compounds derived therefrom, formed via the EM ›rocess arise equally from glucose carbons 1 to 3 and 4 to 6, whereas those lerived via the HMP pathway arise only from carbons 4 to 6 (Blumenthal *t al.*, 1954). These relations can be seen in Fig. 1. Experimentally the ›rocedure consists of carrying out simultaneously, with the same cell prepaation, experiments with glucose-1-C^{14} and glucose-U-C^{14}. A 3-carbon ·ompound such as lactate or pyruvate, or a 2-carbon compound derived herefrom, e.g. acetate and/or ethanol, is isolated from each and is purified ınd assayed for radioactivity. It is unnecessary to recover the intermediates ıuantitatively. When an appropriate intermediate does not accumulate, one ›an be "trapped" by the addition of some of the unlabeled substance in ıuestion, or an inhibitor, such as arsenite, can be added. The data from ›lucose-1-C^{14} provide an indication of the relative incorporation of this ›arbon in the intermediate, and the data from glucose-U-C^{14} provide a cor·ection factor for the endogenous metabolism, whereby "true" values may ›e calculated for the specific activities of compounds derived from glucose-1-carbon.

Referring to Fig. 1, we can see that the metabolism of glucose-1-C^{14} via he EM pathway would yield methyl-labeled pyruvate (pyruvate-3-C^{14} and subsequent decarboxylation of the pyruvate would yield a 2-C compound, such as acetate, which would have only 1 out of 4 carbons labeled with C^{14} whereas the glucose-1-C^{14} contained 1 out of 6. The relative specific activity (RSA) is:

$$\text{RSA} = \frac{(\text{Avg. specific activity of carbon of compound/mmole C})100}{(\text{Avg. specific activity of carbon of glucose/mmole C})} \qquad (6)$$

The RSA of a 2-C compound derived via the EM pathway would therefore be $(1/4 \div 1/6) \times 100 = 150$. Any acetate formed via the HMP pathway would be unlabeled and have an RSA = 0. If both pathways are operative, the RSA should be somewhere between 0 and 150.

The correction for formation of unlabeled acetate from endogenous materials is made with the glucose-U-C^{14}. If there were no endogenous production of acetate, the RSA of the acetate would be the same as that of the starting glucose-U-C^{14}. The "true" or corrected RSA becomes

$$\text{RSA(corrected)} = \frac{\text{RSA(observed) for acetate from glucose-1-C}^{14}}{\text{RSA(observed) for acetate from glucose-U-C}^{14}} \tag{7}$$

Since acetate formed via the EM would have a RSA(corrected) of 150

$$\%\ \text{Acetate via EM} = \frac{\text{RSA(corrected)}}{150} \times 100 \tag{8}$$

The percentage of glucose utilized via the EM pathway is not equal to the percentage of acetate formed via this pathway since the yields of acetate by each pathway are not the same. Assuming that two molecules of acetate are formed via the EM pathway and only one molecule of acetate is formed through the operation of the HMP pathway, the following equations can be derived:

$$\%\ \text{Glucose via EM pathway} = \frac{\%\ \text{acetate via EM}}{200 - \%\ \text{acetate via EM}} \tag{9}$$

$$\%\ \text{Glucose via HMP pathway} = 100 - \%\ \text{glucose via EM} \tag{10}$$

These equations provide an estimate of the percentage participation of the EM and HMP pathways for glucose yielding a derivative, acetate in this case, but does not include any glucose that does not reach the triose phosphate stage, such as glucose that is directly converted into polysaccharide without prior catabolism. The corrected RSA of 3-C compounds, such as pyruvate, derived via the EM pathway would be 100 and would thus be equal to the percentage of 3-C compounds formed via the EM pathway. Therefore Eqs. (9) and (10) are used by just substituting the percentage of pyruvate (or 3-C compound) for percentage of acetate (or 2-C compound).

Equations (7–9) are based on the assumption that there is no recycling in the HMP pathway, i.e., no re-formation of hexose-P from the pentose-P formed. Since pentose-P formed from glucose-1-C^{14} via the HMP will be unlabeled, any hexose-P formed by recycling would also be unlabeled When this unlabeled glucose-P is catabolized, it will then dilute the 2-C o 3-C intermediates previously formed. Consequently Eq. (9) represents a minimal value for the EM pathway, and Eq. (10) a maximal value for the HMP.

To compensate for such recycling, Dawes and Holms (1958, 1959) calculated the amount of dilution one could expect if the hexose-P formed through cyclic operation of the HMP is degraded via the HMP and EM pathways in the same proportion as the original glucose: *via* HMP = 1 glucose → 2/3 hexose + 1/3 pyruvate + CO_2; *via* EM = 1 glucose → 2 pyruvate.

The results with glucose-1-C^{14}, the labeled glucose considered most likely to give a true picture of recycling, were presented graphically as the apparent percentage of total pyruvate molecules bearing C^{14} label versus the percentage of glucose molecules oxidized via the HMP pathway. The former term is equivalent to pyruvate RSA(corrected)/2 so that if one has determined the corrected RSA of 3-C intermediates such as pyruvate or lactate, the graph can be used to correct for recycling. When this is done, we find, for example, that 30% HMP without recycling (Eq. 9) is reduced to 22% with recycling. An enlarged version of the published graph was kindly provided by Dr. Dawes. Even this 8% correction may be too high since it was assumed in the recycling calculations that there was no drainage of the intermediates of the pentose cycle for biosynthetic functions, a condition that may not exist even in resting cells.

H. G. Wood *et al.* (1963) have performed extensive calculations on the problem of recycling. They calculate the percentage of glucose utilized via the recycling HMP on the basis of the end products formed from the inflowing glucose so that their values are different from those of Dawes and Holms (1958), who use a definition based on the hexose-P turned over. Although the values are interconvertible, I prefer the usage of Dawes and Holms (1958).

One of the advantages of methods based on the isolation of C^{14}-intermediates such as pyruvate is that the compound can be degraded to determine whether the distribution of C^{14} is appropriate for the pathways utilized. From Fig. 1 it can be seen that the distribution of C^{14} in pyruvate after the metabolism of glucose-1-C^{14} would be very different if the EM or ED pathways were used. In fact, this was the way that the ED pathway was originally discovered in *Pseudomonas saccharophila.*

One can also make estimations of the percentage of glucose utilized via the HMP and ED pathways based on the RSA of isolated pyruvate and the distribution of C^{14} in the pyruvate. Such estimates have been made in bacteria (Lewis *et al.,* 1955; Jyssum and Joner, 1962; White and Wang, 1964) and fungi (Ramachandran and Gottlieb, 1963).

It is possible to estimate the glucose catabolic pathways using both the $C^{14}O_2$ method of Wang *et al.* (1958a) and the C^{14}-intermediates method of Blumenthal *et al.* (1954) in a single experiment. Comparisons of two different methods in a single experiment are ideal since the conditions are

identical. Only glucose-1-, -6- and -U-C^{14} are needed whereas each method separately requires two labeled glucoses, with glucose-1-C^{14} common to both. Such a comparison was performed using resting cell suspensions of *Bacillus subtilis* previously grown in two different media; the results are presented in Table II (Goldman and Blumenthal, 1963).

It was stated earlier that the value for the HMP pathway was considered minimal using Eq. (3) for the $C^{14}O_2$ method, while the value of this pathway was considered maximal using Eq. (9) for the C^{14}-intermediates method. The results in Table II seem to bear this out since the values for the HMP pathway are higher than those by the $C^{14}O_2$ method using both types of cells. Considering the fact that the two methods are based on a number of different assumptions, they yield remarkably similar estimates. The "correct" answer probably lies somewhere between these two values, and the recent refinements for cycling in the intermediates (Dawes and Holms, 1958) and CO_2 (Wang and Krackov, 1962) methods may indeed be slightly overcompensatory. Under anaerobic conditions, there was no significant recycling in *B. subtilis* (Goldman and Blumenthal, 1963).

The Cleveland group has claimed: "Calculations which are based on models which do not permit recycling are considered infeasible and almost certainly will give unreliable results" (H. G. Wood *et al.*, 1963). Horecker (1962a), commenting on the possibility of recycling in cells utilizing hexoses, suggested that a true cyclic operation of the HMP was exceedingly unlikely: "Recycling of pentose phosphate back to hexose phosphate does occur in some species, but appears to be a relatively limited phenomenon." Cells utilizing pentoses as their main carbon source do employ a cyclic operation of the HMP, and evidence for such an operation has been obtained in fungi (van Sumere and Shu, 1957; Arnstein and Bentley, 1956). Cheldelin *et al.* (1962) also believe that the route of glucose metabolism in intact cells is largely unidirectional leading to eventual formation of CO_2, thereby minimizing randomization of labeling patterns through the operation of reversed catabolic sequences, such as the HMP pathway.

In fungi, as in most cells, when the EM and HMP pathways are present together it is the EM pathway that is usually the major one (Tables IV and V). Under such circumstances, it is unlikely that HMP recycling will affect the calculations in microorganisms in a major way. However, when glucose is catabolized by the combined ED and HMP pathways, as it is in certain oxidative bacteria, there is likely to be extensive recycling that cannot be ignored. Thus White and Wang (1964), employing a technique similar to that devised by Dawes and Holms (1958), estimated that there is recycling of 80–90% of the triose-P formed by *Acetobacter xylinum* from glucose via the HMP and ED pathways.

Several promising isotopic methods for the estimation of glycolytic path-

ways in living cells have recently been described. Two of the methods employ glucose specifically labeled with the nonradioactive isotopes deuterium (Rose, 1961) and O^{18} (Rittenberg and Ponticorvo, 1962). In addition, H. G. Wood *et al.* (1963) have proposed several modifications of techniques for the evaluation of pathway participation employing C^{14}-labeled glucoses that may also prove to be very useful.

III. OCCURRENCE OF PATHWAYS IN FUNGI

A. Embden-Meyerhof (EM) Pathway

Although fungi are commonly thought of as being strictly aerobic, the relationship between growth and oxygen supply varies considerably in different species. Many fungi can utilize carbohydrates anaerobically, producing "typical" fermentation products, although they cannot grow without some oxygen. The formation of lactate from glucose is restricted almost entirely to the phycomycetes although ethanol formation is rather common among the fungi, especially in the mucorales and fusaria. Although the genus *Aspergillus* is ordinarily considered to be strongly oxidative, *A. clavatus* and certain other species have extremely high capacity for alcoholic fermentation (cf. Foster, 1949; Cochrane, 1958).

There has been an unfortunate tendency by some to equate glycolysis or anaerobic glycolysis with the utilization of the EM pathway, and oxidative glycolysis with the HMP pathway. However, both pathways can operate under anaerobic or aerobic conditions, only the electron acceptors being varied. In bacteria, including *Leuconostoc mesenteroides* and *Zymomonas mobilis* (*Pseudomonas lindneri*), glycolytic systems are operative anaerobically with the formation of typical fermentation products. Yet, it has been shown that the EM pathway is *not* operative in either organism, the glycolytic pathways actually used being modified HMP and ED pathways, respectively (cf. Horecker, 1962a).

Most of the enzymes of the EM pathway are common to the HMP and ED pathways (Table I) and are widely distributed in fungal mycelia and spores (Table III). Only phosphofructokinase can be considered as being specifically associated with the EM pathway. The lack of phosphofructokinase in *Penicillium chrysogenum* and *Microsporum canis* (Table III) is probably due to the fact that this kinase has often been reported to be quite labile. With *P. chrysogenum*, at least, there exists supplementary evidence that the EM pathway is operative in the intact cell (Table IV).

In some instances the apparent lack of an enzyme may be due to the presence of an "inhibitor," such as the hexokinase and phosphoglucomutase inhibitors present in crude *Aspergillus niger* spore extracts (Khanna and

TABLE IV

QUANTITATIVE ESTIMATIONS OF PATHWAYS OF GLUCOSE CATABOLISM

Microorganism	Pathway participation: Embden-Meyerhof (%)	Pathway participation: Hexose monophosphate (%)	Pathway participation: Entner-Doudoroff (%)	Method	References
Aspergillus niger	78	—	—	Product C^{14} distribution	Shu *et al.* (1954)
Caldariomyces fumago	—	35	65	Product C^{14} distribution and specific activity	Ramachandran and Gottlieb (1963)
Candida utilis	50–96	4–50	—	Product specific activity	Blumenthal *et al.* (1954)
Claviceps purpurea	90–96	4–10	—	$C^{14}O_2$ yield	McDonald *et al.* (1960)
Fusarium lini	83	17	—	$C^{14}O_2$ specific activity	Heath *et al.* (1956)
Neurospora crassa, mycelium	88–99	1–12	—	$C^{14}O_2$ yield	Blumenthal (1962)
Neurospora crassa, conidia	90	10	—	$C^{14}O_2$ yield	Blumenthal (1962)
Penicillium chrysogenum	56–70	30–46	—	Product specific activity	Lewis *et al.* (1954)
Penicillium chrysogenum	42	58	—	$C^{14}O_2$ specific activity	Heath and Koffler (1956)
Penicillium chrysogenum	77	23	—	$C^{14}O_2$ yield	Wang *et al.* (1958a)
Penicillium digitatum	77–83	17–23	—	$C^{14}O_2$ yield	Wang *et al.* (1958a), Reed and Wang (1959)
Rhizopus oryzae	100	—	—	Product C^{14} distribution	Gibbs and Gastel (1953)
Rhizopus MX	100	—	—	Product C^{14} distribution and specific activity	Margulies and Vishniac (1961)
Saccharomyces cerevisiae	83–100	0–27	—	Product specific activity	Blumenthal *et al.* (1954)
Saccharomyces cerevisiae	88	12	—	$C^{14}O_2$ yield	Wang *et al.* (1956, 1958a)
Saccharomyces cerevisiae	94–96	4–6	—	$C^{14}O_2$ yield	Chen (1959a)
Streptomyces griseus	90–97	3–10	—	$C^{14}O_2$ yield	Wang *et al.* (1958a,b), Gilmour *et al.* (1959)
Tilletia caries, mycelium	66	34	—	$C^{14}O_2$ yield	Newburgh and Cheldelin (1958)
Tilletia caries, spores	—	—	100	$C^{14}O_2$ yield	Newburgh and Cheldelin (1958)
Tilletia contraversa, spores	33	67	—	$C^{14}O_2$ yield	Newburgh and Cheldelin (1959)
Verticillium alboatrum	48	52	—	$C^{14}O_2$ yield	Brandt and Wang (1960)

Tewari, 1963). Claims for the lack of an EM pathway on the basis of the inability to demonstrate some enzymes are not proper. Negative evidence, such as not being able to isolate phosphoglyceric acid from living fusaria metabolizing glucose in the presence of fluoride, or the inability to detect phosphoglycerate mutase [see Foster (1949) and Cochrane (1956, 1958) for summaries], was used by Nord and Weiss (1951) to support their claim of the lack of an EM pathway in *Fusarium*. The subsequent demonstration of the presence of the EM pathway in intact *Fusarium* cells (Table IV) by Cochrane (1956) and Heath *et al.* (1956), in addition to the evidence for the presence of EM enzymes in cell extracts (Table III), finally settled this problem. A similar situation developed over a question of the presence of the EM pathway in bacterial spores and also was settled in the affirmative (Goldman and Blumenthal, 1964a). These findings serve to emphasize the usefulness of the estimations of the pathways of glucose catabolism in intact cells as a complement to studies with cell-free extracts, and vice versa.

As mentioned earlier, many cultural and physiological conditions affect the enzymatic constitution of fungi. Bertrand and deWolf (1959) found Zn^{++} to be necessary in *A. niger* for the synthesis of phosphofructokinase and triose-P dehydrogenase. They also suggested that Zn^{++} was involved in the activity of aldolase, glucose-6-P and 6-phosphogluconate dehydrogenases. Geser (1962) employed radioactive Zn^{65} to aid in his studies of *A. niger*. He found aldolase activity to be profoundly inhibited during zinc deficiency whereas 6-phosphogluconate dehydrogenase activity increased and glucose-6-P dehydrogenase activity was somewhat depressed. Apparently there was greater utilization of glucose via the HMP pathway during zinc deficiency. In the same fungus Franke *et al.* (1963) found that the cultural conditions affected the relative amounts of glucose oxidase and three enzymes representative of the EM pathway, including hexokinase, aldolase, and triose-P dehydrogenase. As stated earlier, however, these three enzymes are not specifically associated with the EM pathway. Franke *et al.*, demonstrated that increasing the glucose concentration in the growth medium from 5% to 30% greatly increased the content of glucose oxidase in the mycelium, with a concomitant decrease in the three EM enzymes.

A tabulation of the major and minor pathways for glucose catabolism in fungi and related microbes using only estimates made with intact cells is presented in Table V. In those instances where a major pathway could be listed in fungi or actinomycetes, not including the two yeasts, the EM pathway was the major route used in fifteen organisms, the HMP pathway in five, and the ED in two. In all five instances where the HMP pathway was the major one, the EM pathway was the minor one,

TABLE V

PATHWAYS OF GLYCOLYSIS IN FUNGI

Microorganism	Pathway participation[a]		References
	Major	Minor	
Aspergillus flavus-oryzae[b]	(EM)	(HMP)	Bentley (1962)
Aspergillus niger	EM	HMP	Shu *et al.* (1954), Wang *et al.* (1958a), McDonough and Martin (1958)
Aspergillus terreus	EM	HMP	Bentley and Thiessen (1957)
Caldariomyces fumago	ED	HMP	Ramachandran and Gottlieb (1963)
Candida utilis	EM	HMP	Blumenthal *et al.* (1954)
Clavi ceps purpurea	EM	HMP	McDonald *et al.* (1960)
Fusarium lini	EM	HMP	Heath *et al.* (1956), Cochrane (1956)
Neurospora crassa (conidia and mycelium	EM	HMP	Blumenthal (1962)
Penicillium charlesii	HMP	EM	Bentley *et al.* (1962)
Penicillium chrysogenum	EM	HMP	Lewis *et al.* (1954), Heath and Kofller (1956), Sih *et al.* (1957), Wang *et al.* (1958a)
Penicillium cyclopium	EM	HMP	Bentley and Keil (1962)
Penicillium digitatum	EM	HMP	Wang *et al.* (1958a), Reed and Wang (1959)
Penicillium urticae[b]	(HMP)	(EM)	Bu'Lock *et al.* (1964)
Rhizopus oryzae	EM	—	Gibbs and Gastel (1953)
Rhizopus MX	EM	—	Margulies and Vishniac (1961)
Saccharomyces cerevisiae	EM	HMP	Blumenthal *et al.* (1954), Wang *et al.* (1956, 1958a), Chen (1959a)
Streptomyces coelicolor	HMP	EM	Cochrane *et al.* (1953)
Streptomyces griseus	EM	HMP	Wang *et al.* (1958a,b), Gilmour *et al.* (1959)
Streptomyces olivaceus[b]	(EM)	(HMP)	Maitra and Roy (1959)
Streptomyces reticuli	HMP	EM	Cochrane *et al.* (1953)
Tilletia caries, mycelium	EM	HMP	Newburgh and Cheldelin (1958)
Tilletia caries, spores	ED	—	Newburgh and Cheldelin (1958)
Tilletia contraversa, spores	HMP	EM	Newburgh and Cheldelin (1959)
Ustilago maydis	HMP	EM	McKinsey (1959)
Ustilago maydis	EM		Boothroyd *et al.* (1955)
Verticillium alboatrum	EM	HMP	Brandt and Wang (1960)
Zygorhynchus moelleri	EM	HMP	Bartlett and Moses (1957)

[a] EM = Embden-Meyerhof pathway; ED = Entner-Doudoroff pathway; HMP = hexose monophosphate pathway.

[b] Both EM and HMP pathways were operative, but extent of participation was not estimated.

hereby underlining the importance of the EM pathway in fungal glycolysis. Only *Tilletia caries,* among the fungi, appears to have the capability of utilizing all three pathways. In this instance, though, the ED pathway alone is found in the spore stage. Apparently the presence of the ED pathway in spores is not a common occurrence since spores of *T. conraversa* and *Neurospora crassa* do not employ it.

B. Hexose Monophosphate (HMP) Pathway

The HMP pathway is known, or has been known, under a wide variety of names including the hexose monophosphate oxidative shunt, the Warburg-Lipmann-Dickens pathway, the pentose or pentose phosphate cycle, the Horecker cycle, the "direct" oxidative pathway, or the phosphogluconate oxidative scheme.

According to the list in Table I, there are five enzymes that are specifically associated with the HMP pathway. These are phosphogluconate dehydrogenase, ribose-P isomerase, ribulose-P epimerase, transketolase, and transaldolase. Glucose-6-P and 6-phosphogluconate dehydrogenases appear to be common in the fungi (Table III). A recent survey demonstrated glucose-6-P dehydrogenase in all of nine fungi examined (Dowler *et al.,* 1963). Transketolase and transaldolase have been demonstrated in *Streptomyces olivaceus* (Maitra and Roy, 1959) *Ustilago maydis* (Gottlieb and Caltrider, 1963), *Aspergillus niger* (McDonough and Martin, 1958), *Claviceps purpurea* (McDonald *et al.,* 1960), *Penicillium chrysogenum* (Sih *et al.,* 1957), and *Tilletia caries* (Newburgh *et al.,* 1955). Horecker (1962a) suggests that transketolase and transaldolase are universal in their distribution, having been detected in all cells that have been adequately studied.

NADP is the usual coenzyme involved in the oxidative reactions of the HMP pathway although there are a few microbes that use NAD in its place under anaerobic conditions. Horecker (1962b), while considering the evolutionary development of the different carbohydrate metabolic pathways, comments that the oxidation of glucose-6-P by NAD was a very early development, perhaps arising alongside of the EM pathway as an alternate or additional fermentation mechanism. In any case, Hochster (1957) reported the interesting observation that there apparently are both NAD- and NADP-linked glucose-6-P and 6-phosphogluconate dehydrogenases in *Aspergillus flavus-oryzae.* He measured the activities of all four enzymes over a 14-day growth period and found that both of the NADP-linked dehydrogenases were present in the earliest samples examined and that their activities did not change with time. In contrast to this, there was no detectable NAD-linked dehydrogenases before the third day, after

which they gradually reached levels quite similar to those of the NADP-linked dehydrogenases.

The HMP pathway is common in fungi, usually accounting for less than 40% of the glucose dissimilated (Table IV). The level of NADP appears to be the limiting factor in the utilization of the HMP pathway in fungi and other microbes (Eagon, 1963). In intact *Neurospora crassa* conidia and mycelium, the HMP pathway is minor at all times studied (Blumenthal, 1962), including mycelium grown on Westergaard-Mitchell medium containing citrate, which reportedly increases the production of glucose-6-P dehydrogenase very markedly (Turian, 1962).

Studies of both fungal and bacterial spores indicate that as the spores develop into vegetative cells there are profound changes in their metabolic pathways. Enzymes of the HMP and TCA cycles are lacking in *Ustilago maydis* spores until 12 hours after germination (Gottlieb and Caltrider, 1963). In developing bacterial spores, the HMP pathway increases in activity before returning to the low level characteristic of the vegetative cell. In addition, the TCA cycle is almost completely absent in the spores (Goldman and Blumenthal, 1964a,b). Sussman (1961) has observed an initial block in the TCA cycle of germinating ascospores of *Neurospora tetrasperma,* and conidia of *N. crassa* are reported to be deficient in some TCA enzymes (Turian, 1962). Finally, intact ungerminated *Fusarium solani* spores increase their 2% conversion of glucose C-6 to CO_2 about 6-fold after germination (Cochrane *et al.,* 1963). This increase may be interpreted as due in part to a release of TCA cycle inhibition following germination. Thus both bacterial and fungal spores may have the same initial defect in their TCA cycle. In *Bacillus cereus* the continued repression of the TCA cycle during development of the spores into vegetative cells is aided by corepressors in the dialyzable components of a casein enzymatic digest medium, and by the presence of glucose or lactate as carbon sources. In this instance the degree to which the HMP pathway is used during glucose catabolism is not directly related to the activity of the TCA cycle (Blumenthal, 1965).

There are several different pathways that a fungus can use in order to convert glucose to 6-phosphogluconate, and these are outlined in Fig. 3. They can be considered to be minor variations of the HMP or ED pathways. Thus, it is possible for an organism to be deficient in hexokinase if it has glucose oxidase and gluconokinase. It is possible for a fungus to form 6-phosphogluconate even if it lacks hexokinase and glucose-6-P dehydrogenase. This indirect pathway involves gluconate and 2-ketogluconate followed by a phosphorylation involving 2-ketogluconate kinase (DeLey and Vandamme, 1955). The resulting 2-ketogluconate-6-P can then be

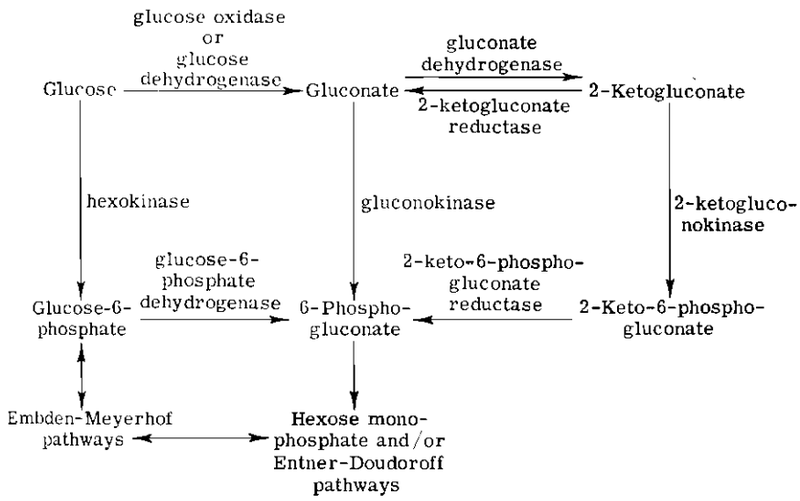

FIG. 3. Interrelationships in the metabolism of hexoses and hexonic acids.

reduced to 6-phosphogluconate by a specific reductase (DeLey and Verhofstede, 1957). It has been suggested that such a bypass for the formation of 6-phosphogluconate exists in *Caldariomyces fumago* (Ramachandran and Gottlieb, 1963).

C. Entner-Doudoroff (ED) Pathway

The ED pathway has two enzymes that are specifically associated with it, namely, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (Table I). This pathway seems to be relatively scarce in nature, having been reported only in some Gram-negative bacteria and in two fungi (Tables IV and V). There are reports of the absence of the ED enzymes in *Aspergillus niger* (McDonough and Martin, 1958; Geser, 1962).

In *Caldariomyces fumago* the ED is the major glycolytic pathway, with the HMP as a minor one. The mycelium lacks hexokinase (Table III), and the fungus apparently uses one of the sequences that bypass hexokinase and lead to the formation of 6-phosphogluconate (Fig. 3).

Tilletia caries spores apparently use the ED pathway as the sole glycolytic pathway. *T. caries* mycelium, however, does not catabolize glucose this way. This situation is somewhat reminiscent of that in *Pseudomonas natriegens* (Eagon and Wang, 1962) where glucose is metabolized via the

EM and HMP pathways, whereas gluconate is catabolized via the ED or HMP pathways. Apparently the simultaneous operation of the ED and EM pathways is incompatible, although the reasons for this are unknown.

D. Glucuronate-Xylulose Pathway

There is increasing evidence for the presence in animals of a new hexose-pentose cycle called the glucuronate-xylulose pathway or cycle, or more briefly the glucuronic acid cycle. The reaction scheme, which uses part of the HMP pathway, is a follows: D-glucose → D-glucuronate (through a series of known phosphorylated intermediates) → L-gulonate (inversion of the carbon chain) → xylitol → D-xylulose (inversion of the carbon chain) → D-xylulose-P → HMP cycle → glucose (cf. Hollmann, 1964). The quantitative significance of the cycle is unknown, but it appears to be minor. However, in this cycle, the C-6 of glucose is preferentially lost just as C-1 is lost preferentially via the HMP cycle. Ascorbic acid is derived from L-gulonate, one of the components of the cycle. In *Aspergillus niger*, addition of glucuronolactone to the growth medium resulted in a threefold increase (2.6% yield) in the ascorbic acid content of the mycelium in 10 days, as well as an 18% increase in mycelial yield. This is very indirect evidence that the glucuronate cycle is present in fungi (Sastry and Sarma, 1957). In the differentiating slime mold, there are two developmental stages which yield more $C^{14}O_2$ from glucose-6-C^{14} than from glucose-1-C^{14} (Wright, 1963a), and the glucuronate-xylulose cycle is the only metabolic sequence known that will yield such results. However, attempts to demonstrate certain key enzymes yielded negative results.

Some of the enzymes of the glucuronate cycle, other than the HMP enzymes, also are known to be present in fungi. Recent studies have disclosed the existence of an unusual reductive pathway for pentose metabolism in *Penicillium chrysogenum* and other filamentous fungi (Chiang and Knight, 1960) and in yeasts (Horecker, 1962a). This pathway is able to convert an L-pentose, such as L-arabinose, to D-xylulose-5-P via xylitol. The xylitol → D-xylulose → D-xylulose-5-P sequence in this fungal pentose pathway is also part of the glucuronate cycle.

Another instance in fungal carbohydrate metabolism which involves the preferential oxidation of a hexose C-6 has been reported by Avigad *et al.* (1961). *Polyporus circinatus* possesses a D-galactose oxidase that oxidizes the C-6 position of D-galactose, yielding D-galactodialdose, instead of the C-1 position as with glucose oxidase. The further metabolism of this compound has not been described.

However suggestive the foregoing evidence is of the presence of a glucuronate-xylulose cycle in fungi, further proof is needed.

IV. SIGNIFICANCE OF GLYCOLYTIC PATHWAYS

A. Roles of NAD and NADP

There has been some tendency to consider the HMP pathway as just an alternative to the EM sequence in the formation of pyruvate. However, the HMP pathway is not really such an alternative because it performs an entirely different function. In the EM pathway NADH functions as the specific coenzyme of glycolysis and also is involved as a coenzyme in the TCA cycle (with the exception of isocitric dehydrogenase which yields NADPH) and the related terminal respiratory sequences. On the other hand, the oxidative reactions of the HMP pathway are major sources of NADPH in aerobic cells. What are the cellular functions of NADH, the coenzyme product of the EM pathway, and of NADPH, that of the HMP pathway?

In all biological systems studied so far, it is NADH, not NADPH, that is involved in energy generation by oxidative phosphorylation through the cytochrome system (cf. Lindenmayer, Chapter 12). Therefore, a primary distinction between the EM and HMP pathways is that aerobic glycolysis releases energy only via the former, in conjunction with the TCA cycle. What then are the functions of NADPH formed by the glucose-6-P and 6-phosphogluconate dehydrogenases of the HMP and the isocitric dehydrogenase of the TCA cycle? If we examine the reactions involving NADPH in the cell, it becomes evident that, with the exception of the three reactions just mentioned, all NADPH-linked reactions proceed in the direction of NADPH oxidation with the concomitant formation of a reduced product. For example, these reactions include fatty acid synthesis, glycogen synthesis via the reductive carboxylation of pyruvate and, particularly, the reductive animation of α-ketoglutarate leading to the formation of glutamate (Horecker, 1962a). The latter reaction, catalyzed by glutamate dehydrogenase, is the key reaction interconnecting glycolysis and amino acid biosynthesis (cf. Nicholas, Chapter 13).

The fact that NADPH-linked reactions are involved in biosynthetic reactions in fungi has been emphasized recently in studies on two glutamic dehydrogenases found in *Neurospora crassa*. Sanwal and Lata (1961) found that the NAD-linked glutamate dehydrogenase served only as a degradative route, i.e., leading to the formation of NADH, whereas the NADPH-linked glutamate dehydrogenase was the one linked to a biosynthetic role. The same situation exists in the fungus *Pyricularia oryzae* (Kato *et al.*, 1962).

Therefore, the primary function of NADPH seems to be as a source of

reduced coenzymes for reductive reactions, a function separate from that of NADH, which is to transfer electrons to oxygen during the release of energy. A cell which possesses both coenzymes is able to obtain energy rapidly under aerobic conditions without losing the ability to synthesize reduced cell components. NAD is found largely in the oxidized form in cells whereas the reverse is true for NADP, which is present almost entirely as NADPH (cf. Horecker, 1962a). This suggests that transhydrogenase does not usually catalyze the oxidation of NADPH by NAD in the cell and that NADPH is not oxidized by the cytochrome system. This would further suggest that the availability of NADP would be a limiting factor for the operation of the HMP and/or ED pathway. How, then, do microbes that use these pathways as their primary glycolytic routes obtain enough NADP?

Eagon (1963) has recently investigated this problem using ten microorganisms, including *Saccharomyces cerevisiae, Penicillium chrysogenum,* and *Streptomyces griseus,* whose routes of glucose catabolism are known quantitatively. He found a strong correlation between the presence of both NADPH oxidase and transhydrogenase, and those microorganisms predominantly utilizing the HMP and/or ED pathways. Although NADH oxidase was present in all extracts, little or no NADPH oxidase or transhydrogenase was detected in extracts from those microbes predominantly utilizing the EM pathway, among the fungi tested. Another mechanism for regenerating NADP under anaerobic conditions is available to some cells. They can utilize a NADP-linked lactate dehydrogenase to reduce pyruvate to lactate while oxidizing NADPH (Evans and Karnovsky, 1962).

B. Role of HMP Pathway in Synthetic Reactions

In the introduction, the statement was made that the function of glycolytic pathways is to satisfy three major requirements of cells: (1) energy, (2) precursors or components for synthesis, (3) oxidation or reduction reactions necessary for converting these precursors to the appropriate intermediates or end products. The importance of the EM-TCA sequence as a source of energy, and the special role of the HMP in providing NADPH for biosynthetic reduction reactions, have been mentioned. What about the supply of precursors? If we consider the special units needed for a growing cell, i.e., units other than those, such as triose-P, that are provided by both the EM and HMP cycles it becomes apparent that the HMP has a special role (cf. Cheldelin *et al.,* 1962).

The pentose requirement of growing cells points directly to involvement of the HMP cycle. Pentose-P can be formed by the oxidative reactions of the HMP cycle, and/or by the cycle operating in the reverse direction,

the so-called nonoxidative pentose cycle involving transketolase and transaldolase reactions. The results of experiments with specifically labeled glucose indicate that both arms of the HMP "cycle" contribute to the ribose-P of nucleotides and ribonucleic acid.

Erythrose-4-P is a second important component for synthetic reactions that is provided by the HMP pathway. This intermediate in the HMP pathway is an important precursor in the biosynthesis of aromatic amino acids.

C. Factors Affecting Pathways

There have been no systematic studies concerned with the effect of different physical and physiological conditions on the utilization of glucose catabolic pathways. Alterations in the relative degree of utilization of the glycolytic pathways in germinating spores have been mentioned previously. Presumably other instances of such biochemical alterations will be discovered in cells undergoing morphogenesis.

Comparisons of proliferating and resting cell suspensions show a slight increase in the percentage of glucose utilized via the HMP pathway in growing cells. Thus a 10% increase in the HMP, from a value of 40% to a value of 50%, was observed in *Candida utilis* (Blumenthal *et al.*, 1954). Similar increases in the extent of utilization of the HMP have been observed in the fungi *Claviceps purpurea* (McDonald *et al.*, 1960) and *Neurospora crassa* (Blumenthal, 1962). Such results are not surprising since the biosynthetic processes, which require NADPH, pentoses, etc., would be expected to be more active in growing cells than in resting cells.

The effects of the degree of aerobiosis on the utilization of the several pathways have also been studied to a limited extent. Thus, the percentage of glucose utilized via the HMP pathway aerobically and anaerobically in *Saccharomyces cerevisiae* was 26 and 10%, respectively, whereas the corresponding figures in *C. utilis* were 41 and 4% (Blumenthal *et al.*, 1954), and in *Bacillus subtilis* about 38 and 26% (Goldman and Blumenthal, 1963). Qualitatively similar changes have also been observed in *Fusarium lini* (Heath *et al.*, 1956) and *Verticillium alboatrum* (Brandt and Wang, 1960). In the latter organism, however, there was unexpectedly less utilization of the HMP pathway in an atmosphere of pure oxygen compared with air. This is not a universal response in microorganisms since it was observed that the percentage of glucose catabolized via the HMP pathway by a resting suspension of bacilli could be varied, reaching a high of 40% with relatively dilute suspensions of bacilli in oxygen, and a low of 2% with relatively dense cell suspensions in air (Pepper and Costilow, 1964). On the other hand, *S. cerevisiae* used the HMP to ap-

proximately the same extent under three different degrees of aeration (Chen, 1959a). The addition of oxidation-reduction mediators such as methylene blue or pyocyanine may increase the HMP pathway through increased aerobic NADPH reoxidation (Evans and Karnovsky, 1962).

The fact that microbes use a greater percentage of glucose via the HMP under aerobic conditions than under anaerobic conditions is to be expected. Because the energy derived from NADH though oxidative phosphorylation cannot be obtained anaerobically, the cell would be expected to use more glucose anaerobically (Pasteur effect), obtaining the small amount of energy available by oxidation of two triose-P molecules via the EM pathway. Only one triose-P would be obtained per molecule of glucose via the HMP pathway.

There has been a suggestion that the glucose concentration may affect the extent to which the pathways are used (Katz and Wood, 1963). Such effects apparently are not common for animal cells (Evans and Karnovsky, 1962) or microbes (Chen, 1959a; Goldman and Blumenthal, 1963). However, there has been a recent report of an effect of glucose concentration on the C-6:C-1 ratio from slime molds at different developmental stages (Wright, 1963a) so that the effect of glucose concentrations should be considered.

Increased levels of P_i might be thought to stimulate the EM pathway and inhibit the HMP pathway in living cells (cf. D'Adamo, 1963) since P_i is both a substrate in the triose-P dehydrogenase reaction and an inhibitor of the glucose-6-P dehydrogenase and the transaldolase reactions. Although such an effect of P_i has been demonstrated in cell extracts (Kravitz and Guarino, 1958), it apparently is small in intact cells. Thus, *Bacillus subtilis* grown on limiting amounts of P_i utilized the EM pathway to about the same extent as cells grown with normal P_i levels, even though the rate of glucose utilization was considerably inhibited. Furthermore, addition of P_i to such phosphorus-deficient cells only increased the extent of participation of the EM pathway by about 10% (Goldman and Blumenthal, 1963). Similarly, the P_i concentration did not affect the glycolytic pathways of *Escherichia coli* (Paege and Gibbs, 1961). In *E. coli*, furthermore, varying the pH from 5.0 to 8.0 did not alter the pathways even though the character of the fermentation products was changed. A similar lack of effect of pH on the glucose pathways was observed in dwarf bunt spores (Newburgh and Cheldelin, 1959).

The analysis of glycolytic pathways is even more complicated when we deal with the interactions in a host-parasite system. Apparently the metabolism of wheat, bean, and safflower tissues infected with obligate fungal parasites is changed, a marked increase in the HMP pathway resulting (Daly *et al.*, 1961). However, the infection of *Streptomyces griseus* with

an actinophage does not appreciably alter the extent of the participation of the HMP pathway (Gilmour *et al.,* 1959).

The mechanisms involved in the regulation of glucose catabolism are not known although great advances can be expected in the near future. The general problem of metabolic regulation is covered by Zalokar (Chapter 14) and has been the subject of symposia (cf. Holzer, 1961; Wright, 1963b).

V. ENDOGENOUS METABOLISM

Among the filamentous fungi, the problem of high rates of endogenous respiration, which is often a useful measure of endogenous metabolism, has been particularly troublesome. This problem was briefly mentioned in Section II C, on manometric methodology. Starvation or prolonged aeration have often been used with fungal cells before respirometric studies in attempts to reduce the endogenous respiration. However, the differences in the properties of starved and fresh cells often are not fully appreciated. The effects of starvation on the metabolism of glucose by *Zygorhyncus moelleri* have been well documented (Moses, 1959), and differences in the effect of substrates on the endogenous metabolism of starved or unstarved mycelia have also been noted in *Neurospora crassa* (Blumenthal, 1963). In respirometric studies, the absence of an increase in the rate of respiration upon addition of a substrate, or even decreased respiration, may not mean that the substrate is not being oxidized. The Crabtree effect, or the inhibition of respiration upon the addition of hexoses, is not uncommon in animal tissues, and in these instances the substrate is oxidized. Perhaps this effect may also be found in microorganisms, especially those with high endogenous respiration.

Endogenous metabolism in microorganisms has been the subject of a recent review (Dawes and Ribbons, 1962) and of a symposium (Lamanna, 1963), the latter including reviews of the endogenous metabolism of filamentous fungi (Blumenthal, 1963), yeast (Eaton, 1963), and slime molds (Wright, 1963a).

The availability of radioisotopes has made possible quantitative studies of the endogenous respiration of fungi, including *Penicillium chrysogenum* and *Neurospora crassa,* during the concomitant utilization of exogenously added glucose and/or acetate (Blumenthal, 1963). The extent to which the endogenous respiration of *N. crassa* was inhibited was influenced by many factors, such as the nature and concentration of the exogenous substrate, the age of the cells, the growth temperature, and whether or not the cells were starved. The respiration of the mycelium was inhibited about 15–40% during glucose oxidation and about 50–100% during acetate

oxidation. The results of experiments with glucose and acetate as cosubstrates suggested that each was inhibiting the utilization of different endogenous substrates. Extension of these studies to *N. crassa* ascospores and conidia revealed differences in the response of the sexual and asexual spores. The conidia behaved similarly to the mycelium in that the endogenous respiration was completely inhibited by acetate, while glucose inhibited about 70% of the endogenous respiration. On the other hand, the endogenous respiration of germinating ascospores whose primary endogenous energy source is known to be trehalose (Sussman, 1961) was inhibited only to an extent of about 35% by either substrate (Blumenthal, 1962). The reasons for the differences in behavior of the two spore types are not known.

In germinated *Fusarium solani* macroconidia the rate of glucose oxidation was inversely proportional to spore density, but the rate of endogenous respiration and the extent of utilization of glucose was invariant with density. These data were interpreted as evidence that glucose does not suppress or accelerate the endogenous respiration (Cochrane *et al.*, 1963). However, the interpretation of such results obtained with manometric data alone is difficult and may lead to erroneous conclusions (Blumenthal, 1963).

In a recent study with *Aspergillus sojae* mycelium, it was reported that the endogenous substrate(s) varied depending on the relative amounts of carbon and nitrogen in the growth medium (Mizunuma, 1963). In media with a high C:N ratio, carbohydrate or lipid was the major endogenous substrate at first, the utilization of nitrogenous materials with accompanying liberation of ammonia, occurring at a later time. When a low C:N ratio was used, endogenous utilization of carbohydrate and fat was low and pool amino acids, protein, and nucleic acids were the main source of energy. These results are in agreement with earlier ones indicating that there appears to be more than a single endogenous substrate.

It is clear that the response of the endogenous metabolism of a cell during the metabolism of an exogenous substrate is complex. The previous history of an organism, perhaps by regulating the quantitative distribution of enzymes and/or endogenous reserves of the cell, does affect the way in which the endogenous respiration behaves subsequently in the presence of an added substrate.

Eaton (1963) has used a clever technique to obtain a rough estimate of the glycolytic pathways used by yeast in the metabolism of their reserve glycogen deposits. He grew the yeast on glucose-1-C^{14} as the sole carbon source, a procedure that yields glycogen whose glucose units are labeled only in C-1 (cf. Chen, 1959b). The release of $C^{14}O_2$ from resting suspensions of these cells was measured in the presence or absence of suffi-

cient malonate to completely inhibit the TCA cycle. Using these data he estimated that about 39–44% of the endogenous glucose from glycogen was metabolized through the HMP pathway as compared to 24% for exogenously supplied glucose-1-C^{14}. These results certainly indicate, at least in a semiquantitative manner, that the EM-TCA pathway is the major route for endogenous "glucose," as well as for exogenous glucose.

REFERENCES

Arnstein, H. R. V., and R. Bentley. (1956). *Biochem. J.* **62**:403–411.

Avigad, G., C. Asensio, D. Amaral, and B. L. Horecker. (1961). *Biochem. Biophys. Res. Commun.* **4**:474–477.

Bartlett, G. W., and V. Moses. (1957). *J. Gen. Microbiol.* **16**:550–560.

Bentley, R. (1962). *Ann. Rev. Biochem.* **31**:589–624.

Bentley, R., and J. G. Keil. (1962). *J. Biol. Chem.* **237**:867–873.

Bentley, R., and C. P. Thiessen. (1957). *J. Biol. Chem.* **226**:673–687.

Bentley, R., D. S. Bhate, and J. G. Keil. (1962). *J. Biol. Chem.* **237**:859–866 .

Bertrand, D., and A. deWolf. (1959). *Bull. Soc. Chim. Biol.* 41:545–554.

Bloom, B., and D. Stetten, Jr. (1953). *J. Am. Chem. Soc.* **75**:5446.

Blumenthal, H. J. (1962). Unpublished data.

Blumenthal, H. J. (1963). *Ann. N.Y. Acad. Sci.* **102**:688–706.

Blumenthal, H. J. (1965). *In* "Spores III" (H. O. Halvorson, ed.), pp. 222–236. Am. Soc. Microbiol., Ann Arbor, Michigan.

Blumenthal, H. J., K. F. Lewis, and S. Weinhouse. (1954). *J. Am. Chem. Soc.* **76**:6093–6097.

Boothroyd, B., J. A. Thorn, and R. H. Haskins. (1955). *Can. J. Biochem. Physiol.* **33**:289–296.

Brandt, W. H., and C. H. Wang. (1960). *Am. J. Botany* **47**:50–53.

Bu'Lock, J. D., D. Hamilton, and A. J. Powell. (1964). *Biochem. J.* 90:26P–27P.

Cantino, E. C., and G. Turian. (1959). *Ann. Rev. Microbiol.* **13**:97–124.

Casselton, P. J., and P. J. Syrett. (1962). *Ann. Botany (London)* [N.S.] **26**:83–94.

Chattaway, F. W., C. C. Thompson, and A. J. E. Barlow. (1960). *J. Gen. Microbiol.* **22**:649–656.

Cheldelin, V. H. (1961). "Metabolic Pathways in Microorganisms," 91 pp. Wiley, New York.

Cheldelin, V. H., C. H. Wang, and T. E. King. (1962). *In* "Comparative Biochemistry" (M. Florkin and H. S. Mason, eds.), Vol. 3, Part A, pp. 427–502. Academic Press, New York.

Chen, S. L. (1959a). *Biochim. Biophys. Acta* **32**:470–479

Chen, S. L. (1959b). *Biochim. Biophys. Acta* **32**:480–484.

Chiang, C., and S. G. Knight. (1960). *Biochem. Biophys. Res. Commun.* **3**:554–559.

Cochrane, V. W. (1955). *J. Bacteriol.* **69**:256–263.

Cochrane, V. W. (1956). *Mycologia* **48**:1–12.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Cochrane, V. W., H. D. Peck, Jr., and A. Harrison. (1953). *J. Bacteriol.* **66**:17–23.

Cochrane, V. W., S. J. Berry, F. G. Simon, J. C. Cochrane, C. B. Collins, J. A. Levy, and P. K. Holmes. (1963). *Plant Physiol.* **38**:533–541.

Cohen, S. S. (1963). *Science* **139**:1017–1026.

D'Adamo, A. F. (1963). *J. Theoret. Biol.* **4**:142–144.
Daly, J. M., A. A. Bell, and L. R. Krupka. (1961). *Phytopathology* **51**:461–471.
Damodaran, M., V. Jagannathan, and K. Singh. (1955). *Enzymologia* **17**:199–206.
Davidson, E. A. (1960). *J. Biol. Chem.* **235**:23–25.
Dawes, E. A., and W. H. Holms. (1958). *Biochim. Biophys. Acta* **29**:82–91.
Dawes, E. A., and W. H. Holms. (1959). *Biochim. Biophys. Acta* **34**:551–552.
Dawes, E. A., and D. W. Ribbons. (1962). *Ann. Rev. Microbiol.* **16**:241–264.
DeLey, J., and J. Vandamme. (1955). *J. Gen. Microbiol.* **12**:162–171.
DeLey, J., and S. Verhofstede. (1957). *Enzymologia* **18**:47–62.
Dickens, F. (1958). *Ann. N.Y. Acad. Sci.* **75**:71–94.
Dowler, W. M., P. D. Shaw, and D. Gottlieb. (1963). *J. Bacteriol.* **86**:9–17.
Duff, R. B., and D. M. Webley. (1959). *Biochim. Biophys. Acta* **34**:398–406.
Eagon, R. G. (1963). *Biochem. Biophys. Res. Commun.* **12**:274–279.
Eagon, R. G., and C. H. Wang. (1962). *J. Bacteriol.* **83**:879–886.
Eaton, N. R. (1963). *Ann. N.Y. Acad. Sci.* **102**:678–687.
Evans, W. H., and M. L. Karnovsky. (1962). *Biochemistry* **1**:159–166.
Foster, J. W. (1949). "Chemical Activities of Fungi," 648 pp. Academic Press, New York.
Foster, J. W. (1958). *Texas Rept. Biol. Med.* **16**:79–100.
Fraenkel, D., M. J. Osborn, B. L. Horecker, and S. M. Smith (1963). *Biochem. Biophys. Res. Commun.* **11**:423–428.
Franke, W., G. Eichhorn, and J. Bertram. (1963). *Arch. Mikrobiol.* **46**:96–116.
Geser, G. (1962). *Arch. Mikrobiol.* **41**:408–440.
Ghosh, S., H. J. Blumenthal, E. Davidson, and S. Roseman. (1960). *J. Biol. Chem.* **235**:1265–1273.
Gibbs, M., and R. Gastel. (1953). *Arch. Biochem. Biophys.* **43**:33–38.
Gilmour, C. M., V. Evenson, M. Stern, and C. H. Wang. (1959). *Bacteriol. Proc.* pp. 82–83.
Godin, P. (1955). *Antonie van Leeuwenhoek, J. Microbiol. Serol.* **21**:83–93.
Goldman, M., and H. J. Blumenthal. (1963). *J. Bacteriol.* **86**:303–311.
Goldman, M., and H. J. Blumenthal. (1964a). *J. Bacteriol.* **87**:377–386.
Goldman, M., and H. J. Blumenthal. (1964b). *J. Bacteriol.* **87**:387–390.
Goldstein, A., and E. C. Cantino. (1962). *J. Gen. Microbiol.* **28**:689–699.
Gottlieb, D., and P. G. Caltrider. (1963). *Nature* **197**:916–917.
Heath, E. C., and H. Koffler. (1956). *J. Bacteriol.* **71**:174–181.
Heath, E. C., D. Nasser, and H. Koffler. (1956). *Arch. Biochem. Biophys.* **64**:80–87.
Higgins, E. S., and W. G. Chambers. (1963). *Arch. Biochem. Biophys.* **103**:87–93.
Hochster, R. M. (1957). *Arch. Biochem. Biophys.* **66**:499–501.
Hochster, R. M., and J. H. Quastel, eds. (1963). "Metabolic Inhibitors," Vol. 1, 669 pp. Academic Press, New York.
Hollmann, S. (1964). "Non-Glycolytic Pathways of Metabolism of Glucose" (Transl. and rev. by O. Touster), 275 pp. Academic Press, New York.
Holzer, H. (1961). *Cold Spring Harbor Symp. Quant. Biol.* **26**:277–288.
Horecker, B. L. (1962a). "Pentose Metabolism in Bacteria," 100 pp. Wiley, New York.
Horecker, B. L. (1962b). *Comp. Biochem. Physiol.* **4**:363–369.
Horváth, I., and A. Szentirmai. (1962). *Acta Microbiol. Acad. Sci. Hung.* **9**:105–116.
Jagannathan, V., and K. Singh. (1953). *Enzymologia* **16**:150–160.
Jyssum, K., and P. E. Joner. (1962). *Acta Pathol. Microbiol. Scand.* **55**:335–341.

Kato, K., S. Koike, K. Yamada, H. Yamada, and S. Tanaka. (1962). *Arch. Biochem. Biophys.* **98**:346–347.
Katz, J., and H. G. Wood. (1960). *J. Biol. Chem.* **235**:2165–2177.
Katz, J., and H. G. Wood. (1963). *J. Biol. Chem.* **238**:517–523.
Khanna, R., and K. K. Tewari (1963). *Arch. Mikrobiol.* **45**:398–406.
Kravitz, E. A., and A. J. Guarino. (1958). *Science* **128**:1139–1140.
Kuo, M. H., and H. J. Blumenthal. (1961). *Biochim. Biophys. Acta* **52**:13–29.
Lal, M., and P. M. Bhargava. (1962). *Biochim. Biophys. Acta* **58**:628–630.
Lamanna, C., ed. (1963). *Ann. N. Y. Acad. Sci.* **102**:515–793.
Lamanna, C., and M. F. Mallette. (1959). "Basic Bacteriology," 2nd ed., pp. 624–651. Williams & Wilkins, Baltimore, Maryland.
Lewis, K. F., H. J. Blumenthal, C. E. Wenner, and S. Weinhouse. (1954). *Federation Proc.* **13**:252.
Lewis, K. F., H. J. Blumenthal, R. S. Weinrach, and S. Weinhouse. (1955). *J. Biol. Chem.* **216**:273–286.
Lynen, F. G., and H. P. Hoffmann-Walbeck. (1948). *Ann. Chem.* **559**:153–168.
McDonald, J. K., V. H. Cheldelin, and T. E. King. (1960). *J. Bacteriol.* **80**:61–71.
McDonough, M. W., and S. M. Martin. (1958). *Can. J. Microbiol.* **4**:329–333.
McKinsey, R. D. (1959). *Am. J. Botany* **46**:566–571.
Maitra, P. K. and S. C. Roy. (1959). *J. Biol. Chem.* **234**:2497–2503.
Margulies, M., and W. Vishniac. (1961). *J. Bacteriol.* **81**:1–9.
Maruyama, Y., and M. Alexander. (1962). *J. Bacteriol.* **84**:307–312.
Mizunuma, T. (1963). *Agr. Biol. Chem. (Tokyo)* **27**:88–98.
Moses, V. (1959). *J. Gen. Microbiol.* **20**:184–196.
Newburgh, R. W., and V. H. Cheldelin. (1958). *J. Bacteriol.* **76**:308–311.
Newburgh, R. W., and V. H. Cheldelin. (1959). *Can. J. Microbiol.* **5**:415–417.
Newburgh, R. W., and C. A. Claridge, and V. H. Cheldelin. (1955). *J. Biol. Chem.* **214**:27–35.
Nord, F. F., and S. Weiss. (1951). *In* "The Enzymes" (J. B. Sumner and K. Myrbäck, eds.), Vol. 2, Part 1, pp. 684–790. Academic Press, New York.
Paege, L. M., and M. Gibbs. (1961). *J. Bacteriol.* **81**:107–110.
Pepper, R. E., and R. N. Costilow. (1964). *J. Bacteriol.* **87**:303–310.
Pette, D., W. Luh, and T. Bücher. (1962). *Biochem. Biophys. Res. Commun.* **7**:419–424.
Ramachandran, S., and D. Gottlieb. (1963). *Biochim. Biophys. Acta* **69**:74–84.
Reed, D. J., and C. H. Wang. (1959). *Can. J. Microbiol.* **5**:59–66.
Rittenberg, D., and L. Ponticorvo. (1962). *J. Biol. Chem.* **237**:PC2709–PC2710.
Rose, I. A. (1961). *J. Biol. Chem.* **236**:603–609.
Sanwal, B. D., and M. Lata. (1961). *Biochem. Biophys. Res. Commun.* **6**:404–409.
Sastry, K. S., and P. S. Sarma. (1957). *Nature* **179**:44–45.
Seydoux, J., and G. Turian. (1962). *Pathol. Microbiol.* **25**:752–756.
Shu, P., A. Funk, and A. C. Neish. (1954). *Can. J. Biochem. Physiol.* **32**:68–80.
Sih, C. J., and S. G. Knight. (1956). *J. Bacteriol.* **72**:694–699.
Sih, C. J., and P. B. Hamilton, and S. G. Knight. (1957). *J. Bacteriol.* **73**:447–451.
Strauss, B. S., and S. Pierog. (1954). *J. Gen. Microbiol.* **10**:221–235.
Sussman, A. S. (1961). *In* "Spores II" (H. O. Halvorson, ed.), pp. 198–213. Burgess, Minneapolis, Minnesota.
Tsao, M. U. (1962). *Science* **136**:42–43.
Turian, G. (1962). *Neurospora Newsletter* **2**:15.

van Sumere, C. F., and P. Shu. (1957). *Can. J. Biochem. Physiol.* **35**:445–448.
Wang, C. H., and J. H. Krackov. (1962). *J. Biol. Chem.* **237**:3614–3622.
Wang, C. H., and C. T. Gregg, I. A. Forbusch, B. E. Christensen, and V. H. Cheldelin. (1956). *J. Am. Chem. Soc.* **78**:1869–1872.
Wang, C. H., I. Stern, C. M. Gilmour, S. Klungsoyr, D. J. Reed, J. J. Bialy, B. E. Christensen, and V. H. Cheldelin. (1958a). *J. Bacteriol.* **76**:207–216.
Wang, C. H., J. J. Bialy, S. Klungsoyr, and C. M. Gilmour. (1958b). *J. Bacteriol.* **75**:31–37.
White, G. A., and C. H. Wang. (1964). *Biochem. J.* **90**:424–433.
Wood, H. G. (1955). *Physiol. Rev.* **35**:841–859.
Wood, H. G., J. Katz, and B. R. Landau. (1963). *Biochem. Z.* **338**:809–847.
Wood, W. A. (1961). *In* "The Bacteria" (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. 2, pp. 59–149. Academic Press, New York.
Wright, B. E. (1963a). *Ann. N. Y. Acad. Sci.* **102**:740–754.
Wright, B. E., ed. (1963b). "Control Mechanisms in Respiration and Fermentation," 343 pp. Ronald Press, New York.

CHAPTER 11

Carbohydrate Metabolism

2. Tricarboxylic Acid Cycle

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I. INTRODUCTION

In the last twenty-five years, a great deal of effort has been directed toward establishing the ubiquity of the enzymatic machinery of living cells concerned with the combustion of carbohydrate to CO_2 and water. It is now generally accepted that the tricarboxylic acid cycle (citric acid cycle, Krebs cycle, TCA cycle) serves as a major route of pyruvate utilization in both animal and plant cells. It is further established that the functional significance of this cyclic process relates to the provision of carbon moieties for the biosynthesis of cell constituents as well as to the coupling of the pertinent dehydrogenase systems to the respiratory chain. It is the latter which provides the link to molecular oxygen and during the process of hydrogen (electron) transfer considerable energy is made available to the cell. While these generalizations appear valid for animal and plant systems, until a few years ago the notion of a functional TCA cycle in fungi, and microorganisms in general, was in serious doubt. This chapter will therefore describe the study of the TCA cycle in the true fungi (Eumycetes) and, wherever possible, draw attention to the difficulties inherent in this work. The occurrence and role of the TCA cycle will be discussed in relation to the glyoxylate cycle and ancillary reactions in fungi. Recent nomenclatorial revisions which relate to these enzymes are listed in Table I.

TABLE I

NOMENCLATURE

Old trivial name	New trivial name[a]	New systematic name[a]
Coenzyme I, DPN	NAD	Nicotinamide-adenine dinucleotide
Coenzyme II, TPN	NADP	Nicotinamide-adenine dinucleotide phosphate
Condensing enzyme	Citrate synthetase	Citrate oxalacetate-lyase (CoA-acetylating) 4.1.3.7
Aconitase	Aconitate hydratase	Citrate (isocitrate) hydro-lyase 4.2.1.3
Aconitic hydrase	Citrate dehydratase	Citrate hydro-lyase 4.2.1.4
Isocitric dehydrogenase (DPN-linked)	Isocitrate dehydrogenase	L_S isocitrate: NAD oxidoreductase (decarboxylating) 1.1.1.4.1
Isocitric dehydrogenase (TPN-linked)	Isocitrate dehydrogenase	L_S isocitrate: NADP oxidoreductase (decarboxylating) 1.1.1.4.2
α-Ketoglutaric dehydrogenase	Oxoglutarate dehydrogenase	2-Oxoglutarate: lipoate oxidoreductase (acceptoracylating) 1.2.4.2
Succinic dehydrogenase	Succinate dehydrogenase	Succinate: (acceptor) Oxidoreductase 1.3.99.1
Fumarase	Fumarate hydratase	L-Malate hydro-lyase 4.2.1.2
Malic dehydrogenase	Malate dehydrogenase	L-Malate: NAD oxidoreductase 1.1.1.37
Isocitritase, isocitric lyase	Isocitrate lyase	L_S Isocitrate glyoxylate-lyase 4.1.3.1
Malate synthetase	Malate synthetase	L-Malate glyoxylate-lyase (CoA-acetylating) 4.1.3.2

[a] Report on the Commission on Enzymes of the International Union of Biochemistry (1961).

II. THE TRICARBOXYLIC ACID CYCLE

A. Conceptual Development of a TCA Cycle

Early interest concerning the intermediate stages of carbohydrate oxidation stemmed largely from animal studies. Thus, a variety of oxidations were demonstrated with animal tissue by the early work of Thunberg. In addition, succinate, fumarate, malate, and oxalacetate were also shown to have catalytic effects on biological oxidation (cf. Krebs, 1937). However, it remained for Krebs and Johnson (1937) to provide data, obtained largely through the use of specific respiratory poisons, which led to the concept of a cyclic mechanism. These workers noted that the catalytic effect of citrate on muscle tissue oxidation was unaffected by arsenite but under these conditions the overall oxygen consumption was lowered and α-ketoglutaric acid accumulated in considerable amounts. Malonate likewise did not affect the rate of citrate oxidation, but in this instance, suc-

· acid accumulated. Finally, if citrate was acting catalytically, some hanism must exist through which it could eventually be regenerated. ariety of intermediates were examined, but only oxalacetate acid gave to citric acid, and this could be demonstrated only anaerobically (i.e., :r conditions that arrested further citrate oxidation). From these ex- nents, and the data of previous investigators, a cyclic mechanism for pathway of carbohydrate oxidation in animal tissues was envisaged . 1). The citric acid cycle was shown to be operative in a variety of ial tissues, but was reported absent in yeast and bacteria in these ies (Krebs and Johnson, 1937) and also in later work by Krebs 43).

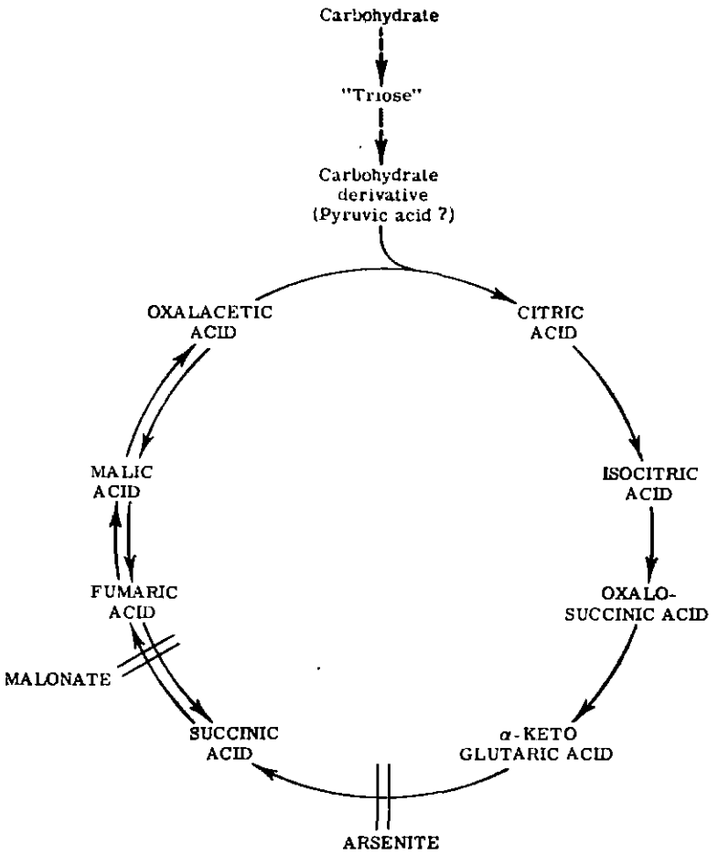

FIG. 1. Citric acid cycle (TCA cycle) of Krebs and Johnson.

Since this historic work, a great many details of the original citric acid cycle have been elaborated. In particular, the mechanism of citric acid synthesis from oxalacetic acid has received considerable attention. It is now well established that acetyl CoA (derived from the oxidative decar-

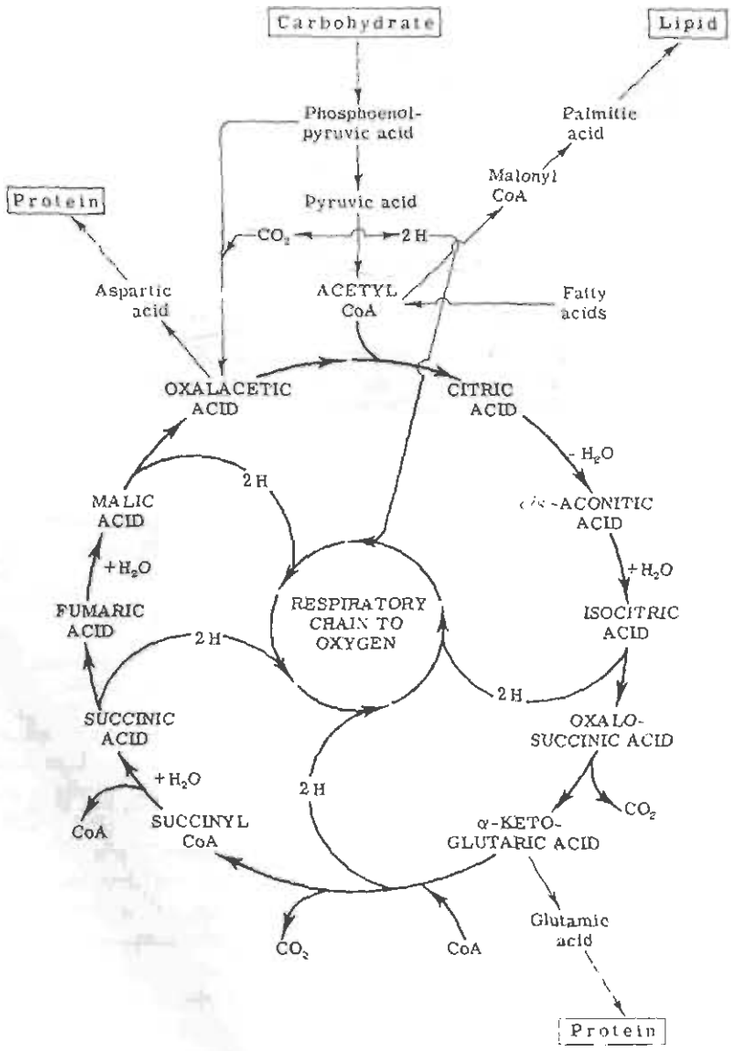

FIG. 2. Tricarboxylic acid (TCA) cycle as currently conceived.

boxylation of pyruvic acid) is the active unit which condenses with oxalacetic acid to form citric acid (cf. Lipmann, 1953). The present version of the TCA cycle differs very little from the original scheme proposed by Krebs and Johnson (1937); it is shown in Fig. 2.

B. Biological Significance of the TCA Cycle

While the TCA cycle was originally proposed as a mechanism for the terminal oxidation of carbohydrate, later studies indicated its utility in supplying amino acids through either transamination or reductive amination. These substances may then serve as intermediates for protein synthesis as well as to provide precursors for the synthesis of purines, pyrimidines, and porphyrins. Thus, the link between carbohydrate and protein synthesis appears well established. In addition, acetyl CoA proved to be a key intermediate for the biosynthesis of fatty acids (cf. Gibson, 1963). There are also several mechanisms for CO_2 fixation which function through certain intermediates of the TCA cycle (cf. Utter, 1961). Since the biosynthesis of cell constituents is accompanied by a withdrawal of carbon moieties from the TCA cycle, the latter mechanisms during growth on glucose (i.e., growth conditions under which CO_2 acceptors such as pyruvate or phosphopyruvate are available) may serve to replenish cell carbon. Although the central role of the TCA cycle would then appear to be to provide carbon intermediates for biosynthesis, another vitally important role of the cycle is the coupling of dehydrogenases with the respiratory chain. It is further recognized that the latter plays a common functional role in energy generation in a variety of animal and plant systems. In addition, all the TCA cycle reactions are localized in mitochondria. Some of these generalizations are illustrated in Fig. 2.

The purported lack of a functional TCA cycle in the true fungi has led to a great deal of controversy, and the time is now appropriate to reevaluate the situation. Therefore, our initial concern will relate to early studies dealing with the presence of a TCA cycle in fungi.

C. Characterization of the TCA Cycle in Fungi

1. Intact Cells

a. Growth. The occurrence of a functional TCA cycle among fungi such as *Aspergillus* and *Penicillium* was long suspected since these forms accumulate considerable amounts of citric acid under certain conditions of culture. In fact, Raistrick and Clark (1919) noted that Wehmer gave the name *Citromyces* to the particular group of fungi which produced citric acid from carbohydrate. In addition, other organic acids of the TCA cycle

are formed in considerable amounts by certain fungi, and these include succinic acid, fumaric acid, acetic acid, and, to a lesser extent, malic acid (see Cochrane, 1958). However, these data indicate an interruption of a cyclic process rather than an operative TCA cycle, and later work by Ramakrishnan *et al.* (1955) and Neilson (1956) have strengthened this interpretation.

Another hint that a TCA cycle may be present in fungi came from nutritional studies. Several yeasts utilize various intermediates of the TCA cycle as sole carbon sources for growth (see Barnett and Kornberg, 1960). However, the finding that other closely related forms cannot grow on these substrates does not necessarily rule out a functional TCA cycle and may simply indicate that permeability barriers exist.

A further clue to the possible role of dicarboxylic acids in fungi came from studies involving the mode of action of iodoacetic acid on the growth of *Neurospora crassa*. In particular, respiration and growth were inhibited appreciably by physiological levels of iodoacetate; inhibition of the former was reversed by succinic acid, while that of the latter was relieved by succinic acid, malic acid, fumaric acid, and pyruvic acid (Ryan *et al.*, 1944). This indicated that the inhibition by iodoacetate, presumably at the level of triose phosphate dehydrogenase, could be released by appropriate intermediates of the TCA cycle, whose function was to provide carbon moieties for growth and biosynthesis. Subsequently, certain single-gene mutants of *N. crassa* were reported to utilize for growth substrates associated with the TCA cycle (R. W. Lewis, 1948).

b. Respiration. Since these early nutritional studies provided no substantial proof of a functional TCA cycle in fungi, physiological experiments were attempted along lines applied successfully to animal tissues. These included the effects of TCA cycle intermediates as well as the action of specific respiratory poisons on the basal respiration of fungi. Although this approach was satisfactory with animal systems, serious difficulties were encountered with the fungi. In fact, before the advent of tracer techniques, the commonly encountered high endogenous respiration of fungal mycelium usually precluded further analysis of exogenous substrate utilization, even after aeration in sterile buffer. When this method does reduce endogenous activity, often a similar decrease in respiratory capacity is observed with exogenous substrate (Mickelson, 1950; Bentley, 1953; Bonner and Machlis, 1957). This may be alleviated by limiting the starvation period to just a few hours of aeration before the respiratory measurements (Moses, 1955; Litchfield and Ordal, 1958), but this is not always the case (Bentley, 1953). One procedure which has helped decrease the endogenous respiration is to reduce the carbohydrate level in the growth medium; this appears to prevent the accumulation of reserve materials

(Bonner and Machlis, 1957). The use of acetone powders of mycelium to reduce endogenous respiration may promote serious damage to the respiratory apparatus and therefore may provide no advantage over the study of intact cells (Bentley, 1953). An additional complication is the ability of certain fungi to assimilate rather significant proportions of various exogenous substrates (see Clifton, 1946).

While certain sugars commonly stimulate respiratory activity of some fungi, this is not always the case with intermediates of the TCA cycle. Although some success has been obtained with the latter (Leonard, 1949; Mickelson and Schuler, 1953; Moses, 1955; N. D. Davis, 1958; Litchfield and Ordal, 1958; Al-Doory, 1959; Ramachandran and Gottlieb, 1963), often incomplete substrate patterns are reported for these compounds (Levine and Novak, 1950; Hirsch, 1952; Hockenhull *et al.,* 1954; Krebs, 1954; Bacila *et al.,* 1955; Garrison, 1961). In addition, the respiratory rates observed with organic acids may be quite low in comparison to those with carbohydrate, and this has led to serious doubt concerning the quantitative significance of TCA cycle intermediates in normal respiration (Leonard, 1949; Levine and Novak, 1950; Al-Doory, 1959; Garrison, 1961; Newcomb and Jennison, 1962). The finding that these intermediates are, in fact, metabolized by cell-free extracts of fungi indicates that permeability barriers may play a significant role (Barron and Ghiretti, 1953; Clark and Wallace, 1958; Litchfield and Ordal, 1958). In certain cases, the permeability barriers of fungi can be overcome in an acid medium; this appears to favor the undissociated form of the organic acid (Barron *et al.,* 1950; Moses, 1955). However, sometimes TCA cycle intermediates fail to stimulate respiration even under these conditions, and those which are active may do so at rates far below those required to account for the respiratory capacity of the fungal cell.

A possible solution to this problem has come from the employment of esters of biologically important substrates; these apparently pass through cell membranes over a wide range of pH. Studies dealing with intact cells of *Penicillium chrysogenum* have indicated that while succinic acid failed to promote basal respiration, diethylsuccinate applied at pH 6 significantly increased oxygen consumption (Beevers *et al.,* 1952). However, one drawback to this method is the possibility that the organism may also respond to the alcohol moiety of the ester, and appropriate controls must be used. Another method which has proved somewhat effective in decreasing permeability barriers is alternate freezing and thawing (Krebs *et al.,* 1952; Moses, 1955). This procedure may also promote serious damage to the respiratory enzymes. A less drastic method of decreasing permeability barriers in fungal mycelium is drying over P_2O_5 in the cold, and this method has been used successfully with *Schizophyllum commune* (Wessels, 1959). More re-

cently, Kovac (1961) has demonstrated an apparent change in the permeability of bakers' yeast by employing deoxycholate. However, the possibility exists that enzymes may, in fact, have been liberated from the yeast cells by this drastic treatment. Consequently, these difficulties indicate that the failure of TCA cycle intermediates to promote fungal respiration cannot be taken as conclusive evidence against the presence of a functional cycle *in vivo*.

Similar problems often are encountered with the use of specific respiratory poisons in fungi. In particular, malonic acid often fails to arrest respiration of fungi (Levine and Novak, 1950; Shu *et al.*, 1954; Chattaway and Thompson, 1956; Clark and Wallace, 1958; Hilton and Smith, 1959; Garrison, 1961; Ramachandran and Gottlieb, 1963) yet proves to be an effective inhibitor of succinic dehydrogenase isolated in cell-free extracts of some of these organisms (Chattaway *et al.*, 1960; Clark and Wallace, 1958; Hilton and Smith, 1959). In contrast, strong malonate inhibition has been obtained with intact cells of *Rhodotorula gracilis* (Litchfield and Ordal, 1958). Some success has also been obtained with malonic acid as a respiratory inhibitor at low pH. Acetate oxidation by intact cells of a yeast was inhibited significantly by malonic acid applied at pH 4 and considerable amounts of succinic acid accumulated (Barron and Ghiretti, 1953). However, under approximately the same conditions (i.e., at acid pH), malonic acid was without effect on *Merulius niveus*, *Rhizopus nigricans* [*R. stolonifer*] (Barron and Ghiretti, 1953) and *Pullularia* [*Aureobasidium*] *pullulans* (Clark and Wallace, 1958). This apparent permeability barrier to malonic acid has been overcome in yeast (Krebs *et al.*, 1952) and in *Zygorhynchus moelleri* (Moses, 1955) by treating the cells with liquid nitrogen or dry ice. The use of diethylmalonate has also proved effective in respiratory studies of fungi. In particular, acetate oxidation by *Penicillium chrysogenum* was unaffected by malonic acid (0.1 *M*, final) at pH 6 while diethylmalonate was strongly inhibitory under these conditions (Beevers *et al.*, 1952). Similar precautions must be exercised here as with esters of biologically important substrates. An additional complication arises with malonic acid for it may also stimulate respiration as well as serve as a carbon source for the growth of fungi (Clark and Wallace, 1958; Novak, 1959a,b; Pedersen, 1963). These findings may relate to the fact that malonyl-CoA is a key intermediate in fatty acid biosynthesis.

In contrast to these difficulties, the employment of arsenite as a potential inhibitor of α-ketoacid oxidation by intact cells of fungi has met with a fair degree of success. This may require the use of a rather high level of arsenite (e.g., 0.01 *M*). Nevertheless, α-ketoacid accumulation is readily demonstrated with this inhibitor, and arsenite-sensitive respiratory systems have been reported in many fungi (Pickett and Clifton, 1943; Hockenhull

et al., 1951; Walker *et al.*, 1951; Shu *et al.*, 1954; Chattaway and Thompson, 1956; Goldschmidt *et al.*, 1956; Bonner and Machlis, 1957; Ramachandran and Gottlieb, 1963).

Monofluoroacetic acid has proved to be a very potent inhibitor of the TCA cycle in animal systems (Peters, 1952). The use of this poison with fungi has again led to difficulty. While significant inhibition of respiration was obtained with fluoroacetate in the case of *N. crassa* (Strauss, 1955) and yeast (Kalnitsky and Barron, 1947), Goldschmidt *et al.* (1956) noted that prior incubation with the poison for at least one hour was required with *P. chrysogenum*. Here also, it appears that acid pH increases the concentration of the undissociated form of the inhibitor and thereby favors the penetration of this compound (Black and Hutchens, 1948; Aldous and Rozee, 1956). Similar permeability barriers have been described in yeast with fluoride, iodoacetate, and 3:5-dinitro-*o*-cresol (Simon and Beevers, 1952). Taking these findings into consideration, one cannot, therefore, rely entirely on negative inhibitor data obtained with intact cells of fungi to substantiate the absence of a functional TCA cycle.

c. Isotope Incorporation. The application of isotopic tracer techniques to the study of the TCA cycle in fungi, and in particular, yeast, has also met with difficulty. Since these difficulties may be inherent in intact cells of fungi generally, and appear to have been resolved to a certain degree with yeast, our discussion will be concerned primarily with the latter. While early experiments performed with intact cells and extracts of yeast were suggestive of a functional TCA cycle *in vivo*, evidence obtained with labeled substrates, including glucose and acetate, was not always conclusive. Early studies by Weinhouse and Millington (1947) involved the oxidation of carboxyl-C^{13}-labeled magnesium- or barium-acetate by bakers' yeast. These investigators isolated the citric acid which accumulated under these conditions and found that the C^{13} content and distribution in citrate was in accord with a functional TCA cycle. However, not all the C_4 acids were formed from the cycle, and an independent mechanism was invoked for their formation from acetate. Later investigations by Ehrensvard *et al.* (1951) with *Torulopis* [*Candida*] *utilis* and Krebs *et al.* (1952) with yeast also suggested the need for another mechanism, in addition to the TCA cycle, to account for acetate oxidation in fungi. Moreover, it was apparent from these data and others (Wang *et al.*, 1952, 1953) that certain reactions of the TCA cycle in yeast were related to the provision of amino acids for biosynthesis. Thus, while there was little doubt as to the presence of a functional TCA cycle in yeast, the quantitative significance of the cycle in terminal respiration remained unclear. This led to the notion that the primary role of the TCA cycle in yeast is to supply intermediates for biosynthesis, rather than to provide energy to the cell (Krebs *et al.* 1952). In

retrospect, this appears reasonable, for only recently have cell-free extracts been obtained from yeast and other fungi which are capable of oxidative phosphorylation (Nossal *et al.*, 1956; Bonner and Machlis, 1957; Utter *et al.*, 1958; Iwasa, 1960; Vitols and Linnane, 1961; Linnane *et al.*, 1962).

A somewhat different approach was used by DeMoss and Swim (1957). These workers studied the labeling pattern of intracellular intermediates in large amounts of yeast after the addition of C^{14}-labeled acetate. In this work, isotope appeared in citrate, α-ketoglutarate, succinate, fumarate, and malate, and the isotope distribution found in citrate and succinate was consistent with the view that the TCA cycle is a major pathway of acetate oxidation by bakers' yeast. However, still unexplained was the origin of some oxalacetic acid, formed possibly by a C_2 unit (containing C^{14} from acetate) condensing with either two C_1 units or a single C_2 unit of endogenous origin. Therefore, while a great deal of evidence indicated a functional TCA cycle in yeast, no mechanism was documented for a second point of entry of acetate into the TCA cycle at this time. The elucidation of the latter came primarily from studies dealing with acetate metabolism in bacteria.

The success of experiments with [2-C^{14}] acetate incorporation by bacteria was due primarily to following the fate of radioactive compounds formed after very short periods of incubation (e.g., 3 seconds to 5 minutes) along lines established by Calvin (1951). In addition, prior growth of the microorganism on acetate as the sole carbon source led to the elucidation of an additional metabolic pathway related to the net synthesis of C_4-dicarboxylic acids. Such a pathway appeared in acetate-grown *Pseudomonas fluorescens* KB1 and *Corynebacterium* sp. (Kornberg, 1958). In these investigations, cells incubated with [2-C^{14}] acetate showed incorporation of isotope into intermediates consistent with an operative TCA cycle. However, initially, a great deal of the total isotope appeared in C_4 compounds. This indicated that label was, in fact, introduced into malate without first passing through citric acid and clearly pointed to a second portal of entry for acetate into the TCA cycle. Previous investigations had indicated that the combined action of isocitritase (R. A. Smith and Gunsalus, 1954) and malate synthetase (Wong and Ajl, 1955) could result in a net synthesis of C_4-dicarboxylic acids, and this metabolic scheme has since been designated as the "glyoxylate cycle" (Kornberg and Krebs, 1957). The latter provides a mechanism for a second point of entry of acetate into the TCA cycle as indicated in Fig. 3.

The studies described with bacteria have been extended to yeast, and a similar situation is encountered here (Barnett and Kornberg, 1960). In addition, isocitritase (Olson, 1959) and malate synthetase (Dixon *et al.*, 1960) have been partially purified from yeast, and the presence of these

enzymes is now documented in various fungi (Kornberg and Collins, 1958; Collins and Kornberg, 1960; Gottlieb and Ramachandran, 1960; Heberling *et al.*, 1960; McCurdy and Cantino, 1960; Frear and Johnson, 1961; Turian, 1961). All these data are consistent with the view that fungi can

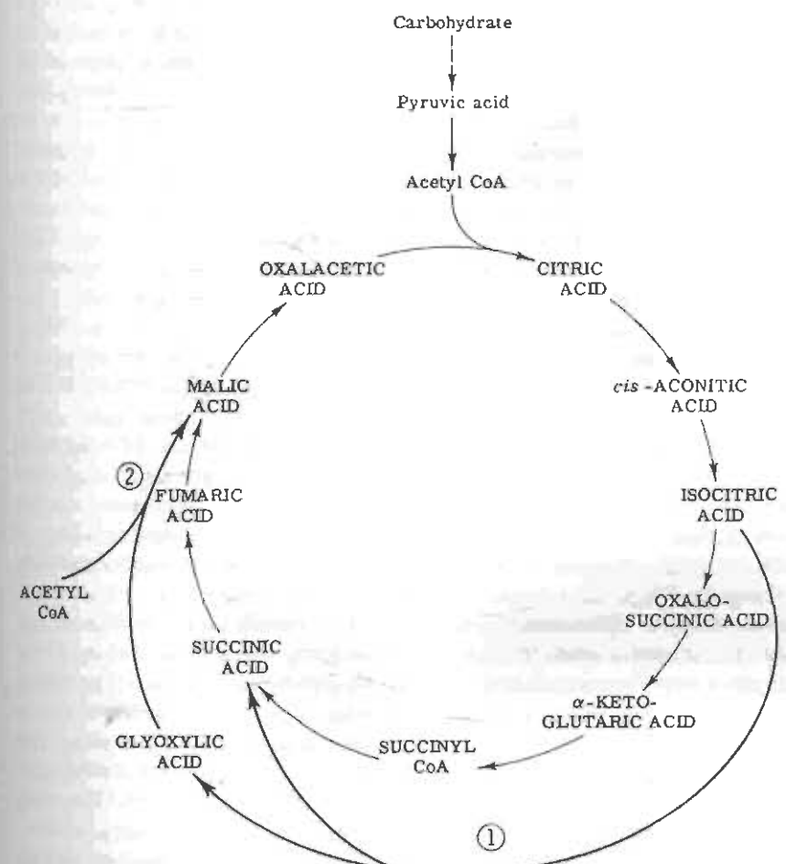

FIG. 3. Relationship of TCA cycle to glyoxylate pathway. Enzymes catalyzing numbered reactions are (*1*) isocitritase and (*2*) malate synthetase.

oxidize acetate largely via a functional TCA cycle, and furthermore, the glyoxylate pathway may supplement the TCA cycle during growth on acetate by providing a mechanism for the net synthesis of C_4-dicarboxylic acids. In addition, the glyoxylate pathway may provide an explanation (see Kornberg and Madsen, 1957) for the accumulation of fumaric acid by

Rhizopus stolonifer (Foster *et al.*, 1949) and citric acid by *Aspergillus niger* (K. F. Lewis and Weinhouse, 1951a; Carson *et al.*, 1951).

2. Cell-Free Extracts

a. Subcellular Localization of Specific Enzyme Activities. It is now well established that, with but two exceptions, the main intracellular site of the reactions of the TCA cycle in animal and plant tissues is the mitochondrion. The apparent exceptions to this generalization are isocitric dehydrogenase (Hogeboom and Schneider, 1950; Plaut and Plaut, 1952; Ernster and Navazio, 1956; Lowenstein, 1961; Tager, 1961) and malic dehydrogenase (see Kun, 1963). These enzymes may also occur in soluble form in the cytoplasm of various tissues. In addition, multiple forms of isocitric dehydrogenase have been reported in yeast (Kornberg and Pricer, 1951) and in *A. niger* (Ramakrishnan and Martin, 1955). Interestingly, the mitochondrion-bound enzyme appears to differ in its coenzyme specificity (i.e., utilizes NAD rather than NADP) and in other salient features (see Plaut, 1963). It is important to note that NADP-linked isocitric dehydrogenase activity has been reported in various fungi (Barron and Ghiretti, 1953). The functional role of the latter enzyme remains to be elucidated.

Studies dealing with the intracellular distribution of the TCA cycle in fungi might, therefore, have been expected to draw upon the extraction procedures important in the isolation of intact, functional mitochondria from higher forms. Unfortunately, this has not always been the case. In addition, the employment of cell-free extracts of fungi to substantiate the occurrence of a TCA cycle has likewise been confronted with serious methodological difficulties. Early cytochemical studies provided an indication of respiratory granules in yeast (Lindegren, 1949; Mudd *et al.*, 1951; Sarachek and Townsend, 1953; Mundkur, 1953; Hartman and Liu, 1954; Ephrussi and Slonimski, 1955; Yotsuyanagi, 1955; Bautz, 1955; Williams *et al.*, 1956), and this work has now been adequately supported by fine structure studies of mitochondria in a variety of fungi (see Moore and McAlear, 1963b; and pp. 336–337 and 399 of this volume). However, fractionation procedures of the type employed with animal tissues to concentrate specific enzyme activities (e.g., acetone fractionation) as well as the drastic breakage methods necessitated for yeast and the filamentous fungi virtually precluded any further analysis of the subcellular localization of the TCA cycle enzymes in these forms. The latter may, in part, explain why past investigators utilized only crude homogenates of fungi to study these reactions. While the study of homogenates of fungi obtained after low speed centrifugation (400–600 *g*, 5–10 minutes) certainly provided basic information concerning the qualitative aspects of the TCA cycle (Cantino and Hyatt, 1953a; McDonald *et al.*, 1960), competing reactions present

in crude extracts may seriously interfere with quantitative measurements of specific enzyme activities. For example, extracts of mycelium (Kaplan *et al.*, 1951) and, in particular, conidia (Zalokar and Cochrane, 1956) of *N. crassa* possess a potent diphosphopyridine nucleotidase whereas homogenates of *P. chrysogenum* contain significant levels of phosphatase (Casida and Knight, 1954). The latter readily hydrolyzed adenosine triphosphate, flavine adenine dinucleotide, NAD and NADP, thereby presenting a formidable problem to the assay of phosphorylated cofactor-linked enzymes of the TCA cycle. High-speed supernatant fractions (10,000–24,000 *g*, 10–45 minutes) have also been employed to study the TCA cycle in fungi and in these cases a considerable fraction of the respiratory particles may have been sedimented and discarded (Jensen *et al.*, 1957; Joshi and Ramakrishnan, 1959; Heberling *et al.*, 1960; Chandra and Shanmugasundarum, 1961; Ramananda Rao *et al.*, 1962).

Particulate matter which appeared to have lipoprotein had early been obtained from cell-free extracts of yeast (Nyman and Chargaff, 1949) and was characterized by certain enzyme activities usually associated with mitochondria isolated from animal and plant tissues (Brachet and Jeener, 1943; Chantrenne, 1943; Slonimski and Ephrussi, 1949; Novelli and Lipmann, 1950; Foulkes, 1951; L. Smith, 1954). However, only a few TCA cycle reactions were demonstrated *in vitro*, and the dehydrogenase systems were rarely capable of coupling to molecular oxygen in the absence of artificial electron carriers. The drastic breakage procedures utilized very likely led to denaturation of many of the enzymes of the TCA cycle and the respiratory chain. Additional confusion arose during studies dealing with the intracellular localization of certain TCA cycle enzymes in yeast. While aconitase, fumarase, and NAD-linked isocitric dehydrogenase activities are associated largely with particulate fractions isolated from animal tissues, these enzyme activities were reported in the supernatant fraction of cell-free extracts of yeast (Hirsch, 1952). Thus, even though certain enzymes of the TCA cycle were isolated from yeast, their subcellular distribution was not in accord with the data obtained with animal tissues.

These discrepancies have now been resolved to some extent and the success of later work may be attributed to the elaboration of milder breakage procedures as well as appropriate isolation media for the preparation of functional mitochondria from yeast and other fungi. With the aid of a highly rapid, mechanical disintegrator Nossal (1954a) prepared particulate fractions from yeast which oxidized all members of the TCA cycle with the consumption of molecular oxygen. Further studies along these same lines (Nossal, 1954b; Nossal *et al.*, 1957; Utter *et al.*, 1958) demonstrated that prolonged disintegration periods lead to the progressive loss of certain enzyme activities of the TCA cycle. Moreover, particulate-bound enzymes,

including aconitase and fumarase, actually appeared in the supernatant fraction after prolonged disintegration. Thus, there was further assurance that yeast cells do, in fact, contain native respiratory granules which had previously been designated as mitochondria purely by cytochemical criteria.

The elaboration of appropriate isolation media was also of considerable importance. This involved the choice of a suitable buffer, the inclusion of sucrose to preserve mitochondrial integrity, and the use of ethylenediamine tetraacetate (Slater and Cleland, 1952), presumably to remove harmful metals and to prevent swelling of the mitochondria (Hunter *et al.*, 1959) and the loss of bound NAD (Lester and Hatefi, 1958). Considerable success has also been achieved by the inclusion of sucrose, bovine serum albumin, and a variety of cofactors in the primary assay medium. Thus, there have been isolated from yeast highly active, respiring mitochondria which stain with Janus Green B and oxidize the principal members of the TCA cycle (Linnane and Still, 1955). Particulate matter containing various enzymes of the TCA cycle have likewise been isolated from *Allomyces macrogynus* (Bonner and Machlis, 1957), *Candida albicans* (Nozu *et al.*, 1958), *Fusarium lini* (Kikuchi and Barron, 1959), *Aspergillus oryzae* (Imamoto *et al.*, 1959), *Myrothecium verrucaria* (Hilton and Smith, 1959), *Schizophyllum commune* (Wessels, 1959; Niederpruem and Hackett, 1961), *Puccinia graminis* (White and Ledingham, 1961), *Fusarium oxysporum* (Maruyama and Alexander, 1962), *Neurospora crassa* (Haskins *et al.*, 1953), and other fungi (Dowler *et al.*, 1963).

Once breakage procedures, isolation media, and cofactor requirements were established, attempts were made to isolate functional mitochondria from fungi which were capable of oxidative phosphorylation with appropriate intermediates of the TCA cycle. Earlier reports of phosphorylation by respiratory granules of yeast (Nossal *et al.*, 1956; Utter *et al.*, 1958) have now been supported by the work of Hodges and Marx (1959) and Vitols and Linnane (1961). In addition, respectable P:O ratios have also been obtained with *A. macrogynus* (Bonner and Machlis, 1957) and *A. oryzae* (Iwasa, 1960). Moreover, electron transport particles similar to the type prepared from beef heart (Crane *et al.*, 1956) and bacteria (Bruemmer *et al.*, 1957) have been purified from yeast (Mackler *et al.*, 1962). It is therefore not unreasonable to assume that yeast and filamentous fungi possess respiratory granules which are the functional equivalents of mitochondria in animal and plant systems.

While most of this recent work agrees in essence with intracellular distribution studies of extracts prepared from higher forms, many serious problems remain with the fungi. The breakage procedures used often yield several metabolically active particulate fractions from cell-free extracts of yeast after differential centrifugation (Nossal, 1954b; Utter *et al.*, 1958;

Vitols and Linnane, 1961). There are also persistent reports of soluble enzyme fractions obtained after ultracentrifugation of crude extracts of fungi which still contain certain TCA cycle enzymes (White and Ledingham, 1961; Maruyama and Alexander, 1962; Dowler *et al.*, 1963). However, the significance of these data is questionable in view of the possibility of preparative artifacts and must be viewed with caution until they are confirmed using less drastic breakage procedures. Finally, the bewildering array of metabolically uncharacterized organelles (e.g., lomasomes, dictyosomes, etc.) recently reported in fungi (Moore and McAlear, 1961, 1963a) lends added complexity to this situation (cf. pp. 95–105 of this volume).

b. Purified Enzymes. The purification and crystallization of TCA cycle enzymes from fungi have lagged considerably behind animal studies. This may be due in part to the low recovery of enzyme activity after the harsh breakage procedures required. In addition, only a few TCA cycle enzymes have, in fact, been crystallized from animal sources, and these include the condensing enzyme (Ochoa, 1948; Ochoa and Weisz-Tabori, 1948) and fumarase (Massey, 1952). This is because most of the TCA enzymes are structurally bound (i.e., localized on the mitochondrion) and they must be solubilized before any extensive purification can be initiated. Moreover, once these enzymes are liberated from the mitochondria, stability problems in aqueous media become paramount. The present discussion will, therefore, be confined to those TCA cycle enzymes which have been partially purified from fungi and, wherever possible, comparisons will be made to their counterparts in higher forms.

Condensing enzyme. The formation of citrate by an aldol condensation involving acetyl coenzyme A and oxalacetate is mediated by the condensing enzyme (Eq. 1).

$$\text{Acetyl coenzyme A} + \text{oxalacetate} + H_2O \rightleftharpoons \text{citrate} + \text{coenzyme A} + H^+ \qquad (1)$$

The condensing enzyme appears to be the first enzyme of the TCA cycle obtained in crystalline form from animal tissue (Ochoa *et al.*, 1951), and there are no reports of an enzyme preparation of this degree of purity from fungi. A partial purification of condensing enzyme has been achieved by ammonium sulfate fractionation with cell-free extracts of yeast (Novelli and Lipmann, 1950) and *A. niger* (Ramakrishnan and Martin, 1955). In contrast to the requirement for magnesium usually exhibited by condensing enzyme isolated from other tissues, the partially purified enzyme prepared from *A. niger* does not require magnesium and, in fact, appears to be inhibited by it. The suspicion that citridesmolase was present as a contaminant and was responsible for this inhibition was disproved because the enzyme was still sensitive to magnesium after extensive purification

(Ramakrishnan, 1958). Thus, the condensing enzyme of *A. niger* may differ significantly from its mammalian counterpart in this regard. The presence of condensing enzyme in crude extracts of yeast (Ochoa *et al.*, 1951) and other fungi is now adequately documented. Considerably higher yields of crystalline condensing enzyme from pig heart have recently been obtained by ethanolic-KOH extraction (Srere and Kosicki, 1961) and perhaps this procedure may prove helpful with extracts of fungi.

Aconitase. The enzyme aconitase is concerned primarily with the interconversion of the three tricarboxylic acids (Eq. 2).

$$\text{Citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \text{isocitrate} \qquad (2)$$

Aconitase has been partially purified from animal tissues (see Anfinsen, 1955) and is activated by ferrous ions and reducing agents (Dickman and Cloutier, 1951; Morrison, 1954). Cofactor requirements of this type have likewise been demonstrated for aconitase preparations from *A. niger*, *Penicillium purpurogenum*, and *Saccharomyces cerevisiae* (Rahatekar and Raghavendra Rao, 1963). A great deal of attention has been paid to the possibility that the above reactions may be mediated by more than one enzyme (see Krebs and Lowenstein, 1960). While crystalline aconitase is not yet available from animal tissues, there seems to be some agreement that only one enzyme is involved. Aconitase has been partially purified from yeast by fractionation with acetone and ammonium sulfate (Racker, 1950). More definitive biochemical studies of aconitase in *A. niger* indicate that at least two enzymes are operative here (Ramakrishnan, 1954; Neilson, 1955), and these may be separated by conventional ammonium sulfate fractionation. One of the components is associated with a high molecular weight protein and is concerned primarily with the formation of citrate from *cis*-aconitate and is designated as aconitic hydrase (Neilson, 1956). The latter is also present in *P. chrysogenum* (see Neilson, 1962). Aconitic hydrase is not affected by ferrous ions and the reducing agents reported to act on aconitase. The other component is associated with a low molecular weight protein and produces both citrate and isocitrate from *cis*-aconitate and, therefore, resembles the aconitase of animal tissues. While both enzyme activities appear in mycelium grown on a complex medium, only aconitic hydrase is detected during growth on a defined medium containing manganese (3 μg%). Aconitase activity also appears to be lost in *A. niger* during growth in a citrate-accumulating molasses medium (Ramakrishnan *et al.*, 1955). The mechanisms concerned with the nutritional regulation of aconitase production in fungi remain to be elucidated.

Isocitric dehydrogenase (NADP specific). Reference has already been made to the occurrence of two separate enzymes which mediate the steps leading from isocitrate to α-ketoglutarate (see Section II, C, 2). These

dehydrogenases are distinguished by their cofactor specificities and other important features. The NADP-linked enzyme is described here, for it appears to be widely distributed in fungi. In addition, the latter activity occurs largely in the soluble, extramitochondrial fraction of the cell and may play an important role in the maintenance of reducing power (Lowenstein, 1961). The reaction sequence catalyzed by the NADP specific isocitric dehydrogenase is as follows:

$$\text{Isocitrate} + \text{NADP}^+ \rightleftharpoons \text{oxalosuccinate} + \text{NADPH} + \text{H}^+ \quad (3)$$

$$\text{Oxalosuccinate} + \text{H}^+ \overset{\text{Mn}^{++} \text{ or } \text{Mg}^{++}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + \text{CO}_2 \quad (4)$$

$$\textit{Sum:}\ \text{Isocitrate} + \text{NADP}^+ \overset{\text{Mn}^{++} \text{ or } \text{Mg}^{++}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \quad (5)$$

Attempts to separate the two reactions have not met with success. The NADP-specific isocitric dehydrogenase has been partially purified from various microorganisms (Kornberg and Pricer, 1951; Barban and Ajl, 1952; Ramakrishnan and Martin, 1955; Agosin and Weinback, 1956; Goldman, 1956). Recent purification procedures have indicated the importance of chelating agents in the isolation and assay media to stabilize the enzyme preparation (see Plaut, 1962). The NADP-specific isocitric dehydrogenase of yeast has been partially separated from the NAD-linked enzyme by ammonium sulfate fractionation (Kornberg and Pricer, 1951). Since the latter activity is usually more labile than the NADP-specific enzyme, further purification of the yeast enzyme was obtained by a brief heat treatment (60°C, 5 minutes), followed by ethanol fractionation. The yeast system resembles preparations from animal tissue in that it catalyzes the decarboxylation of oxalosuccinate, the reductive carboxylation of α-ketoglutarate, and the reduction of oxalosuccinate. Similarly, enzyme systems isolated from both sources show a requirement for divalent metals (e.g., Mn^{++} or Mg^{++}). A partial separation and purification of two isocitric dehydrogenases have also been achieved with extracts of *A. niger* (Ramakrishnan and Martin, 1955); the NADP-linked enzyme isolated from this filamentous fungus likewise showed a requirement for divalent metals.

Isocitric Dehydrogenase (NAD-specific). The NAD-specific isocitric dehydrogenase has been purified from various animal sources (Plaut and Sung, 1954) and has been partially separated from the NADP-linked enzyme in yeast (Kornberg and Pricer, 1951) and *A. niger* (Ramakrishnan and Martin, 1955). The reaction catalyzed by this enzyme is as follows:

$$\text{Isocitrate} + \text{NAD}^+ \rightarrow \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (6)$$

While the NADP-linked enzyme occurs largely in the cytoplasm, the NAD-specific isocitric dehydrogenase appears to be bound to the mitochondria.

There are several lines of evidence which suggest that two different enzymes are involved. Although the NAD-linked enzyme also requires Mg^{++} or Mn^{++}, it is more labile than the NADP-specific isocitric dehydrogenase. In addition, the former has not been shown to catalyze the reduction of oxalosuccinate nor the reductive carboxylation of α-ketoglutarate when prepared from either yeast or heart tissue (Kornberg and Pricer, 1951). Moreover, the NAD-linked enzyme isolated from yeast and *A. niger* requires an additional cofactor, adenosine 5'-phosphate, for maximum activity. While orthophosphate is reported to stimulate the NAD-linked enzyme from *A. niger* (Ramakrishnan and Martin, 1955), neither orthophosphate nor adenosine 5'-phosphate are required with the heart tissue enzyme (Plaut and Sung, 1954).

α-Ketoglutaric dehydrogenase system. The oxidative decarboxylation sequence which leads from α-ketoglutaric acid to succinic acid is catalyzed by a multienzyme system which requires Mg^{++}, diphosphothiamine, α-lipoic acid (6,8-thioctic acid), NAD, coenzyme A, and perhaps other yet unidentified cofactors. This complex is localized in mitochondria, and therefore serious difficulties have been encountered during the resolution of this system. The reaction sequence may be formulated (Sanadi *et al.*, 1956) as follows:

$$\alpha\text{-Ketoglutarate} + NAD^+ + HSCoA \rightarrow \text{succinyl-S CoA} + CO_2 + NADH + H^+ \quad (7)$$

$$\text{Succinyl-S CoA} + GDP + P \rightleftharpoons HSCoA + GTP + \text{succinate} \quad (8)$$

These expressions may be an oversimplification of the mechanisms involved, and this matter is discussed in detail by Sanadi (1963). The biological role of lipoic acid in the oxidative decarboxylation of α-keto acids has been substantiated from various lines of evidence including the arsenite sensitivity of these reactions (see Reed, 1960). Since the inhibitory effect of the latter is reversed by dithiols, but not by monothiols, the presence of an essential dithiol structure is indicated. In this connection, arsenite-sensitive respiratory systems have been encountered in several fungi (see Section II, C, 1). While a great deal of information is now available concerning the α-ketoglutaric dehydrogenase systems of animal tissue and bacteria, very little is known about the details of this complex system in fungi. A partial purification of α-ketoglutaric oxidase from *A. niger* has been achieved by acetic acid precipitation (Ramakrishnan, 1954). Recently, Holzer *et al.*, (1963) have obtained a soluble α-ketoglutaric oxidase system from mitochondria of bakers' yeast by acetone extraction. This preparation required NAD, coenzyme A, and diphosphothiamine, and was inhibited by arsenite.

Succinic dehydrogenase. The mitochondrial enzyme which catalyzes the

reversible oxidation of succinic acid to fumaric acid is succinic dehydrogenase; this reaction is described as follows:

$$\text{Succinate} \rightleftharpoons \text{fumarate} + 2H^+ + 2e \qquad (9)$$

In contrast to the other enzymes of the TCA cycle isolated from fungi, a great deal of information is available concerning succinic dehydrogenase. This is due largely to the contributions of Singer and co-workers, who have extensively purified succinic dehydrogenase from a variety of tissues as well as yeast. It is interesting to note that the procedures used successfully with animal tissues did not yield active succinic dehydrogenase preparations from yeast particles. However, particulate fractions from yeast have been solubilized by rapid desiccation with *n*-butanol, followed by extraction of the powder with tris (hydroxymethyl) aminomethane (Tris) buffer (pH 8.9) (Singer *et al.*, 1957). Subsequent fractionation with protamine sulfate, ammonium sulfate, and calcium phosphate gel adsorption led to preparations that were 65% pure by ultracentrifugal analysis. Considerably higher purifications have been obtained with animal tissues (Singer *et al.*, 1956). Nevertheless, the degree of purity obtained with the yeast enzyme permitted certain comparisons to be made with the mammalian system. Both soluble enzymes have a similar sedimentation velocity (i.e., 6.5 S for bovine heart versus 7.7 S for yeast), and an approximate molecular weight of 200,000. In addition, both proteins contain 4 atoms of non-heme iron per mole of flavine and the yeast flavine resembles the flavine peptides liberated from the bovine heart preparation. Moreover, both enzyme activities are dependent upon the presence of essential sulfhydryl groups and are inhibited in a competitive manner by malonate, oxalacetate, and pyrophosphate. The particulate yeast enzyme is distinguished from the mammalian protein by its sensitivity to acetone, a slightly higher pH optimum, and failure to be activated by preincubation with substrates or competitive inhibitors. An elaborate discussion of the comparative biochemistry of succinic dehydrogenase has appeared recently (Singer and Kearney, 1963).

Partial purification of succinic dehydrogenase from crude extracts of *Aspergillus niger* (Martin, 1954), *Neurospora crassa* (Shepherd, 1951), and *Myrothecium verrucaria* (Hilton and Smith, 1959) has been achieved by centrifugation. Although no further fractionations were reported, each of these systems appeared to be sensitive to malonate. Crude extracts of *Penicillium chrysogenum* have been fractionated with ammonium sulfate to concentrate succinic dehydrogenase activity (Godzeski and Stone, 1955). Although no data were provided for the recovery and degree of purity, succinic dehydrogenase was characterized by a pH optimum of pH 6.6,

formed fumarate as a reaction product, and was inhibited by malonate. The presence of either citrate or Versene appeared to stimulate succinate oxidation markedly. The lability of this material may have prevented further purification. In contrast to these systems, preliminary work by King *et al.* (1960) indicated that the succinic dehydrogenase of *Claviceps purpurea* is extremely stable. Taking advantage of this property, McDonald *et al.* (1963) have extensively purified the soluble succinic dehydrogenase from *C. purpurea*. Like its mammalian counterpart, the *C. purpurea* enzyme has a pH optimum at pH 7.7 and is inhibited in a competitive manner by fumarate, pyrophosphate, and malonate. Unlike the mammalian succinic dehydrogenase, which is apparently stimulated markedly by phosphate (Kearney, 1957; Kearney *et al.*, 1955), only a slight enhancement was noted with phosphate for the *Claviceps* enzyme. Finally, noncompetitive inhibition of the latter by the iron-chelating agent *o*-phenanthroline stands in contrast to data obtained with the yeast succinic dehydrogenase, which is inhibited competitively by this substance (Singer *et al.*, 1957).

Fumarase. The enzyme fumarase brings about the reversible hydration of fumaric acid to L-malic acid (Eq. 10).

$$\text{Fumarate} + H_2O \rightleftharpoons \text{L-malate} \tag{10}$$

The enzyme has been isolated and crystallized from pig heart muscle (Massey, 1952) and is highly specific, acting only on L-malate or fumarate. Fumarase is extremely sensitive to anions (Mann and Woolf, 1930) and is activated only by divalent and trivalent anions; monovalent anions (Cl^-, Br^-, I^-, CNS^-) may actually inhibit activity depending upon the pH. Purification procedures leading to crystalline preparations from mammalian systems have now been established (Frieden *et al.*, 1954; Massey, 1955). Many compounds inhibit fumarase in a competitive manner while thiocyanate and other substances are noncompetitive inhibitors of the enzyme (Massey, 1953). The mammalian enzyme is not affected by sulfhydryl inhibitors. Fumarase has been partially purified from *A. niger* by ammonium sulfate fractionation (Ramakrishnan, 1954). Favelukes and Stoppani (1958) have described the properties of fumarase purified from yeast by acetone extraction, calcium phosphate gel adsorption, and ammonium sulfate fractionation. In contrast to the fumarase of mammalian systems, yeast fumarase requires thiol groups for activity and appears sensitive to low levels of selective sulfhydryl reagents. However, in other respects, the enzyme isolated from yeast closely resembles the fumarase of mammalian systems. More recently, Hayman and Alberty (1961) have demonstrated two molecular forms of fumarase in *Candida utilis*. Although the two enzymes have not been crystallized, adequate separation has been achieved by column electrophoresis. The two fumarases differ with respect

to their electric charge, pH optima, K_m values, and acid-ionization constants and do not resemble the mammalian enzyme in any of these features. Although multiple forms of certain enzymes are now well documented, it is not clear whether both fumarases exist in the same yeast cell.

Malic dehydrogenase. Kun (1963) has drawn attention to the distinction between the two types of enzymatic dehydrogenation of L-malate (e.g., "simple" dehydrogenase versus "decarboxylating" dehydrogenase). In this treatise, our interest will concern only the "simple" malic dehydrogenase which catalyzes the following conversion:

$$\text{L-Malate} + \text{NAD}^+ \rightleftharpoons \text{oxalacetate} + \text{NADH} + \text{H}^+ \qquad (11)$$

Malic dehydrogenase has been purified from animal tissue by Straub (1942) and Wolfe and Neilands (1956). It is apparently unique among the pyridine nucleotide-linked dehydrogenases in having the lowest molecular weight. While early work indicated that only L-malate could serve as a substrate, later studies by Davies and Kun (1957) indicated that a broader range of substrates was oxidized and the enzyme actually appears to be an α-hydroxydicarboxylic acid dehydrogenase. This is true also of malic dehydrogenase isolated from pea epicotyls (Davies, 1961). Thorne (1960) has purified malic dehydrogenase approximately 100- to 200-fold from the supernatant fraction (10,000 *g*, 30 minutes) of cell-free extracts of yeast by conventional chemical fractionation and chromatography on Amberlite IRC-50 resin and diethylaminoethylcellulose. Malic dehydrogenase from yeast was compared with the respective enzymes from mitochondria of horse heart, pig heart, and rat liver as well as with the supernatant fraction (10,000 *g*, 30 minutes) of rat liver by several kinetic criteria. The mitochondrial enzymes resembled each other whereas they differed significantly from the soluble malic dehydrogenases of rat liver and yeast.

Early studies dealing with the intracellular distribution of malic dehydrogenase in animal preparations suggested its localization in both the mitochondria and supernatant fraction of cell extracts (cf. Kun, 1963) which has led to a great deal of interest in the physical properties of these enzymes. Moreover, starch gel electrophoresis of pig heart mitochondrial malic dehydrogenase showed at least six separate bands (Thorne *et al.*, 1963). Different molecular forms of enzyme proteins which have the same enzymatic specificity have been termed "isozymes" (Markert and Møller, 1959). The fungi appear to be no exception in this regard. Tsao (1962) has subjected homogenates of *N. crassa* to electrophoresis and has provided evidence for at least four separate malic dehydrogenases. These findings were not influenced by the particular strain of *N. crassa* employed nor by the culture history. In addition, Staples and Stahmann (1963) have demonstrated three isozymes of malic dehydrogenase in extracts of uredo-

TABLE II

OCCURRENCE OF TCA CYCLE REACTIONS IN FUNGI

Organism	References
1. Phycomycetes	
Allomyces arbuscula	Leonard (1949)
Allomyces macrogynus	Bonner and Machlis (1957)
Blastocladiella emersonii	Cantino and Hyatt (1953a)
Phycomyces nitens	Dowler *et al.* (1963)
Phytophthora infestans	Oksenova (1961)
Pythium debaryanum	Dowler *et al.* (1963)
Rhizophlyctis rosea	Cantino and Hyatt (1953b)
Rhizopus stolonifer	Barron and Ghiretti (1953)
Rhizopus oryzae	Al-Doory (1959)
Zygorhynchus moelleri	Moses (1955, 1957)
2. Ascomycetes	
Ashbya gossypii	Mickelson and Schuler (1953)
Claviceps purpurea	King *et al.* (1960); McDonald *et al.* (1960, 1963)
Gibberella zeae	Dowler *et al.* (1963)
Glomerella cingulata	Dowler *et al.* (1963)
Hansenula anomala	N. D. Davis (1958); Barnett and Kornberg (1960)
Monilinia [Sclerotina] fructicola	Dowler *et al.* (1963)
Neurospora crassa	Shepherd (1951); Haskins *et al.* (1953); Strauss (1955). Chandra and Shanmugasundarum (1961); Tsao (1962)
Neurospora tetrasperma	Cheng (1954); Sussman *et al.* (1956)
Saccharomyces cerevisiae[a]	Slonimski and Ephrussi (1949); Racker (1950); Foulkes (1951); Hirsch (1952); Krebs *et al.* (1952); Nossal (1954b); Linnane and Still (1955)
Saccharomyces drosophilarum	Barnett and Kornberg (1960)
Saccharomyces marxianus	Barnett and Kornberg (1960)
3. Basidiomycetes	
Merulius niveus	Barron and Ghiretti (1953)
Merulius tremellosus	Barron and Ghiretti (1953)
Polyporus palustris	Newcomb and Jennison (1962)
Puccinia graminis	Staples (1957); White and Ledingham (1961)
Puccinia recondita	Staples (1957)
Schizophyllum commune	Wessels (1959); Niederpruem and Hackett (1961); Dowler *et al.* (1963)
Uromyces appendiculatus	Staples and Weinstein (1959); Staples and Stahmann (1963)
Ustilago maydis	Gottlieb and Caltrider (1963)
4. Deuteromycetes	
Aspergillus fumigatus	Dowler *et al.* (1963)
Aspergillus niger[a]	K. F. Lewis and Weinhouse (1951a); Martin (1954); Neilson (1956)
Aspergillus oryzae	Imamoto *et al.* (1959)
Blastomyces dermatitidis	Levine and Novak (1950)
Caldariomyces fumago	Ramachandran and Gottlieb (1963)

TABLE II (*Continued*)

OCCURRENCE OF TCA CYCLE REACTIONS IN FUNGI

Organism	References
Candida albicans	Nozu *et al.* (1958); Ramananda Rao *et al.* (1962)
Candida krusei	Barnett and Kornberg (1960)
Candida utilis	Ehrensvard *et al.* (1951); Jackson and Johnson (1961); Hayman and Alberty (1961); Linnane *et al.* (1962)
Fusarium lini	Kikuchi and Barron (1959)
Fusarium oxysporum	Maruyama and Alexander (1962)
Fusarium solani	Cochrane *et al.* (1963)
Histoplasma capsulatum	Cozad *et al.* (1958); Garrison (1961)
Microsporum canis	Chattaway and Thompson (1956); Chattaway *et al.* (1960)
Myrothecium verrucaria	Hilton and Smith (1959)
Penicillium chrysogenum	Godzeski and Stone (1955); Goldschmidt *et al.* (1956)
Penicillium digitatum	Noble *et al.* (1958); Reed and Wang (1959)
Penicillium purpurogenum	Rahatekar and Raghavendra Rao (1963)
Pullularia [*Aureobasidium*] *pullulans*	Clark and Wallace (1958)
Rhizoctonia sp.	Dowler *et al.* (1963)
Rhodotorula gracilis	Litchfield and Ordal (1958)
Stemphylium solani	Dowler *et al.* (1963)
Trichophyton mentagrophytes	Jensen *et al.* (1957)
Verticillium alboatrum	Brandt and Wang (1960)

[a] Complete references in text.

spores of *Uromyces phaseoli* [*U. appendiculatus*]. The degree of resolution obtained with electrophoretic techniques may provide a powerful tool for studies dealing with the role of the TCA cycle enzymes during growth and development of fungi.

III. OCCURRENCE OF THE TCA CYCLE IN FUNGI

The presence of a functional TCA cycle in representatives of all the major groups of true fungi has now been adequately documented. Data substantiating the occurrence of TCA cycle reactions in fungi are summarized in Table II. In those instances where data obtained with intact cells or extracts are inconclusive, other explanations should first be sought before elaborate regulatory mechanisms are evoked. In addition, the possible existence of the glyoxylate pathway must be considered in the evaluation of isotope data obtained from studies of intact cells.

Although so few fungi have been examined for metabolic activities related to the TCA cycle, Cantino (1955) has drawn upon the data at hand to provide an evaluation of the phylogenetic significance of the TCA cycle in the Phycomycetes. The primitive uniflagellate forms appear to be characterized by a homofermentative metabolism (i.e., they form mostly lactic

acid from glucose) and contain only a weakly functional TCA cycle. Thus, the energy requirements for growth and biosynthesis would be satisfied largely by glycolysis. While these notions are applicable to *Blastocladia* and *Blastocladiella,* some difficulty is encountered with *Allomyces.* Although the former can grow at very low oxygen tensions, the latter is a strict aerobe. In addition, strong evidence has been provided for a functional TCA cycle in *A. macrogynus* (Bonner and Machlis, 1957). On the other hand, among the biflagellate forms, neither the Saprolegniales nor the Leptomitales form organic acids during growth and are strict aerobes. Thus, these groups may be characterized by an oxidative rather than a fermentative type of metabolism (see Cantino, 1955).

IV. ROLE OF THE TCA CYCLE IN FUNGI

A. Amino Acid Biosynthesis

Much of the early work regarding the possibility of a functional TCA cycle in fungi involved isotopic tracer studies concerned with the fate of labeled acetate or glucose when administered to growing cultures. Ehrensvard *et al.* (1951) studied the synthesis of amino acids by *Candida utilis* when grown in a medium containing labeled acetate and provided evidence that the carbon skeleton of aspartic acid could arise from this substrate. Similar results were obtained with bakers' yeast by Wang *et al.* (1952) and additional physiological studies with yeast led Krebs *et al.* (1952) to conclude that the primary role of the TCA cycle in fungi is to supply carbon intermediates for biosynthesis. In addition, glutamic acid also appeared to be formed predominantly from the TCA cycle in yeast (Wang *et al.,* 1953). These concepts were further supported by the report of transaminase activities in crude extracts of *Neurospora crassa* (Fincham, 1951) and were later extended considerably by studies involving the technique of isotopic competition. Using this procedure, it was not only confirmed that the origin of aspartic and glutamic acids was related to an operative TCA cycle, but also these "parent" compounds could further serve as precursors for the synthesis of "families" of amino acids (Roberts *et al.,* 1953; Abelson *et al.,* 1953; McQuillen and Roberts, 1954). Although these ideas were first established with *Escherichia coli,* they were subsequently supported by studies of *Candida utilis, Neurospora crassa* (Abelson *et al.,* 1953; Anderson-Kotto *et al.,* 1954; Abelson and Vogel, 1955) and *Zygorhynchus moelleri* (Moses, 1957). While microbial systems show overall similarities with respect to the origin of a number of amino acids from the TCA cycle, they differ in several important details concerning their individual biosynthetic pathways. There is also good reason

to believe that elaborate control mechanisms (e.g., repression, feedback inhibition) are involved in the regulation of these biosynthetic pathways (cf. Stadtman, 1963). Thus, there is substantial evidence which indicates that one function of the TCA cycle in actively growing fungi is the provision of amino acids.

B. Carbon Dioxide Assimilation

The formation of "parent" amino acids by various enzymatic reactions involving intermediates of the TCA cycle during active growth and protein biosynthesis may lead to a serious drainage of carbon moieties from the cycle. This would necessitate ancillary reactions to provide carbon fragments vital to the continuous function of the TCA cycle. It has long been realized that carbon dioxide is necessary for growth of bacteria, yeasts, and filamentous fungi (Rockwell and Highberger, 1927). Later work indicated that assimilation of carbon dioxide was not necessarily restricted to photosynthetic organisms, but occurred also in heterotrophic systems including yeast (Ruben and Kamen, 1940), *Rhizopus stolonifer* (Foster *et al.*, 1941), *Candida utilis* (Ehrensvard, 1948), *Hansenula anomala, Blastocladia pringsheimii, Allomyces arbuscula* (Lynch and Calvin, 1952) and *Aspergillus niger* (Foster *et al.*, 1941; K. F. Lewis and Weinhouse, 1951b; Mosbach *et al.*, 1952). In many of these studies the fixed carbon dioxide was found in aspartic and glutamic acids and also in certain intermediates of the TCA cycle. However, in comparing the utilization of labeled acetate and pyruvate by yeast it was realized that the radioactivity of yeast protein was significantly higher with the latter (Labbe *et al.*, 1952).

Further work along these lines (Wang *et al.*, 1952, 1953) showed that the intramolecular distribution of C^{14} in aspartic and glutamic acids which arose during the administration of labeled pyruvate to yeast cells might be accounted for by a $C_3 + C_1$ condensation, possibly by fixation of metabolic CO_2. Subsequent investigations with yeast growing in the presence of labeled CO_2 and unlabeled pyruvate allowed the determination of the order in which CO_2 entered the amino acids and from these studies it was learned that glutamic and aspartic acids became radioactive very early. Therefore, these acids must have equilibrated rapidly with the primary products of $C^{14}O_2$ fixation (J. W. Davis *et al.*, 1956).

More definitive information as to the mechanism of CO_2 fixation in fungi has been provided by Stoppani and co-workers. Preliminary studies with yeast indicated that oxalacetate may be the initial product of CO_2 fixation (Stoppani *et al.*, 1957), possibly via a β-carboxylation of phosphoenol pyruvate (Stoppani *et al.*, 1958). The latter has also been con-

sidered as a possible mechanism for CO_2 fixation by uredospores of *Puccinia recondita* and *Uromyces appendiculatus* (Staples and Weinstein, 1959). This idea has been supported by the isolation and 200-fold purification of a phosphopyruvate carboxylase from yeast (Cannata and Stoppani, 1959, 1963a,b). The enzyme carboxylates phosphoenol pyruvate in the presence of CO_2, adenosine diphosphate, and Mn^{++}, and leads to the formation of oxalacetate and adenosine triphosphate. A similar enzyme system has been isolated from the mycelium of *Aspergillus niger* (Woronick and Johnson, 1960). These workers also noted the existence of an additional system for the synthesis of C_4-dicarboxylic acids in *A. niger* which required pyruvate, and adenosine triphosphate. Further studies of the pyruvate carboxylase of *A. niger* indicated that this enzyme system converts pyruvate, adenosine triphosphate, and CO_2 into oxalacetate, adenosine diphosphate, and inorganic phosphate (Bloom and Johnson, 1962). Thus, the continual drainage of carbon moieties from the TCA cycle for biosynthesis may be balanced by CO_2 fixation reactions of the types described in yeast and filamentous fungi.

C. Energy Production

While the studies cited above clearly indicate the utility of the TCA cycle in providing carbon skeletons for growth and biosynthesis in fungi, there is now reasonable justification for assuming that the dehydrogenase systems of the cycle couple to the respiratory chain and thereby release energy during hydrogen (electron) transfer to molecular oxygen. In this regard, cytochrome respiratory pigments of the type reported in yeast have been observed in mycelia of 45 species of fungi (Boulter and Derbyshire, 1957, and see pp. 302–313 of this volume). Moreover, particulate fractions capable of oxidative phosphorylation have likewise been isolated from a variety of fungi (see Section II, C, 2, a).

REFERENCES

Abelson, P. H., and H. J. Vogel. (1955). *J. Biol. Chem.* **213**:355–364.

Abelson, P. H., E. Bolton, R. Britten, D. B. Cowie, and R. B. Roberts. (1953). *Proc. Natl. Acad. Sci. U.S.* **39**:1020–1026.

Agosin, M., and E. D. Weinback. (1956). *Biochim. Biophys. Acta* **21**:117–126.

Al-Doory, Y. (1959). *Mycologia* **51**:851–854.

Aldous, J. G., and K. R. Rozee. (1956). *Biochem. J.* **62**:605–610.

Anderson-Kotto, I., G. Ehrensvard, G. Hogstrom, L. Reio, and E. Saluste. (1954). *J. Biol. Chem.* **210**:455–463.

Anfinsen, C. B. (1955). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, pp. 695–698. Academic Press, New York.

., D. Amaral, and H. Medina. (1955). *Arquiv. Biol. Tecnol., Inst. Biol. is Tecnol.* 10:37–46.
, and S. Ajl. (1952). *J. Bacteriol.* 64:443–453.
A., and H. L. Kornberg. (1960). *J. Gen. Microbiol.* 23:65–82.
S. G., and F. Ghiretti. (1953). *Biochim. Biophys. Acta* 12:239–249.
S. G., M. I. Ardao, and M. Hearon. (1950). *J. Gen. Physiol.* 34:211–224.
(1955). *Naturwissenschaften* 42:49–50.
L., E. P. Goldschmidt, and H. Koffler. (1952). *Arch. Biochem. Biophys.* 238.
L. L. (1953). *J. Gen Microbiol.* 8:365–377.
and J. O. Hutchens. (1948). *Arch. Biochem.* 17:211–223.
J., and M. J. Johnson. (1962). *J. Biol. Chem.* 237:2718–2720.
A., and L. Machlis. (1957). *Plant Physiol.* 32:291–301.
., and E. Derbyshire. (1957). *Exptl. Botany* 8:313–318.
, and R. Jeener. (1943). *Enzymologia* 11:196–212.
. H., and C. H. Wang. (1960). *Am. J. Botany* 47:50–53.
, J. H., P. W. Wilson, J. L. Glenn, and F. L. Crane. (1957). *J. Bacteriol.* 116.
. (1950–1951). *Harvey Lectures* 46:218–251.
J. J. B., and A. O. M. Stoppani. (1959). *Biochim. Biophys. Acta* 32:284–
. J. B., and A. O. M. Stoppani. (1963a). *J. Biol. Chem.* 238:1196–1207.
. J. B., and A. O M. Stoppani (1963b). *J. Biol. Chem.* 238:1208–1212.
. C. (1955). *Quart. Rev. Biol.* 30:138–149.
. C., and M. T. Hyatt. (1953a). *J. Bacteriol.* 66:712–720.
. C., and M. T. Hyatt. (1953b). *Am. J. Botany* 40:688–694.
. F., and E. H. Mosbach, and E. F. Phares. (1951). *J. Bacteriol.* 62:235–
E, Jr., and S. G. Knight. (1954). *J. Bacteriol.* 67:658–661.
T., and E. R. B. Shanmugasundarum. (1961). Enzymologia 23:347–352.
ıe, H. (1943). *Enzymologia* 11:213–221.
, F. W., and C. C. Thompson. (1956). *Biochem. J.* 63:648–656.
, F. W., C. C. Thompson, and A. J. E. Barlow. (1960). *J. Gen. Microbiol.* 656.
C. (1954). *Plant Physiol.* 29:458–467.
S., and R. H. Wallace. (1958). *Can. J. Microbiol.* 4:125–139.
. E. (1946). *Advan. Enzymol.* 6:269–308.
V. W. (1958). *In* "Physiology of Fungi," pp. 223–224. Wiley, New York.
V. W., J. C. Cochrane, J. M. Vogel, and R. S. Coles, Jr. (1963). *J. Bac-* 6:312–319.
F., and H. L. Kornberg. (1960). *Biochem. J.* 77:430–438.
. C., H. W. Larsh, and W. R. Romig. (1958). *Proc. Oklahoma Acad. Sci.* 06.
L., J. L. Glenn, and D. E. Green. (1956). *Biochim. Biophys. Acta* 22:475–
. D. (1961). *Biochem. J.* 80:93–99.
. D., and E. Kun. (1957). *Biochem. J.* 66:307–316.
W., V. H. Cheldelin, B. E. Christensen, and C. H. Wang. (1956). *Biochim.* s. *Acta* 21:101–105.
D. (1958). *Mycologia* 50:770–776.

DeMoss, J. A., and H. E. Swim. (1957). *J. Bacteriol.* **74**:445–451.
Dickman, S. R., and A. A. Cloutier. (1951). *J. Biol. Chem.* **188**:379–388.
Dixon, G. H., H. L. Kornberg, and P. Lund. (1960). *Biochim. Biophys. Acta* **41**:217–233.
Dowler, W. M., P. D. Shaw, and D. Gottlieb. (1963). *J. Bacteriol.* **86**:9–17.
Ehrensvard, G. (1948). *Cold Spring Harbor Symp. Quant. Biol.* **13**:81–87.
Ehrensvard, G., L. Reio, E. Saluste, and R. Stjernholm. (1951). *J. Biol. Chem.* **189**:93–108.
Ephrussi, B., and P. P. Slonimski. (1955). *Nature* **176**:1207–1208.
Ernster, L., and F. Navazio. (1956). *Exptl. Cell. Res.* **11**:438–486.
Favelukes, G., and A. O. M. Stoppani. (1958). *Biochim. Biophys. Acta* **28**:654–655.
Fincham, J. R. S. (1951). *Nature* **168**:957–958.
Foster, J. W., S. F. Carson, S. Ruben, and M. D. Kamen. (1941). *Proc. Natl. Acad. Sci. U.S.* **27**:590–596.
Foster, J. W., S. F. Carson, D. S. Anthony, J. B. Davis, W. E. Jefferson, and M. V. Long. (1949). *Proc. Natl. Acad. Sci. U.S.* **35**:663–672.
Foulkes, E. C. (1951). *Biochem. J.* **48**:378–383.
Frear, D. S., and M. A. Johnson. (1961). *Biochim. Biophys. Acta* **47**:419–421.
Frieden, C., R. M. Bock, and R. A. Alberty. (1954). *J. Am. Chem. Soc.* **76**:2482–2484.
Garrison, R. G. (1961). *J. Infect. Diseases* **108**:120–124.
Gibson, D. M. (1963). *In* "Progress in the Chemistry of Fats and other Lipids" (R. T. Holman, W. O. Lundberg, and T. Malkin, eds.), Vol. 6, pp. 117–136. Pergamon Press, New York.
Godzeski, C., and R. W. Stone. (1955). *Arch. Biochem. Biophys.* **59**:132–144.
Goldman, D. S. (1956). *J. Bacteriol.* **71**:732–736.
Goldschmidt, E. P., I. Yall, and H. Koffler. (1956). *J. Bacteriol.* **72**:436–446.
Gottlieb, D., and P. G. Caltrider. (1963). *Nature* **197**:916–917.
Gottlieb, D., and S. Ramachandran. (1960). *Mycologia* **52**:599–607.
Hartman, P. E., and C. Liu. (1954). *J. Bacteriol.* **67**:77–85.
Haskins, F. A., A. Tissières, H. K. Mitchell, and M. B. Mitchell. (1953). *J. Biol. Chem.* **200**:819–826.
Hayman, S., and R. A. Alberty. (1961). *Ann. N.Y. Acad. Sci.* **94**:812–824.
Heberling, R. L., J. J. Berky, and R. W. Stone. (1960). *Arch. Biochem. Biophys.* **86**:102–109.
Hilton, J. L., and F. G. Smith. (1959). *Iowa State Coll. J. Sci.* **33**:279–292.
Hirsch, H. M. (1952). *Biochim. Biophys. Acta* **9**:674–686.
Hockenhull, D. J. D., G. D. Wilkin, and F. G. Winder. (1951). *Nature* **168**:1043.
Hockenhull, D. J. D., M. Herbert, A. D. Walker, G. D. Wilkin, and F. G. Winder. (1954). *Biochem. J.* **56**:73–82.
Hodges, J. M., and W. Marx. (1959). *Arch. Biochem. Biophys.* **83**:563–564.
Hogeboom, G. H., and W. C. Schneider. (1950). *J. Biol. Chem.* **186**:417–427.
Holzer, H., G. Hierholzer, and I. Witt. (1963). *Biochem. Z.* **337**:115–119.
Hunter, F. E. Jr., J. L. Levy, J. Fink, B. Schutz, F. Guerra, and A. Hurwitz. (1959). *J. Biol. Chem.* **234**:2176–2186.
Imamoto, F., K. Iwasa, and K. Okunuki. (1959). *J. Biochem. (Tokyo)* **46**:141–150.
Iwasa, K. (1960). *J. Biochem. (Tokyo)* **47**:445–453.
Jackson, W. T., and M. J. Johnson. (1961). *J. Bacteriol.* **81**:182–188.
Jensen, E. M., H. Altschuller, and R. C. Bard. (1957). *J. Bacteriol.* **74**:656–660.
Joshi, A. P., and C. V. Ramakrishnan. (1959). *Enzymologia* **21**:43–51.

Kalnitsky, G., and E. S. G. Barron. (1947). *J. Biol. Chem.* **170**:83–95.
Kaplan, N. O., S. P. Colowick, and A. Nason. (1951). *J. Biol. Chem.* **191**:473–483.
Kearney, E. B. (1957). *J. Biol. Chem.* **229**:363–375.
Kearney, E. B., T. P. Singer, and N. Zastrow. (1955). *Arch. Biochem. Biophys.* **55**:579–581.
Kikuchi, G., and E. S. G. Barron. (1959). *Arch. Biochem. Biophys.* **84**:96–105.
King, T. E., C. A. Ryan, V. H. Cheldelin, and J. K. McDonald. (1960). *Biochim. Biophys. Acta* **45**:398–400.
Kornberg, H. L. (1958). *Biochem. J.* **68**:535–541.
Kornberg, H. L., and J. F. Collins. (1958). *Biochem. J.* **68**:3P–4P.
Kornberg, H. L., and H. A. Krebs. (1957). *Nature* **179**:988–991.
Kornberg, H. L., and N. B. Madsen. (1957). *Biochim. Biophys. Acta* **24**:651–653.
Kornberg, A., and W. E. Pricer, Jr. (1951). *J. Biol. Chem.* **189**:123–136.
Kovac, L. (1961). *Nature* **191**:698–699.
Krebs, H. A. (1937). *Lancet* **II**:736–738.
Krebs, H. A. (1943). *Advan. Enzymol.* **3**:191–252.
Krebs, H. A. (1954). *In* "Chemical Pathways of Metabolism" (D. M. Greenberg, ed.), Vol. 1, p. 135. Academic Press, New York.
Krebs, H. A., and W. A. Johnson. (1937). *Enzymologia* **4**:148–156.
Krebs, H. A., and J. M. Lowenstein. (1960). *In* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. 1, 129–203. Academic Press, New York.
Krebs, H. A., S. Gurin, and L. V. Eggleston. (1952). *Biochem. J.* **51**: 614–628.
Kun, E. (1963). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 7, pp. 149–160. Academic Press, New York.
Labbe, R. F., R. C. Thomas, V. H. Cheldelin, B. E. Christensen, and C. H. Wang. (1952). *J. Biol. Chem.* **197**:655–661.
Leonard, W. R. (1949). *J. Cellular Comp. Physiol.* **34**:293–309.
Lester, R. L., and Y. Hatefi. (1958). *Biochim. Biophys. Acta* **29**:103–112.
Levine, S., and M. Novak. (1950). *J. Bacteriol.* **60**:333–340.
Lewis, K. F., and S. Weinhouse. (1951a). *J. Am. Chem. Soc.* **73**:2500–2503.
Lewis, K. F., and S. Weinhouse. (1951b). *J. Am. Chem. Soc.* **73**:2906–2909.
Lewis, R. W. (1948). *Am. J. Botany* **35**:292–295.
Lindegren, C. C. (1949). *In* "The Yeast Cell, its Genetics and Cytology," Chapter 6, pp. 10–18. Educational Publ., St. Louis, Missouri.
Linnane, A. W., and J. L. Still. (1955). *Arch. Biochem. Biophys.* **59**:383–392.
Linnane, A. W., E. Vitols, and P. G. Nowland. (1962). *J. Cell Biol.* **13**:345–350.
Lipmann, F. (1953). *Bacteriol. Rev.* **17**:1–16.
Litchfield, J. H., and Z. J. Ordal. (1958). *Can. J. Microbiol.* **4**:205–213.
Lowenstein, J. M. (1961). *J. Biol. Chem.* **236**:1217–1219.
Lynch, V. H., and M. Calvin. (1952). *J. Bacteriol.* **63**:525–531.
McCurdy, H. D. Jr., and E. C. Cantino. (1960). *Plant Physiol.* **35**:463–476.
McDonald, J. K., V. H. Cheldelin, and T. E. King. (1960). *J. Bacteriol.* **80**:61–71.
McDonald, J. K., J. A. Anderson, V. H. Cheldelin, and T. E. King. (1963). *Biochim. Biophys. Acta* **73**:533–549.
McQuillen, K., and R. B. Roberts. (1954). *J. Biol. Chem.* **207**:81–95.
Mackler, B., P. J. Collipp, H. M. Duncan, N. A. Rao, and F. M. Huennekens. (1962). *J. Biol. Chem.* **237**:2968–2974.
Mann, P. J. G., and B. Woolf. (1930). *Biochem. J.* **24**:427–434.
Markert, C. L., and F. Møller. (1959). *Proc. Natl. Acad. Sci. U.S.* **45**:753–763.
Martin, S. M. (1954). *Can. J. Microbiol.* **1**:6–11.

Maruyama, Y., and M. Alexander. (1962). *J. Bacteriol.* 84:307–312.
Massey, V. (1952). *Biochem. J.* 51:490–494.
Massey, V. (1953). *Biochem. J.* 55:172–177.
Massey, V. (1955). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 1, pp. 729–735. Academic Press, New York.
Mickelson, M. N. (1950). *J. Bacteriol.* 59:659–666.
Mickelson, M. N., and M. N. Schuler. (1953). *J. Bacteriol.* 65:297–304.
Moore, R. T., and J. H. McAlear. (1961). *Mycologia* 53:194–200.
Moore, R. T., and J. H. McAlear. (1963a). *J. Cell Biol.* 16:131–141.
Moore, R. T., and J. H. McAlear. (1963b). *J. Ultrastruct. Res.* 8:144–153.
Morrison, J. F. (1954). *Biochem. J.* 58:685–692.
Mosbach, E. H., E. F. Phares, and S. F. Carson. (1952). *Arch. Biochem. Biophys.* 35:435–442.
Moses, V. (1955). *J. Gen. Microbiol.* 13:235–251.
Moses, V. (1957). *J. Gen. Microbiol.* 16:534–549.
Mudd, S., A. F. Brodie, L. C. Winterscheid, P. E. Hartman, E. H. Beutner, and R. A. McLean. (1951). *J. Bacteriol.* 62:729–739.
Mundkur, B. D. (1953). *Nature* 171:793–794.
Neilson, N. E. (1955). *Biochim. Biophys. Acta* 17:139–140.
Neilson, N. E. (1956). *J. Bacteriol.* 71:356–361.
Neilson, N. E. (1962). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 5, pp. 614–616. Academic Press, New York.
Newcomb, H. R., and M. W. Jennison. (1962). *Can. J. Microbiol.* 8:145–156.
Niederpruem, D. J., and D. P. Hackett. (1961). *Plant Physiol.* 36:79–84.
Noble, E. P., D. R. Reed, and C. H. Wang. (1958). *Can. J. Microbiol.* 4:469–476.
Nossal, P. M. (1954a). *Biochim. Biophys. Acta* 14:154–155.
Nossal, P. M. (1954b). *Biochem. J.* 57:62–69.
Nossal, P. M., D. B. Keech, and D. J. Morton. (1956). *Biochim. Biophys. Acta* 22:412–420.
Nossal, P. M., I. A. Hansen, and J. N. Ladd. (1957). *Biochim. Biophys. Acta* 23:103–115.
Novak, E. K. (1959a). *Acta Microbiol. Acad. Sci. Hung.* 6:1–4.
Novak, E. K. (1959b). *Acta Microbiol. Acad. Sci. Hung.* 6:117–123.
Novelli, G. D., and F. Lipmann. (1950). *J. Biol. Chem.* 182:213–228.
Nozu, K., S. Takagi, T. Kamiki, and M. Kashiwara. (1958). *Biken's J.* 1:35–44.
Nyman, M. A., and E. Chargaff. (1949). *J. Biol. Chem.* 180:741–746.
Ochoa, S. (1948). *J. Biol. Chem.* 174:133–157.
Ochoa, S., and E. Weisz-Tabori. (1948). *J. Biol. Chem.* 174:123–132.
Ochoa, S., J. R. Stern, and M. C. Schneider. (1951). *J. Biol. Chem.* 193:691–702.
Oksenova, V. A. (1961). *Novenyved. Tud. Tanacskozas Kozlemen., Budapest, 1960* 1:23–29.
Olson, J. A. (1959). *J. Biol. Chem.* 234:5–10.
Pedersen, T. A. (1963). *Physiol. Plantarum* 16:167–176.
Peters, R. A. (1952). *Proc. Roy. Soc.* B139:143–170.
Pickett, M. J., and C. E. Clifton. (1943). *J. Cellular Comp. Physiol.* 22:146–165.
Plaut, G. W. E. (1962). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 5, pp. 645–651. Academic Press, New York.
Plaut, G. W. E. (1963). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 7, pp. 105–126. Academic Press, New York.
Plaut, G. W. E., and K. A. Plaut. (1952). *J. Biol. Chem.* 199:141–151.

Plaut, G. W. F., and S. Sung. (1954). *J. Biol. Chem.* **207**:305–314.
Racker, E. (1950). *Biochim. Biophys. Acta* **4**:211–214.
Rahatekar, H. I., and M. R. Raghavendra Rao. (1963). *Enzymologia* **25**:292–296.
Raistrick, H., and A. B. Clark (1919). *Biochem. J.* **13**:329–344.
Ramachandran, S., and D. Gottlieb. (1963). *Biochim. Biophys. Acta* **69**:74–84.
Ramakrishnan, C. V. (1954). *Enzymologia* **17**:169–174.
Ramakrishnan, C. V. (1958). *Nature* **182**:1601–1602.
Ramakrishnan, C. V., and S. M. Martin. (1954). *Can. J. Biochem. Physiol.* **32**:434–439.
Ramakrishnan, C. V., and S. M. Martin. (1955). *Arch. Biochem. Biophys.* **55**:403–407.
Ramakrishnan, C. V., R. Steel, and C. P. Lentz. (1955). *Arch. Biochem. Biophys.* **55**:270–273.
Ramananda Rao, G., M. Sirsi, and T. Ramakrishnan. (1962). *J. Bacteriol.* **84**:778–783.
Reed, D. J., and C. H. Wang. (1959). *Can. J. Microbiol.* **5**:59–66.
Reed, L. J. (1960). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 3, pp. 195–223. Academic Press, New York.
Roberts, R. B., D. B. Cowie, R. Britten, E. T. Bolton, and P. H. Abelson. (1953). *Proc. Natl. Acad. Sci. U.S.* **39**:1013–1019.
Rockwell, G. E., and J. H. Highberger. (1927). *J. Infect. Diseases* **40**:438–446.
Ruben, S., and M. D. Kamen. (1940). *Proc. Natl. Acad. Sci. U.S.* **26**:418–422.
Ryan, F. J., E. L. Tatum, and A. C. Giese. (1944). *J. Cellular Comp. Physiol.* **23**:83–94.
Sanadi, D. R. (1963). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 7, pp. 307–344. Academic Press, New York.
Sanadi, D. R., D. M. Gibson, P. Ayengar, and M. Jacob. (1956). *J. Biol. Chem.* **218**:505–520.
Sarachek, A., and G. F. Townsend. (1953). *Science* **117**:31–33.
Shepherd, C. J. (1951). *Biochem. J.* **48**:483–486.
Shu, P., K. G. Tanner, and G. A. Ledingham. (1954). *Can. J. Botany* **32**:16–23.
Simon, E. W., and H. Beevers. (1952). *New Phytologist* **51**:163–190.
Singer, T. P., and E. B. Kearney. (1963). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 7, pp. 383–445. Academic Press, New York.
Singer, T. P., E. B. Kearney, and P. Bernath. (1956). *J. Biol. Chem.* **223**:599–613.
Singer, T. P., V. Massey, and E. B. Kearney. (1957). *Arch. Biochem. Biophys.* **69**:405–421.
Slater, E. C., and K. W. Cleland. (1952). *Nature* **170**:118–119.
Slonimski, P. P., and B. Ephrussi. (1949). *Ann. Inst. Pasteur* **77**:47–63.
Smith, L. (1954). *Arch. Biochem. Biophys.* **50**:285–298.
Smith, R. A., and I. C. Gunsalus. (1954). *J. Am. Chem. Soc.* **76**:5002–5003.
Srere, P. A., and G. W. Kosicki. (1961). *J. Biol. Chem.* **236**:2557–2559.
Stadtman, E. R. (1963). *Bacteriol. Rev.* **27**:170–181.
Staples, R. C. (1957). *Contrib. Boyce Thompson Inst.* **19**:19–31.
Staples, R. C., and M. A. Stahmann. (1963). *Science* **140**:1320–1321.
Staples, R. C., and L. H. Weinstein. (1959). *Contrib. Boyce Thompson Inst.* **20**:71–82.
Stoppani, A. O. M., S. L. S. de Favelukes, L. Conches, and F. L. Sacerdote. (1957). *Biochim. Biophys. Acta* **26**:443–445.
Stoppani, A. O. M., L. Conches, S. L. S. de Favelukes, and F. L. Sacerdote. (1958). *Biochem. J.* **70**:438–455.

Straub, F. B. (1942). *Z. Physiol. Chem.* **275**:63–72.
Strauss, B. S. (1955). *Arch. Biochem. Biophys.* **55**:77–94.
Sussman, A. S., J. R. Distler, and J. S. Krakow. (1956). *Plant Physiol.* **31**:126–135.
Tager, J. M. (1961). *Biochem. J.* **79**:22P.
Thorne, C. J. R. (1960). *Biochem. J.* **76**:4P.
Thorne, C. J. R., L. D. Grossman, and N. O. Kaplan. (1963). *Biochim. Biophys. Acta* **73**:193–203.
Tsao, M. U. (1962). *Science* **136**:42–43.
Turian, G. (1961). *Compt. Rend.* **252**:1374–1376.
Utter, M. F. (1961). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 5, pp. 319–340. Academic Press, New York.
Utter, M. F., D. B. Keech, and P. M. Nossal. (1958). *Biochem. J.* **68**:431–440.
Vitols, E., and A. W. Linnane. (1961). *J. Biophys. Biochem. Cytol.* **9**:701–710.
Walker, T. K., A. N. Hall, and J. W. Hopton. (1951). *Nature* **168**:1042–1043.
Wang, C. H., R. C. Thomas, V. H. Cheldelin, and B. E. Christensen. (1952). *J. Biol. Chem.* **197**:663–667.
Wang, C. H., B. E. Christensen, and V. H. Cheldelin. (1953). *J. Biol. Chem.* **201**:683–688.
Weinhouse, S., and R. H. Millington. (1947). *J. Am. Chem. Soc.* **69**:3089–3093.
Wessels, J. G. H. (1959). *Acta Botan. Neerl.* **8**:497–505.
White, G. A., and G. A. Ledingham. (1961). *Can. J. Botany* **39**:1131–1148.
Williams, M. A., C. C. Lindegren, A. Yuasa, B. Ephrussi, P. P. Slonimski, and Y. Yotsuyanagi. (1956). *Nature* **177**:1041–1042.
Wolfe, R. G., and J. B. Neilands. (1956). *J. Biol. Chem.* **221**:61–69.
Wong, D. T. O., and S. J. Ajl. (1955). *Nature* **176**:970–971.
Woronick, C. L., and M. J. Johnson. (1960). *J. Biol. Chem.* **235**:9–15.
Yotsuyanagi, Y. (1955). *Nature* **176**:1208–1209.
Zalokar, M., and V. W. Cochrane. (1956). *Am. J. Botany* **43**:107–110.

CHAPTER 12

Carbohydrate Metabolism

3. Terminal Oxidation and Electron Transport

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The literature on this subject, as on fungus biochemistry in general, is very unbalanced as to the emphasis the various groups of fungi have received. An overwhelming amount of work has been done on the yeasts, and particularly on a single species, *Saccharomyces cerevisiae,* our well-known bakers' and brewers' yeast. Some of the other lower ascomycetes, such as the aspergilli and penicillia, have also received attention because of their industrial importance, and *Neurospora,* because of the geneticists' interest. The rest of the fungi are still largely unexplored except for a very few.

The attitude reviewers have assumed in view of this lopsided situation has, therefore, tended to two extremes: either they have reviewed the literature on all fungi except for the yeasts, having said, in effect, that yeast belongs in the domain of animal biochemistry; or they have concentrated on yeast and ignored the rest. I have tried in this chapter to strike a balance between these two tendencies, since knowledge of the work on yeast obviously will be important in further investigations of other fungi, and since work on a diversity of fungus material is necessary for elucidating many

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interesting problems (such as flagellar motion, protoplasmic streaming, morphogenetic phenomena) that cannot be studied in yeast.

A further complication arises from the necessity to keep abreast of the rapid advances made on electron transport and phosphorylating mechanisms in animal and bacterial systems. To restrict our attention to work done on fungi would result in a narrow-minded and sterile approach. But obviously only the barest outlines can be given here of the entire field, to serve as a framework within which the fungus studies can be placed in perspective.

Regarding enzyme terminology, the recommendations in the Report of the Commission on Enzymes of the International Union of Biochemistry (1961) are followed whenever possible. The systematic names of enzymes are usually included in brackets. For reduced coenzymes the new names NADH and NADPH are used instead of the old ones DPNH and TPNH. Both the coenzymes together are designated by NAD(P)H. Since the choice of spelling between the alternative forms haem vs. heme, and haemo- vs. hemo- was left open by the Commission, I shall use the latter forms in conformity with most of the American literature.

I. THE CYTOCHROMES (INTRODUCTION)

Cytochromes may be defined (Commission on Enzymes, 1961) as "haemoproteins whose principal biological function is electron and/or hydrogen transport by virtue of a reversible valency change of their haem iron. A haemoprotein is a protein, the prosthetic group of which is a tetrapyrollic chelate of iron."

Figure 1 shows the chemical structure of protoporphyrin 9, the compound that is assumed to be the precursor of most hemoproteins and also

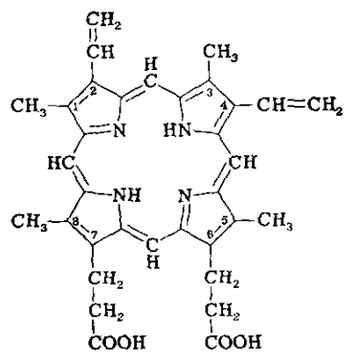


FIG. 1. Protoporphyrin 9.

of the chlorophylls (magnesium porphyrin chelates). Different hemoproteins may have different side chains on their iron-porphyrin groups, and these may be attached to the proteins in different ways. With regard to these properties, cytochromes can be classified into four types: A, B, C, and D. The respective side chains are shown in Table I, following the

TABLE I

CLASSIFICATION OF THE CYTOCHROMES[a]

Type	Prosthetic group	Side chains (positions of carbon atoms on porphyrin ring) 2	4	8
A	Formyl-porphyrin iron	—CHOH—CH_2—R_1	—CH=C(R_2)(R_3)	—C(H)=O
B	Protoporphyrin iron	—CH=CH_2	—CH=CH_2	—CH_3
C	Substituted mesoporphyrin iron with covalent porphyrin-protein linkages	—CH(—S—R)—CH_3	—CH(—S—R)—CH_3	—CH_3
D	Dihydroporphyrin iron	1 vinyl (or alkylvinyl) and 1 α-hydroxyalkyl side chain		—CH_3

[a] Cf. Commission on Enzymes, 1961; Lemberg *et al.*, 1961.

numbering of the carbon atoms around the porphyrin ring shown in Fig. 1. Individual cytochromes are designated by lower case letters with or without numerical subscripts, as cytochrome a, a_3, b, b_2, c, c_1 (*D*-type cytochromes have been found only in bacteria).

Of the six coordination positions of the iron atom in heme, four are filled by the pyrrolic nitrogens, while the other two may be occupied by ligands which are parts of a protein, or by other molecules (H_2O, O_2, CO, pyridine, etc.). In cytochromes, except for those that can react directly with oxygen, both of the extra coordination positions are thought to be occupied by the protein, the heme thus lying in a fold of the protein.

Originally the cytochromes were identified entirely on the basis of their absorption spectra, but some of them have been isolated and purified by the usual methods of protein separation; their prosthetic groups, and even the apoenzymes, were analyzed chemically. The reversible $Fe^{++} \rightleftarrows Fe^{+++}$ valency change by which cytochromes are distinguished from all other hemoproteins (see Section VI) results in two distinct absorption spectra, one for the reduced and the other for the oxidized state of the iron. The

reduced state has sharper absorption bands and is therefore used most for identification. The absorption spectra of these compounds in the visible and ultraviolet regions consist of four main band structures (each of which may be made up of several individual bands), the α, β, γ, and δ bands given in decreasing order of their wavelengths.

Reviews on the cytochromes include those by Chance and Williams (1956), Granick and Mauzerall (1961), Green (1959), Green and Fleischer (1962), Mahler (1961), Mason (1957), Okunuki (1961 Okunuki *et al.* (1958), Paul (1951, 1960), Slater (1958a,b), Smi (1954d, 1961), Stotz *et al.* (1956), Warburg (1949), as well as most of the symposium volume edited by Falk, Lemberg, and Morton (1961) and the important monograph by Lemberg and Legge (1949).

In addition to the "absolute" absorption spectra of the reduced and oxidized cytochromes obtained separately, "difference" absorption spectra have also been studied; these plot the difference in optical densities between the reduced and oxidized pigments at each wavelength (Chance, 1952; Smith, 1954a,b,c). One such difference spectrum is shown in Fig. 2 for aerobically grown cells of bakers' yeast (for examples of absolute spectra, cf. Figs. 5 and 6). Only those pigments appear in the anaerobic-

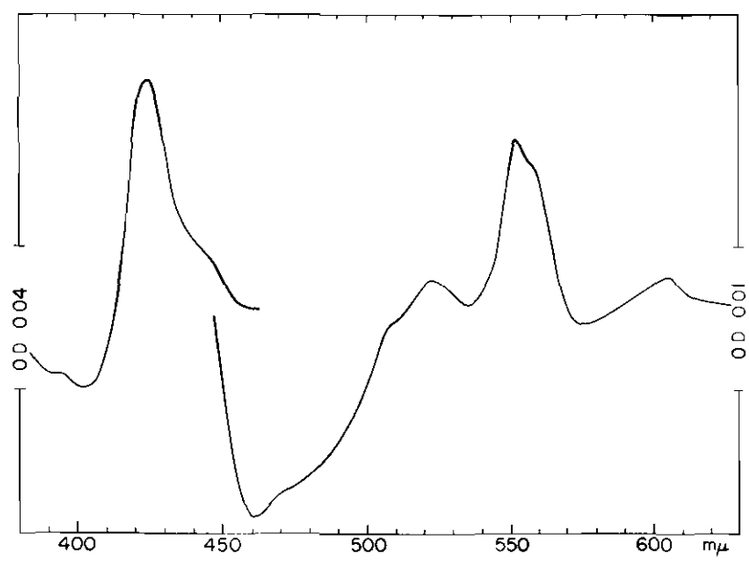


FIG. 2. Difference spectrum of aerobically grown bakers' yeast (strain LK2G12). Reference cuvette was aerated, the other cuvette anaerobic. Optical density scale is different on right- and left-hand side of figure, as indicated on margins. From Lindenmayer and Smith (1964).

minus-aerobic difference spectrum which become oxidized (i.e., steady-state oxidized) in the presence of oxygen and reduced in its absence. The peak at 605 and the fused peaks at 550–560 mμ are due to the α bands of cytochromes a (with some contribution by a_3), b, and c; while that at 525 mμ is due to the β bands of a, b, and c. The large peak at 423 mμ is attributable to the γ bands of b and c, and the shoulder around 445 mμ to the γ band of cytochrome a_3 (with some contribution by a). On the following pages we will discuss each of these components in more detail. A large trough can also be seen in Fig. 2 between 450 and 500 mμ, which can be attributed to the flavoprotein components of the respiratory system (cf. Section VIII). Flavine compounds having higher absorption intensity in the oxidized than in the reduced state, their absorption appears as a trough in this case.

It has been shown (cf. Keilin and Hartree, 1949; Estabrook, 1961) that the spectral bands of cytochromes become narrower and more intense if the biological material in which they occur is suspended in a glycerol solution, cooled to the temperature of liquid air, allowed to warm up for a short time, during which period devitrification takes place, and the spectrum is observed at the temperature of liquid air or nitrogen. The absolute absorption spectrum of intact commercial bakers' yeast cells in the presence of a reducing agent, dithionite ($Na_2S_2O_4$), was obtained in this way and is shown in Fig. 3. The α bands of cytochromes b and c can be seen here, without fusion and somewhat displaced to shorter wavelength, at 559.5 and 547 mμ; and between them a peak at 554 mμ corresponding to cytochrome c_1, which was completely masked in the room-temperature spectrum shown above. The β bands are similarly sharpened in the liquid air spectrum. According to Boulter and Derbyshire (1957) the absolute absorption bands of bakers' yeast reduced with dithionite at room temperature lie at 604, 563.5, 551, and 522 mμ; at the temperature of liquid N_2 they are at 601, 561, 552, 549, 520 mμ.

Spectral studies on the cytochrome system of bakers' yeast have been numerous ever since Fischer and co-workers (1924) demonstrated the presence in this organism of porphyrins and pyridine hemochromogens (ferrohemochromes), and Keilin (1925) observed the reduced cytochrome bands in the absence of oxygen, and their disappearance on aeration. Spectroscopes were first used to detect these bands in intact cells or tissues, as well as in extracts or purified preparations (cf. Hartree, 1955); spectrophotographic methods were also employed; and finally spectrophotometric methods (photo tubes, photomultipliers) are mostly used today.

Boulter and Derbyshire (1957) observed the visible absorption spectra of 45 different species of fungi, listed in Table II, both at room temperature and at −195 C in the presence of $Na_2S_2O_4$, using a Zeiss hand spectro-

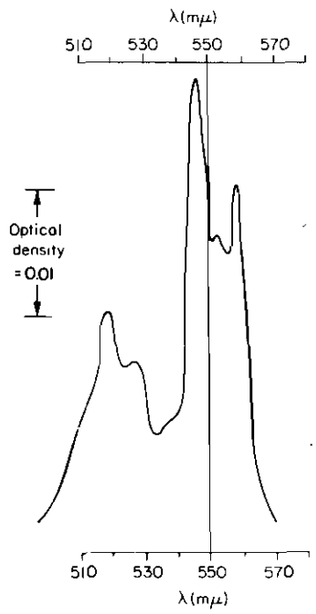


FIG. 3. Absolute absorption spectrum of commercial bakers' yeast at the temperature of liquid air. From Lindenmayer and Estabrook (1958).

scope and a Hartridge reversion spectroscope; they found the spectra to be similar to that of yeast (except for some doubts about cytochrome c_1).

In addition, *Aspergillus oryzae* was investigated by several Japanese workers; it will be further discussed under isolated mitochondria (Section XIII). *Penicillium notatum*, as well as *Neurospora crassa*, were shown to contain a complete and typical cytochrome system (Keilin and Tissières, 1953). The cytochromes of various respiration-deficient mutants of bakers' yeast and *N. crassa* are discussed in Section VII. Typical cytochrome spectra were also found in the asporogenous yeast *Mycoderma vini* (Chauvet, 1943), in the slime mold *Physarum polycephalum* (Ohta, 1954), in the imperfect *Fusarium lini* (Kikuchi and Barron, 1959), and in the smut fungus *Ustilago zeae* [*U. maydis*] (Grimm and Allen, 1954), which is noted for its high production of cytochrome c. The same absorption bands have been observed in *Schizophyllum commune* (Niederpruem and Hackett, 1961).

Generally speaking, the cytochrome system of the fungi is remarkably similar to that of mammalian and avian cells, and unlike the systems found

TABLE II

FUNGI SHOWING AN ABSORPTION SPECTRUM SIMILAR TO THAT OF A REDUCED YEAST SUSPENSION[a]

Phycomycetes	Basidiomycetes
Absidia glauca	*Collybia velutipes*
Absidia cylindrospora	*Coniophora cerebella*
Absidia orchidis	[*Cerebella puteana*]
Achlya radiosa	*Cyathus striatus*
Allomyces javanicus	*Fomes annosus*
Cunninghamella echinulata	*Hypholoma fasciculare*
Mucor hiemalis	*Marasmius androsaceus*
Mucor ramannianus	*Marasmius graminum*
Phycomyces nitens	*Marasmius peronatus*
Pythium ultimum	*Polyporus betulinus*
Rhizopus stolonifer	*Polystictus versicolor*
Saprolegnia sp.	*Sphaerobolus stellatus*
Syncephalastrum spinosa	*Tricholoma nudum*
Thamnidium elegans	*Trametes rubescens*
Zygorhynchus moelleri	
Zygorhynchus vuilleminii	**Deuteromycetes**
Ascomycetes	*Aspergillus nidulans*
	Aspergillus niger
Gelasinospora tetrasperma	*Aspergillus versicolor*
Neurospora crassa	*Botrytis allii*
Sordaria fimicola	*Cladosporium fulvum*
	Cytosporina sp.
Mycelia sterila	*Fusarium culmorum*
	Fusarium oxysporum f. *lini*
Rhizoctonia [*Corticium*] *solani*	*Isaria farinosa*
	Penicillium spinulosum
	Trichoderma viride
	Verticillium sp.

[a] Reproduced by permission of the Oxford Univ. Press (Clarendon), London and New York, from Boulter and Derbyshire (1957).

in bacteria and in green plants. Comparative aspects of electron transport mechanisms have been discussed by Conn (1960) and Dolin (1961).

II. CYTOCHROMES a AND a_3

Originally the enzyme that reacts with oxygen and binds CO and cyanide was called "oxygen-transferring enzyme," and distinguished from the non-autoxydizable cytochromes (Warburg and Negelein, 1934): "Das sauerstoffübertragendes Ferment ist die autoxydable Häminverbindung die sich

durch ihr Verhalten zu Sauerstoff, zu Kohlenoxyd und zu Blausäure, sowie durch die Lage ihrer Absorptionsbanden von dem Cytochrom unterscheidet." [2] It is cytochrome a_3 to which we assign these properties today (Keilin and Hartree, 1939). Several investigators prefer to regard cytochromes a + a_3 as a single entity, however, calling the complex "cytochrome a" (Wainio and Cooperstein, 1956; Okunuki *et al.*, 1958; Green, 1959). By its enzymatic action this complex is also called cytochrome oxidase or cytochrome c oxidase (cytochrome c:O_2 oxidoreductase).

The only kind of prosthetic group in this complex appears to be heme a, a formyl-porphyrin iron compound. Copper has also been regularly found to be part of this enzyme (cf. Lemberg, 1961; Paul, 1960).

The main reason behind the proposal of Keilin and Hartree (1939), that there are in cytochrome oxidase two A-type cytochromes was the different behavior of the α and γ bands at 605 and 445 mμ on addition of CO, cyanide, and azide. While the intensity of the α band is diminished by only about 25% on adding CO (a weak band appears at the same time at 590 mμ), the absorption intensity of the γ band decreases by more than half in the presence of CO (with the concomitant appearance of a large band at 430 mμ). The existence of two pigments was therefore postulated in this complex: cytochrome a, which does not combine with CO, or the other inhibitors, and has an abnormally high α:γ band extinction ratio (this ratio is about 1:10 for the other cytochromes); and cytochrome a_3, which combines with CO, cyanide, and azide, as well as with O_2, and has a normal α:γ ratio. Steady-state data with cholate-treated preparations (Smith, 1955; Yonetani, 1960a,b) provided further evidence for this hypothesis, in that the peak at 445 mμ was always found proportionately more oxidized than the one at 605 mμ. On the other hand, nobody has succeeded in separating a cytochrome oxidase preparation into two active components with the above properties.

We may quote Lemberg (1961) by the way of a summary of the present understanding of this problem: "It would appear that cytochrome *a* is a haemoprotein with haem *a* bound to imidazole nitrogen and inserted in a crevice of the protein . . . . Cytochrome a_3 would appear to be dissimilar to haem *a* compounds with nitrogenous ligands and to contain haem *a* in linkage to hydrophilic lipid inserted between the haem *a* and the protein. Further studies are, however, required to confirm this hypothesis."

The role of the copper atoms in this enzyme is far from being understood. Copper is definitely not present as a porphyrin-chelate, and the question of its reversible oxidation and reduction has not been settled yet

[2] "The oxygen-transferring enzyme is the autoxidizable hemin compound which is distinguishable from cytochrome by its behavior toward O_2, CO, and HCN, as well as by the position of its absorption bands."

(Ehrenberg and Yonetani, 1961; Beinert *et al.*, 1962). That the presence of copper is necessary for the growth of yeast with normal respiratory activity, as shown by Elvehjem (1931), McHargue and Calfee (1931), and Yoshikawa (1937), may partly be due to copper requirements in porphyrin synthesis. The suppression of pigment formation and sporulation in copper-deficient phycomycetous and ascomycetous molds (cf. Cochrane, 1958) is, on the other hand, probably due to a decrease in copper-containing oxidases (tyrosinase, laccase).

The cytochrome a:a_3 concentration ratio has been estimated from extinction coefficient measurements to be between 1 and 2, both in yeast and in heart muscle, the ratio probably not being stoichiometric (Lemberg, 1961). A ratio of 3 was once favored (Ball and Cooper, 1952) for theoretical reasons, i.e., in order to provide an explanation for the 4-electron transfer to O_2 without intermediates, but the value of 3 is not thought necessary any more (George and Griffith, 1959).

The Michaelis constant for cytochrome a_3 with O_2 in yeast was found to be between 0.6 and 0.8×10^{-6} *M*, and the forward velocity constant of the second-order reaction between the enzyme and O_2 was about 1×10^8 (liters per mole) per second, making it one of the most rapid enzyme reactions known (Ludwig and Kuby, 1955).

Claude Bernard in 1857 discovered that cyanide inhibits cell respiration; and the CO- and cyanide-binding property of the "oxygen-transferring enzyme" led to its discovery by Warburg in 1924. Carbon monoxide is not a very effective inhibitor. A CO:O_2 concentration ratio of 10 inhibits about 50% of yeast respiration whereas in the case of hemoglobin a ratio of 0.005 is enough to produce half-displacement of oxygen. The half-value for cyanide inhibition of yeast respiration is 4.5×10^{-6} *M*, compared to that for fermentation which is 1.0×10^{-2} *M*.

The photodissociation of the CO compound of yeast cytochrome a_3 was used to determine its "photochemical action spectrum" by Warburg and Negelein (1929) as well as by Melnick (1941), by Castor and Chance (1955), and by Yonetani and Kidder (1963). The respiratory rate of yeast cells was measured and plotted in the presence of CO when the cells were illuminated by approximately monochromatic light of various wavelengths. The α, β, and γ bands of the a_3-CO compound are at 590–591, 548–549, and 429–430 $m\mu$ in the third study, and the extinction coefficients of the α and γ bands have the normal ratio to each other. The absorption spectrum of the a_3-CO compound of yeast was also directly observed (Keilin and Hartree, 1939; Ball *et al.*, 1951; Chance, 1953a) and was found to be identical to the photochemical action spectrum. So was the photodissociation difference spectrum of the γ band obtained by Chance (1953b).

Cyanide combines with cytochrome a_3 in both oxidized and reduced states. The oxidized a_3-cyanide compound cannot be reduced by the other cytochromes. This is why cyanide is not a competitive inhibitor with respect to O_2, as it was observed by Warburg (1949). The absorption properties of the cyanide compound of oxidized cytochrome a_3 are very similar to that of the oxidized pigment itself; the γ band is shifted from 418 to 424 mμ and somewhat reduced. When cyanide combines with reduced cytochrome a_3, its bands are shifted from 605 to 595–599 mμ, and from 445 to 439 mμ (Yonetani, 1960a; Lemberg, 1961) (see Fig. 4).

It might be mentioned here that respiration-deficient mutants arise with high frequency in yeast in the presence of cyanide (Pett, 1936; Stier and Castor, 1941). The same is not true for yeast grown anaerobically, as shown by Harris (1956), in contrast to Lindegren and Hino (1957).

Cyanide inhibition of O_2 uptake in concentrations not exceeding 10^{-4} *M* was for a long time considered as good evidence for the presence of cytochrome c oxidase in fungi. This implication seems to be still valid today, although the wealth of enzymological assays available to us makes it desirable to obtain more precise and detailed data on any organism under study. The converse of the above statement, namely that lack of cyanide sensitivity implies the lack of cytochrome c oxidase, has already been effectively refuted by the findings of Darby and Goddard (1950) and Kidder (1961) on *Myrothecium verrucaria*. This cellulose-decomposing imperfect fungus has a respiratory system which, as that in *Arum* spadix, is not inhibited by cyanide, yet appears to have a normal cytochrome spectrum and an active cytochrome c oxidase. No explanation has been forthcoming for this anomaly, except for suggestions of a possible great excess of cytochrome c oxidase (for which there is some indication in the spectra) or of the presence of a B-type autoxidizable cytochrome. The statement was also made (Grimm and Allen, 1954) that *Ustilago sphaerogena* has a cyanide-insensitive respiration, in spite of the copious amount of cytochromes produced by this smut fungus. Respiration of *Candida albicans* also appears to be cyanide insensitive (Ward and Nickerson, 1958).

Cyanide-sensitive repiration, on the other hand, was reported in the slime mold *Physarum polycephalum* (Allen and Price, 1950) and in the Phycomycetes, including *Allomyces* (the mitochondria of which are discussed below), *Monoblepharella taylori* (Shoup and Wolf, 1946), *Blastocladiella emersonii* (Cantino and Hyatt, 1953), and *Leptomitus lacteus* (Schade and Thimann, 1940). The same was demonstrated for *Ashbya gossypii* (Mickelson, 1950), *Aspergillus niger* (Mann, 1944; Martin, 1954), *A. oryzae*, (cf. Tamiya, 1942, 1958), and *Penicillium notatum* (Wolf, 1947), the cytochrome spectrum of which has already been mentioned. In the case of *P. chrysogenum*, according to Sih *et al.* (1958),

although respiration was inhibited by cyanide, CO, and azide, and there was a cytochrome c-like spectrum, the CO inhibition was not effectively reversed by light and no evidence for A-type cytochromes was found. In view of the findings on *P. notatum* (Keilin and Tissières, 1953), it would be desirable to have this organism or particular strain reexamined. The cytochromes of *Neurospora crassa* have already been mentioned and will be further discussed. Boulter (1957) showed that *Gelasinospora tetrasperma* respiration was sensitive to cyanide and CO and was light reversible, and that most of the oxygen uptake was mediated by the cytochromes throughout its development, although this organism contains a great deal of tyrosinase in later stages of growth. Boulter and Hurst (1960) also demonstrated the light reversibility of CO inhibition in *N. crassa* and *Polystictus versicolor*.

III. CYTOCHROME c

This cytochrome is soluble and quite heat resistant, which probably accounts for the fact that it is the best known chemically of all the cytochromes. Its prosthetic heme group has saturated side chains and is held by two thioether linkages to cysteine molecules in the protein, in addition to iron-nitrogen bonds to two histidine (or to one histidine and one lysine) residues of the protein (cf. Mahler, 1961). The amino acid sequence of the portion of the protein surrounding the heme was given by Paléus and Tuppy (1961) for yeast cytochrome c. Margoliash and collaborators have recently worked out the entire amino acid sequences for yeast and seven other kinds of cytochrome c (for that of horse heart, see Margoliash, 1962). Clavilier *et al.* (1964) have recently reported the presence of two isozymes of cytochrome c in a haploid strain of yeast.

Armstrong *et al.* (1961) summarized the properties of isolated yeast and bovine heart cytochrome c. Yeast cytochrome c has a molecular weight of 15,000, iron content of 0.38% dry weight, sedimentation constant $S_{20,w} = 1.7$ to 1.9×10^{-13} S, its isoelectric point is at pH 9.85 $\pm$ 0.05, and its redox potential at pH 6.4 is $E_0 = +0.282$ *V*. Okunuki (1961), on the other hand, estimates the molecular weight of native yeast cytochrome c at 12,000.

Cytochrome c was first isolated from yeast in 1930 (Keilin), and first crystallized in 1956 (Hagihara *et al.*, 1956a,b). The room-temperature absorption bands of the reduced pigment lie at 549.3, 520.0, 414.7, and 314.0 mμ. A low-temperature spectrum of reduced yeast cytochrome c was obtained by Keilin and Hartree (1949) and Estabrook (1956); it showed two distinct α peaks, at 546.6 and 536.6 mμ, the former much larger than the latter, as well as at least four β peaks, at 526.2, 518.5, 512.3, and

507.0 mμ. At the same time mammalian cytochrome c was shown to have three α bands and possibly seven β bands.

The extracted cytochrome c contents of yeast grown under anaerobic and aerobic conditions were compared, and that of the former was found to be much lower (Borei and Sjödén, 1943) (see also Section VII). Incorporation of labeled glycine into cytochrome c was observed when anaerobically grown cells were exposed to oxygen, although the utilization of an anaerobically formed precursor of the prosthetic group could not be excluded (Yčas and Drabkin, 1957). Furthermore, Raut's (1953) W-1 mutant of yeast was shown to require glycine or protoporphyrin for the aerobic synthesis of cytochrome c and catalase, or for the anaerobic synthesis of cytochrome $b_1(556)$, but not for growth (Yčas and Starr, 1953).

Neilands (1952) studied the extracted cytochrome c of *Ustilago sphaerogena*, an organism that can produce this pigment in amounts up to 1% of its dry weight when grown in the presence of 1 ppm zinc and 2 ppm thiamine (Grimm and Allen, 1954). Neilands found the molecular weight of this cytochrome c to be 18,000–20,000, the sedimentation constant $S_{20,w} = 1.4 \times 10^{-13}$ *S*, its iron content 0.28%, and its isoelectric point to be at pH 7.

Finally, Yamanaka *et al.* (1962) isolated a C-type cytochrome from *Physarum polycephalum*, which they hesitated to call cytochrome c—in spite of its reactivity with mammalian cytochrome c oxidase and the complete agreement between its absorption spectrum and that of mammalian cytochrome c—because it can be precipitated by saturated ammonium sulfate whereas the latter cannot.

IV. CYTOCHROME c_1

This component of the electron transport system eluded detection, because of the proximity of its absorption bands to those of cytochrome c, until the methods of low-temperature spectra were applied (except for Yakushiji and Okunuki's report in 1940 on the presence of this pigment in mammalian preparations; this was, however, regarded at that time as being due to an artifact). Its low-temperature band at 552 mμ was observed in *Torulopsis* [*Candida*] *utilis* by Keilin and Hartree (1949), and its properties and function in preparations were further discussed by the same authors (1955). The wavelengths of its absorption bands were given as 553–554, 524, and 418 mμ. It is a nonautoxydizable hemoprotein, in which the prosthetic group is probably held to the protein by thioether linkages, but which is more thermolabile than c. Neither cytochrome c nor c_1 reacts with CO or cyanide. Cytochrome c_1 of heart muscle was also

tudied by Ball and Cooper (1957), Bernstein and Wainio (1960), Bomtein *et al.* (1961), and Orii *et al.* (1962).

The 554-mμ low-temperature band was observed in bakers' yeast by .indenmayer and Estabrook (1958). "Frozen steady-state" spectra of akers' yeast showing this band were obtained by Chance and Spencer 1959).

V. CYTOCHROME b

This is the least known of the mitochondrial cytochromes, and its funcion the subject of the most controversies (cf. Slater and Colpa-Boonstra, 961). Purified preparations of cytochrome b have been obtained from ovine heart and studied by Hübscher *et al.* (1954), and Doeg *et al.* 1960); those of cytochrome b and c_1 by Takemori and King (1962). No vork has come to my attention concerning the isolation of cytochrome b rom yeast or any other fungus.

The absorption bands of the mammalian pigment in the reduced state are it 564, 530, and 430 mμ, and in the oxidized state at 554, 500, and 415 mμ. It is reportedly a slowly autoxydizable pigment which does not eact with cyanide or CO. Its prosthetic group is iron-protoporphyrin held by two iron-nitrogen bonds to the protein.

VI. CATALASE, PEROXIDASE, AND HEMOGLOBIN IN FUNGI

A brief discussion of these hemoproteins is included here, although as a rule they do not participate in electron transport (peroxidases might under certain circumstances) and they do not occur in the mitochondria, but their presence in fungi must be taken into account whenever the cytochrome system is studied.

Cytochrome c peroxidase (cytochrome c:H_2O_2 oxidoreductase) has been isolated from yeast by Abrams *et al.* (1940); and yeast catalase (H_2O_2:H_2O_2 oxidoreductase) has been studied by Brown (1953). We may, on the basis of the spectral characteristics, assume that the prosthetic groups of both of these yeast enzymes and of yeast hemoglobin are similar to their counterparts in other materials, particularly to the peroxidases of horseradish, to liver catalase, and to the hemoglobin found in the root nodules of legumes, as well as in blood. The prosthetic groups of all these hemoproteins consist of iron-protoporphyrin 9 (heme), the iron being bound on one side to the protein, and there also may be ester bonds from the propionyl groups of the porphyrin to the protein (Paul, 1960; Theorell, 1951). The sixth coordination position of the iron may be occupied by

H_2O in all cases, or by H_2O_2 in catalase and peroxidase and by O_2 in hemoglobin. Whereas in catalase and in the peroxidases, when active, iron is present in the 3-valent (oxidized) form, in functional hemoglobin it is in the 2-valent (reduced) form.

The absorption spectra of liver catalase, of horseradish peroxidases, and of methemoglobin (oxidized hemoglobin) from blood, and of their various compounds, are very similar to each other (Keilin and Hartree, 1951; Hartree, 1955). The main absorption bands are at 630–640, 500, and 405 mμ, with two weaker bands at 575 and 545 mμ. The cyanide compounds of these oxidized pigments have bands at around 540–560 and 420–425 mμ. The cyanide difference spectrum of commercial bakers' yeast, with both cuvettes anaerobic and with cyanide added to the reference cuvette, is shown in Fig. 4. The peak at 442 mμ is due to the disappearance

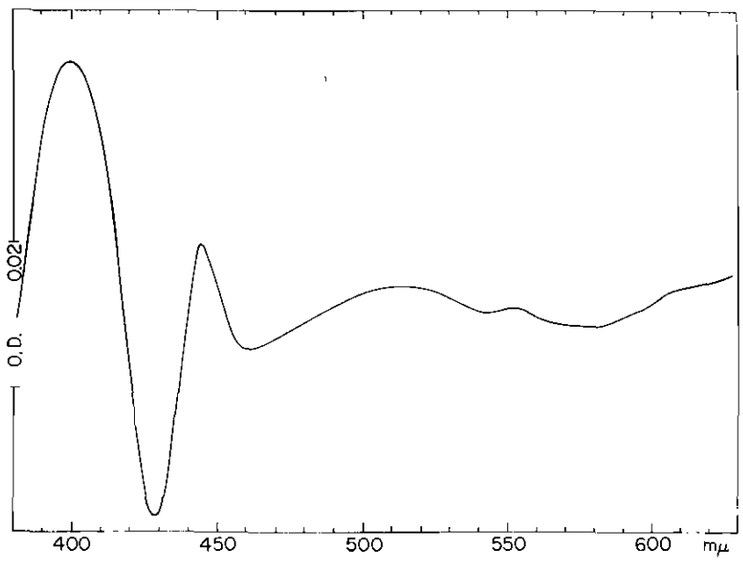


FIG. 4. Cyanide difference spectrum of commercial bakers' yeast. Both cuvettes were anaerobic; cyanide was added to one. Previously unpublished experiment of Lindenmayer and Smith.

of the reduced band of cytochrome a_3, while that around 400 mμ is due to the disappearance of catalase and peroxidase absorption. The large trough at 428 mμ, and the small troughs around 575 and 540 mμ, can be attributed to the cyanide compounds of the latter enzymes.

As for the reduced pigments (chemically reduced peroxidase and regular

hemoglobin), their absorption bands are around 555 and 430 mμ, with a smaller one at 580–590 mμ (catalase cannot be reduced with hydrosulfite or dithionite). The reduced pigments combine with CO, in which case their bands are around 575, 545, and 420–425 mμ.

On the other hand, the O_2 compound of hemoglobin (oxyhemoglobin) has bands at 577 and 542 mμ, which disappear on addition of a reducing agent and are somewhat displaced on addition of CO, but remain unchanged in the presence of cyanide. These were, incidentally, the properties on the basis of which Keilin (1953) and Keilin and Tissières (1953, 1954) postulated the presence of a hemoglobin-like pigment in certain strains of bakers' yeast, *Neurospora*, and *Penicillium*. Similar findings were reported with respect to yeast by Warburg and Haas (1934), and Yčas (1956), and to *N. crassa* by Boulter and Derbyshire (1957).

While both catalase and horseradish peroxidase can catalyze the oxidation of various hydrogen donors (pyrogallol, benzidine, ascorbate, cytochrome c, and H_2O_2 itself) with H_2O_2, yeast cytochrome c peroxidase is specific to cytochrome c only, as shown by Abrams *et al.* (1940, 1942). These investigators found that bakers' yeast contained 250 times as much peroxidase iron as catalase iron. In agreement with them, Smith (1954a) found in a sample of commercial bakers' yeast the following molar concentrations: cytochrome c peroxidase 2.7 μM, catalase 0.085 μM, and cytochrome c oxidase 0.92 μM. The determination of the peroxidatic activity in yeast extracts containing all three enzymes may be done in the presence of carbon monoxide (which inhibits oxidase but not peroxidase) and using CH_3OOH (methyl-hydrogen peroxide which can serve as oxygen donor for peroxidase but not for catalase). Chantrenne (1955a) reported another peroxidase in yeast, particle bound, with benzidine as the donor.

Yeast catalase was first studied by Euler and his collaborators (Euler and Blix, 1919; Euler *et al.*, 1927), who also found that only a small portion of the total catalase activity can be detected in intact yeast cells, while the rest of the activity can best be uncovered by heating or by treatment with solvents (e.g., *n*-butanol). Kaplan (1962) has studied this "Euler effect" in detail and found that the latent enzyme appears to be slowly converted into the "patent" (unmasked) enzyme while yeast cells are kept anaerobic. No new catalase, latent or patent, is formed in yeast during anaerobiosis, in agreement with the findings of Chantrenne and Courtois (1954), Chantrenne (1955b), Chantrenne and Devreux (1959), Chaix (1961), and Bhuvaneswaran and Sreenivasan (1961).

Chantrenne (1955a) claimed that the formation of cytochrome c peroxidase in wild-type and *petite* yeast is induced by oxygen, just as catalase is. Sels (1958a,b) has also shown cytochrome c peroxidase to be inducible in *petite* mutants, but only with a time lag with respect to the

induction of cytochrome c and catalase. In contrast, our observations on cytochrome c peroxidase (Lindenmayer and Smith, 1957, 1964) indicated that in anaerobically grown yeast there was high activity of this enzyme, which did not change appreciably after exposure to oxygen. The discrepancy between our findings and those of the Belgian investigators may be due to the different methods of obtaining cell-free extracts (Chantrenne autolyzed the cells for 24 hours, while we broke the cells by 30 seconds' shaking in a Nossal shaker), or to the particular strains of yeast used.

Catalase and peroxidase have been studied in several other fungi. Cheng (1954) demonstrated catalase and peroxidase activity (with pyrogallol and cytochrome c as donors) in *Neurospora tetrasperma*. Nicholas (1956) showed that catalase and peroxidase, but not oxidase, activity in *N. crassa* was much lower than typical when the mycelium was grown in a molybdenum-deficient medium. Lack of molybdenum may result in a decrease in flavine enzymes which furnish H_2O_2 for the peroxidase. Lenhoff and Kaplan (1956) also studied peroxidase in *poky* and wild-type *N. crassa*. Catalase was shown to be liberated into the medium by *Aspergillus oryzae* (Crewther and Lennox, 1953); and was determined in the spores of *A. niger* (Bhatnagar and Krishnan, 1960). Lyr (1955, 1956) studied the peroxidase that is excreted into the medium by heartwood-destroying basidiomycetes (the sapwood-decomposing species apparently excrete laccase instead). Of 182 species examined, he found peroxidase in the growth medium of 14. The substrate specificity of these peroxidases was similar to horseradish peroxidase. They are presumably active in lignin decomposition, but the question immediately arises where does the H_2O_2 come from which is necessary for their activity. The same question has, of course, been asked with reference to the intracellular role played by peroxidase.

VII. CYTOCHROMES a_1 AND b_1, AND THE ANAEROBICALLY PRODUCED CYTOCHROMES OF YEAST

In the *petite colonie* mutants of bakers' yeast and in the *mi-3* strain of *Neurospora crassa* a pigment was noted with an absorption band in the reduced state around 590 mμ which has been called "cytochrome a_1" in both cases, by analogy to the terminal oxidase of *Acetobacter pasteurianum*. In the *petite* yeast mutants another pigment was observed with a band around 557 mμ; it was called "cytochrome b_1" after the B-type cytochrome found in *Escherichia coli*. In *anaerobically grown* yeast, the same bands may be observed as in the *petite* mutants, and in both cases the cytochromes a, b, and c are missing. In the case of mutant *mi-3* of *N. crassa*, on the other hand, cytochromes b and c are present, and only a is missing. Both the *petite* and *mi-3* mutations are due to cytoplasmic factors, as are the *poky*

(*mi-1*) and *mi-4* mutations of *N. crassa* (the latter causing a leaky cytochrome deficiency). The absorption spectra of normal and *petite* yeast, grown anaerobically or oxygen adapted, are shown in Fig. 5. The spectra of

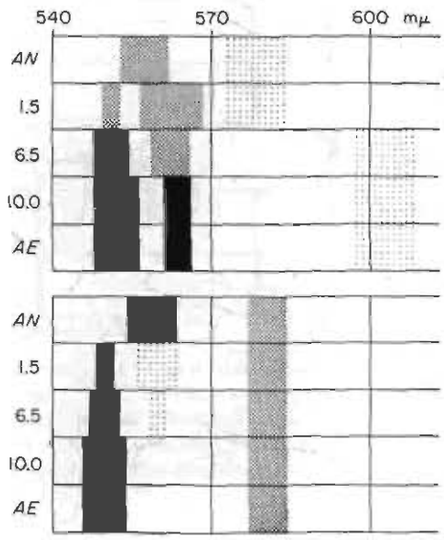


FIG. 5. Absorption spectra in the visible region of wild-type (top of figure) and *petite* (bottom of figure) yeast. *AE*, aerobically grown cells; *AN*, anaerobically grown cells. The other rows show spectra of anaerobically grown cells after they have been aerated for the given number of hours in glucose-phosphate buffer. All spectra were observed in the reduced state. Reproduced, by permission, from Ephrussi (1956).

the various *N. crassa* mutants (C115 and C117 are nuclear mutants) are shown in Fig. 6. For references concerning the *petite* mutants, see Ephrussi (1953, 1956), Slonimski (1953), and Nagai *et al.* (1961). For those on *N. crassa* respiration-deficient strains see Tissières *et al.* (1953), Keilin and Tissières (1953), Tissières and Mitchell (1954), and Pittenger (1956).

The difference in cytochrome composition between top- and bottom-fermenting brewers' yeasts was observed by Euler *et al.* (1927, 1939), and by Fink (1932) and Fink and Berwald (1933). Tamiya (1928) observed a similar difference in cytochrome content between surface and submerged-grown mycelia of *Aspergillus oryzae*. Further references on anaerobically grown yeast include Slonimski (1953, 1956), Chin (1950), Hebb and Slebodnik (1958), Lindenmayer (1959), and Chaix (1961).

Cytochrome a_1 of *Acetobacter pasteurianum* has reduced absorption bands at around 590 and 440 mμ, and the bands of its CO compound lie

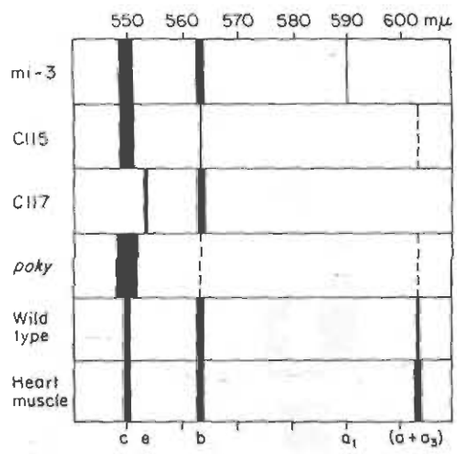


FIG. 6. Absorption spectra in the visible region of various strains of *Neurospora crassa*, compared with that of mammalian heart muscle. All spectra were obtained in the reduced state. The width of the absorption bands indicates relative intensities, although actual differences are much larger: *poky* contains 10–15 times as much cytochrome c as wild-type. Cytochrome e is now called c_1. Reproduced, by permission, from Tissières and Mitchell (1954).

at 590, 540, and 427 mμ (cf. Smith, 1961). The a_1-CO compound dissociates in light. *Neurospora* strain *mi-3* respiration was shown (Tissières and Mitchell, 1954) to be sensitive to cyanide, azide, and CO, and the CO inhibition was reversed by light. Cytochrome c oxidase activity was demonstrated also in cell-free extracts. From these and the spectroscopic observations it may be concluded that cytochrome a_1 is the terminal oxidase in this *Neurospora* mutant, rather than a_3 as in the wild type.

The situation is not so clear cut, however, in the case of cytochrome a_1 of *petite* and anaerobically grown yeast. In both these cases the respiratory rates are very low, so it would be hard to establish CO inhibition and reversibility of light. The fact that cytochromes a_1 and b_1 are reversibly oxidized and reduced in the presence and absence of oxygen can be seen from the anaerobic-minus-aerobic difference spectra (Fig. 7), where the small peak at 585 mμ and the shoulder at 445 mμ are due to reduced-minus-oxidized spectrum of "a_1," and the peaks at around 555, 525, and 423 mμ are due to "b_1." A deep trough can also be seen at 450–480 mμ, which is attributable to flavine.

When it was attempted (Lindenmayer and Smith, 1957, 1964) to find the CO compound of "a_1," the results turned out to be unexpectedly complex. In the presence of CO, anaerobically grown yeast has two large

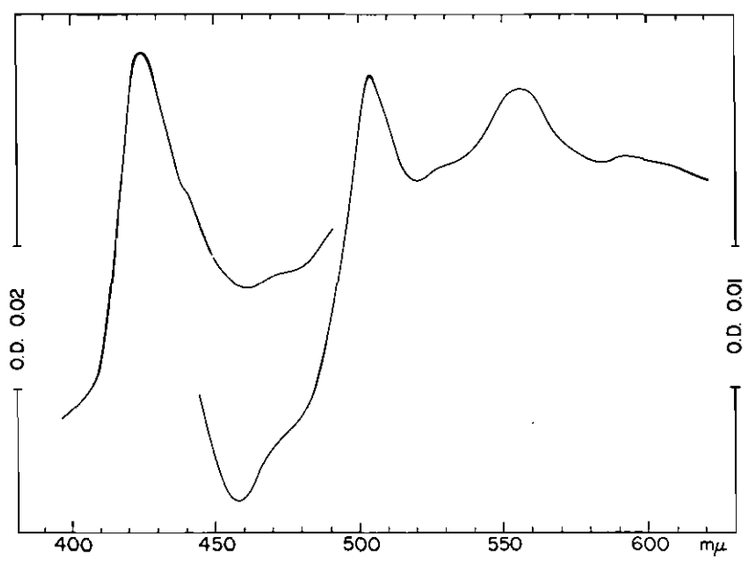


FIG. 7. Difference spectrum of anaerobically grown bakers' yeast (strain LK2G12). Reference cuvette was aerated, the other cuvette anaerobic. Optical density scale is different on right- and left-hand side of figure. From Lindenmayer and Smith, (1964). The peak at 503 mμ is due to an unidentified pigment, present in freshly grown yeast, which disappears on addition of cyanide or dithionite (Lindenmayer and Smith, 1957; Elkind and Sutton, 1957; Lindenmayer, 1959; Nosoh, 1964).

absorption peaks in the violet (Soret) region: one, at 420 mμ, appears slowly on addition of CO, is enhanced by dithionite, and is connected with two other peaks at around 570 and 540 mμ; and the other one, with its principal peak at 450 mμ, appears very rapidly with CO, and seems to be connected to two slight peaks at 590 and 540 mμ.

The absorption characteristics of the "420-CO" pigment are of an iron-protoporphyrin-CO compound, like those of reduced peroxidase and catalase, or hemoglobin in the presence of CO. But its original compound must be present in the oxidized state in those cells since dithionite affects it, so it cannot be peroxidase or catalase. Nor can the original compound be hemoglobin, since we have not observed a band at 583 mμ in our strain that would disappear with dithionite or CO, but not with cyanide.

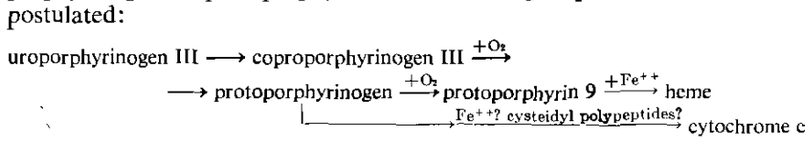
The "450-CO" pigment may be similar to the CO-binding pigment of liver microsomes (see Omura and Sato, 1964), but it could not correspond to the CO compound of cytochrome a_1 like that in *A. pasteurianum*. No peak with absorption around 430 mμ was observed in anaerobically grown yeast with CO.

As a consequence, the correct name for the yeast "cytochrome a_1" should for the present be "cytochrome *590* (*Saccharomyces cerevisiae*)," in accord with the recommendations of the Commission on Enzymes (1961), since its α band in room-temperature absolute spectra is at 590 mμ (Lindenmayer, 1959).

As for "cytochrome b_1" of anaerobically grown wild-type or *petite* yeast, it has been shown that its α band splits into two at low temperatures [557.5 and 551 mμ according to Lindenmayer and Estabrook (1958); 558 and 552.5 mμ according to Heyman-Blanchet *et al.* (1959)] In this respect it is similar to the cytochrome b_1 of *Escherichia coli* (Keilin and Harpley, 1941); but it still seems advisable to call this pigment "cytochrome *556* (*S. cerevisiae*)," the room-temperature absolute absorption maximum of its α band having been observed at 556 mμ. A similar cytochrome with a split α band at 561 and 554 mμ (room temperature) has been reported to be present in *Sclerotinia libertiana* (Yamanaka *et al.*, 1960). This is a soluble, autoxidizable cytochrome, and the organism has no cytochrome c.

Barrett (1961), by acid-solvent extraction, found heme a in anaerobically grown bakers' yeast, which is presumably the prosthetic group of the above-mentioned cytochrome *590*. On aeration of an anaerobically grown brewers' yeast, he observed the appearance of cytochromes b and c, but not of a + a_3. Instead there was an increase in cytochrome *590*. In addition, he found cryptoheme a in aerobically grown cells, but not in anaerobically grown ones. Cryptoheme a is a closely related compound to heme a, the main difference being in the absence of one of the long, undetermined side chains from cryptoheme a (Parker, 1959). Whether cryptoheme a is part of an aerobically produced hemoprotein, or whether it serves as precursor to other hemes, is not known.

This brings up the question how the biosynthesis of anaerobically produced cytochromes is related to the biosynthesis of the cytochrome components of aerobically grown cells. In order to answer this question, the biosynthesis of the various heme prosthetic groups of these enzymes must be taken into account. Granick and Mauzerall (1961) summarized the evidence concerning the biosynthesis of iron-protoporphyrin 9 (heme) from glycine and succinate. In another paper, Sano and Granick (1961) describe the isolation of a coproporphyrinogen oxidase from liver mitochondria, and they also indicate the need for molecular oxygen for the oxidation of protoporphyrinogen to protoporphyrin. The following sequence of reactions is postulated:



This scheme, if found valid for microorganisms, would have interesting implications: (1) It would explain why in yeast cytochromes a, a_3, b, and c are not synthesized under anaerobic conditions (two steps being blocked by lack of O_2). But this would necessitate a separate pathway, not involving O_2, for cytochromes *556* and *590* in yeast, as well as for all the bacterial cytochromes produced anaerobically (*E. coli* has the same cytochromes anaerobically as aerobically). (2) It would explain why the aerobic synthesis of cytochrome c can proceed in certain yeast and *N. crassa* mutants, while the other cytochromes are all blocked (presumably in the steps after the branching to cytochrome c). (3) It would also explain why coproporphyrin III rather than protoporphyrin 9 or uroporphyrin III is excreted by microorganisms in the absence of O_2 [yeast: Kench and Wilkinson (1945), Slonimski (1952), Shavlovskii (1959), Shavlovskii and Bogatchuk (1960); *Bacillus cereus:* Schaeffer (1952); *Micrococcus lysodeikticus:* Townsley and Neilands (1956)]. It does not explain, however, why iron deficiency should always result in coproporphyrin III production as well (cf. Neilands, 1957), unless coproporphyrinogen oxidase itself needs iron for activity.

VIII. MITOCHONDRIAL FLAVINE ENZYMES

Flavoproteins contain one of two main types of prosthetic groups: flavine-mononucleotide (FMN, also known as riboflavine phosphate) or flavine adenine dinucleotide (FAD) (cf. Beinert, 1960). The binding of these compounds to the protein may be different in various enzymes; in many cases they are easily dissociable, whereas in some they can be removed only by tryptic digestion. The absorption spectrum of the free, oxidized compounds are shown in Fig. 8; in this state they are yellow, strongly fluorescent substances. On reduction they lose their absorption between 400 and 500 $m\mu$ and their fluorescence.

The first flavoproteins were isolated from yeast, and the structure of flavines and their role as prosthetic groups were worked out on the old yellow enzyme [NADPH:(acceptor)oxidoreductase], which contains FMN.

For our purposes the two most important flavine enzymes that occur in mitochondria are those involved in the oxidation of NADH and of succinate. Many preparations have been studied from mammalian tissues that exhibit these activities (cf. Slater, 1958a; Singer *et al.*, 1957a; Singer, 1961; Huennekens, 1956; Ernster, 1961). The prosthetic group of the mammalian NADH dehydrogenase [NADH:(acceptor) oxidoreductase] was first thought to be FAD, but recent work indicates that it is probably FMN (Huennekens *et al.*, 1961; King *et al.*, 1962; Ringler *et al.*, 1963). The prosthetic group of mammalian succinate dehydrogenase [succinate:

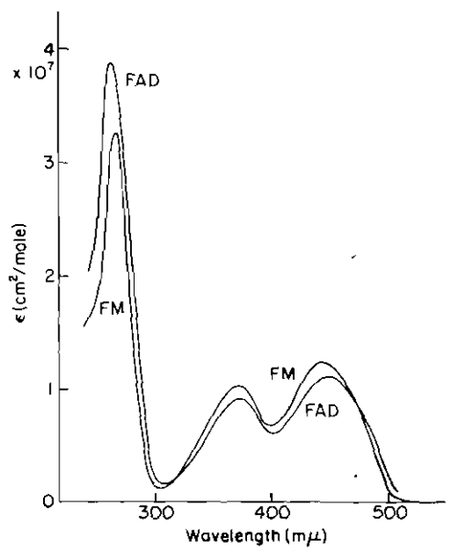


FIG. 8. Absorption spectra of flavine mononucleotide (FM) and of flavine adenine dinucleotide (FAD) in the oxidized state. Reproduced, by permission, from Dixon and Webb (1958).

(acceptor) oxidoreductase] has, on the other hand, been shown to be FAD, bound by covalent bonds to the protein (Kearney, 1960). Both these enzymes contain, in addition, nonheme iron, which is necessary for the activity. According to Green and Fleischer (1962), the stoichiometric ratio of iron to flavine is 5 in both enzymes, but according to Singer *et al.* (1957a) it is 4 in succinate dehydrogenase, and according to Mackler (1961) it is 2 in NADH dehydrogenase. The nonheme iron atoms contribute to absorption of light in the visible region above 520 mμ, whereas the contribution of the flavines is below 500 mμ.

Purified yeast succinate dehydrogenase was studied by Singer *et al* (1957b), who found that it contained 4 atoms of nonheme iron per molecule of flavine, and that the flavine was probably FAD. This was confirmed by Mackler *et al.* (1962), who found that about half of the flavine in yeast "electron transport particles" (see Sections XII and XIII) was peptide bound and half was acid extractable, but both fractions consisted of FAD. The iron:flavine ratio was approximately 1 according to the latter workers.

Although anaerobically grown yeast lacks succinate:cytochrome c oxidoreductase (Slonimski, 1956), it was shown by Hebb *et al.* (1959) that these cells possessed succinate:phenazine-methosulfate oxidoreductase ac-

ity. Similarly, Linnane *et al.* (1962) reported succinate:ferricyanide and ADH:ferricyanide oxidoreductase activities in the same organism.

An FMN-enzyme was isolated from yeast (cf. Haas, 1955) which cata-es the oxidation of NADPH by cytochrome c directly, but it has not en shown whether this enzyme occurs in the mitochondria or in the toplasm. A soluble succinate:phenazine methosulfate oxidoreductase also is found in *Claviceps purpurea* by McDonald *et al.* (1963).

Another set of flavoproteins, the lactate-oxidizing enzymes of yeast, me of which occur in the mitochondria, is discussed in the next section. Many other flavine enzymes have been described from yeast and other ngi, including various dehydrogenases, nitrate and nitrite reductases, ucose and L-amino acid oxidases, but none of these are involved in par-ulate electron transport systems. Riboflavine is known to be widespread ong fungi and abundant in some species; in fact, it is from this source at industry obtains it (cf. Cochrane, 1958).

IX. CYTOCHROME b_2 AND OTHER LACTATE-OXIDIZING FLAVINE ENZYMES OF YEAST

Aerobically grown bakers' yeast contains an L-lactate dehydrogenase -(+)-lactate:cytochrome c oxidoreductase), a flavohemoprotein, also alled cytochrome b_2. This enzyme is partly soluble and partly particulate Vitols and Linnane, 1961), and its ability to react with cytochrome c ncreases markedly as it becomes solubilized (Somlo, 1962). It has been udied very extensively in recent years, mainly because of the great interest n its two prosthetic groups and its resultant physicochemical behavior see five contributions and numerous comments in Falk *et al.* (1961); also Armstrong *et al.* (1963), R. K. Morton (1961), Hasegawa (1962a,b), Mahler and Pereira (1962), Hinkson and Mahler (1963)]. A particular ingle-stranded DNA (consisting of 33 nucleotides) is regularly associated vith this enzyme, even through crystallization. However, the DNA is not ssential for activity. The prosthetic groups are iron-protoporphyrin, FMN, nd magnesium. The electron (or hydrogen) donors are L-lactate, L-α-hy-lroxybutyrate, and glycolate, and the electron acceptors include cyto-chrome c, ferricyanide, phenazine methosulfate, methylene blue, as well as O_2 (cytochrome b_2 being somewhat autoxidizable). The absorption bands of the isolated enzyme in the reduced state at room temperature are at 556.5, 528, 423, and 330 mμ (R. K. Morton *et al.*, 1961), and the α band splits into two at the temperature of liquid air, showing bands at 558 and 553.4 mμ (Chance *et al.*, 1956) or at 557.5 and 552.5 mμ (Lindenmayer and Estabrook, 1958). Cytochrome b_2 does not seem to participate in electron transport in intact aerobically grown yeast cells

(Chance, 1961a) or in isolated electron transport particles of yeast, unless exogenous cytochrome c is added to them (Mahler *et al.*, 1964a). Furthermore, Slonimski (1953) was not able to demonstrate such participation in aerobically grown *petite* cells, although there was lactate:cytochrome c reductase activity in their extracts. Also, Gregolin and Ghiretti-Magaldi (1961) succeeded in isolating normal cytochrome b_2 from *petite* cells. These and other findings suggest that cytochrome b_2 may be primarily in the oxidized state in the cell and remain so even in the presence of exogenous lactate.

There are two other lactate-oxidizing enzymes in aerobically grown yeast: a D(−)-lactate:cytochrome c and a D(−)-lactate:ferricyanide oxidoreductase (Nygaard, 1961a,b, 1963; Singer *et al.*, 1963; Gregolin and Singer, 1963). The former is a stable, particle-bound FAD-zinc-protein (also associated with DNA) which can oxidize D-lactate, D-α-hydroxybutyrate, and other straight-chained D-α-hydroxymonocarboxylic acids with cytochrome c or phenazine methosulfate serving as acceptors. It has recently been shown to transmit electrons directly to the particle bound cytochrome system of yeast [in the presence of Mg^{2+}, (see Gregolin and D'Alberton, 1964)]. The latter enzyme is a very labile, FMN-protein which oxidizes D-lactate with ferricyanide, but not with cytochrome c or phenazine methosulfate as acceptors; this enzyme may actually be a degradation product of the previous one.

In anaerobically grown bakers' yeast, on the other hand, there is a fourth lactate-oxidizing enzyme which oxidizes D(−)-lactate and other D-α-hydroxy acids with ferricyanide, methylene blue, and 2,6-dichlorophenolindophenol, but not with cytochrome c as acceptor (Lindenmayer and Smith, 1957; Slonimski and Tysarowski, 1958; Labeyrie *et al.*, 1959; Iwatsubo and Labeyrie, 1962; Singer *et al.*, 1961, 1963). The prosthetic groups of this enzyme are FAD and zinc, just as in the case of the D(−)-lactate:cytochrome c reductase of aerobic cells, but the two enzymes are distinguishable by the lack of reactivity with cytochrome c on the part of the anaerobic enzyme, as well as by the fact that the anaerobic enzyme is not particle bound (also by differences in thermal stability, Michaelis-constants etc.). In spite of repeated attempts (Boeri *et al.*, 1956), no absorption changes were observed on addition of lactate to intact anaerobically grown yeast cells.

A controversy has been going on whether this last enzyme could serve as precursor in the induced formation of the L-lactate and D-lactate oxidizing enzymes of aerobic cells. Kattermann and Slonimski (1960) have observed that amino acid analogs, which inhibit the synthesis of most enzymes, do not inhibit the induced formation of the L-lactate dehydrogenase (cytochrome b_2); neither do they inhibit the formation of the

D-lactate:cytochrome c reductase according to Nygaard (1961b). On the other hand, Singer *et al.* (1961) and Gregolin and Singer (1961) have claimed that the anaerobic enyme does not necessarily disappear when the other two are formed, as Labeyrie *et al.* (1959) had found. The contradiction between these two sets of observations has not been resolved yet.

Yeast can utilize lactate as the sole source of carbon (Galzy and Slonimski, 1957a,b). Lactate can also be utilized by *Penicillium chrysogenum* (Hockenhull *et al.*, 1954), which has been shown to have a lactate:cytochrome c oxidoreductase, an FMN enzyme (Casida and Knight, 1954).

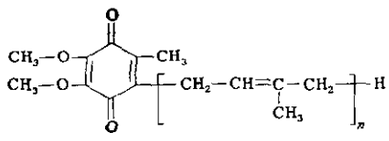
X. MITOCHONDRIAL QUINONES

A great deal of attention has been focused lately on the participation of quinoid compounds in electron transport and oxidative phosphorylation. Their biosynthesis and function has been the subject of a Ciba symposium (1961).

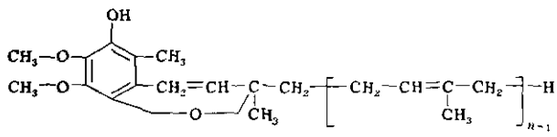
Ubiquinone (coenzyme Q), discovered in 1958 independently by R. A. Morton and by F. L. Crane and their co-workers in rat liver and bovine heart, is the common name of a group of benzoquinone derivatives with isoprenoid side chains of various lengths. As such, they are related chemically to the vitamin K group of naphthoquinone compounds, as well as to the benzoquinone vitamin E compounds and to plastoquinone (Fig. 9). The K and E vitamins also have been implicated in mitochondrial electron transport, while plastoquinone may be involved in photosynthetic electron transport (cf. Bishop, 1961; Dam and Søndergaard, 1960; Boyer, 1960; R. A. Morton, 1961; Redfearn and Friend, 1961; Slater, 1962). Ubiquinones in ethyl alcohol solution absorb in the ultraviolet at 275 mμ, whereas the corresponding reduced compounds have a weaker absorption band at 290 mμ. The extinction coefficients at these wavelengths, as well as the melting points, vary with the length of the side chain (from 6 to 10 five-carbon isoprene units in naturally occurring materials) [see Crane (1961)]. In the following, the various ubiquinones will be distinguished by indicating the number of isoprene units rather than the number of carbon atoms in the side chain.

In contrast to the large amount of information available concerning the concentration and role of ubiquinones in mammalian mitochondria, our knowledge concerning them in fungi is restricted to reports of their occurrence in whole cells or mycelia. Table III lists the fungi in which the ubiquinone species and content has been determined. For comparison it may be mentioned that the ubiquinone found in mammals and higher plants is UQ_{10}, in insects and algae it is UQ_9, and in protozoa UQ_8 (Crane, 1962). The original finding of Lester and Crane (1959) to the effect that

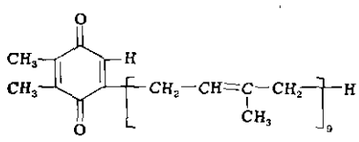
Ubiquinone (UQ_n) $6 \leq n \leq 10$



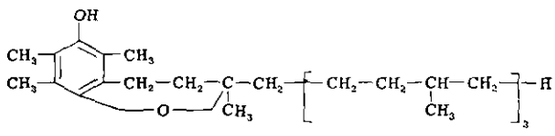
Ubichromenol (UC_n) $6 \leq n \leq 10$



Plastoquinone (PQ_9)



Vitamin E (α-tocopherol)



Vitamin K_1

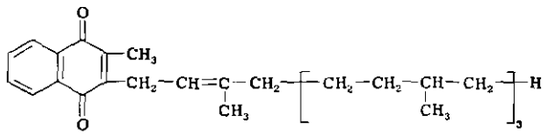


FIG. 9. Quinones.

TABLE III

UBIQUINONE IN THE FUNGI

Organism	Type of UQ	Amount (μmole/g dry wt.)	References
Mucor [Absidia] corymbifer	UQ_9	0.20	Lester and Crane (1959), Lester *et al.* (1958, 1959), Gloor *et al.* (1958)
Saccharomyces cerevisiae			
Grown aerobically	UQ_6	0.35	Lester and Crane (1959), Lester *et al.* (1958, 1959), Gloor *et al.* (1958)
Grown aerobically	UQ_6	0.14	Mahler *et al.* (1964b)
Grown aerobically	UQ_6	0.52	Sugimura *et al.* (1964)
Grown anaerobically	—	0	Lester and Crane (1959), Lester *et al.* (1958, 1959), Sugimura and Rudney (1960)
Respiration-deficient mutants, grown aerobically	UQ_6	0.11	Mahler *et al.* (1964b)
Respiration-deficient mutants, grown aerobically	UQ_6	0.08	Sugimura *et al.* (1964)
Candida utilis	UQ_7, UQ_9	—	Lester and Crane (1959), Lester *et al.* (1958, 1959), Stevenson *et al.* (1962), McHale *et al.* (1962)
Aspergillus fumigatus	UQ_{10}	0.6	Packter and Glover (1960)
Aspergillus flavus	Tetrahydro-UQ_{10}	—	Lavate *et al.* (1962)
Aspergillus flavus-oryzae			Lavate *et al.* (1962)
Aspergillus terreus			Lavate *et al.* (1962)
Penicillium stipitatum			Lavate *et al.* (1962)
Neurospora crassa			Lavate *et al.* (1962)
Ustilago maydis	UQ_{10}	0.20	Erickson *et al.* (1960)
Ustilago maydis	UQ_9	0.02	Erickson *et al.* (1960)
Agaricus campestris	UQ_9	0.05	Erickson *et al.* (1960)

basidiomycetes contain a basidioquinone, different from the ubiquinones, has been rescinded in favor of the later findings of Erickson *et al.* (1960), shown in Table III. On the other hand, Lavate *et al.* (1962) did claim the existence of a different compound, tetrahydro-UQ_{10}, in penicillia and aspergilli and in *Neurospora*. Ubichromenols (UC), isomers of the ubiquinones, have been found in dried food yeast (*Torulopsis* [*Candida*] *utilis*), including mostly UC_7 and some UE_9, in addition to UQ_7 and UQ_9 (Edwin *et al.*, 1961). However, freshly grown food and bakers' yeasts contain no UC compounds (Diplock *et al.*, 1961; Stevenson *et al.*, 1962;

McHale *et al.*, 1962). In addition, α-tocopherol has also been reported as being present in bakers' yeast (Diplock *et al.*, 1961), although fungi have generally been assumed to be devoid of E vitamins.

The lack, or low level, of UQ in anaerobically grown yeast, and its synthesis on the admission of oxygen, was also reported by Sugimura and Rudney (1960) and Rudney and Sugimura (1961). They found that adding UQ_6 and a lipid fraction of aerobic particles to extracts of anaerobically grown yeast did not result in succinate:cytochrome c reductase activity. Neither did the addition of UQ_1 or UQ_{10} result in a stimulation of the oxidative rate of NADH in such extracts (Lindenmayer and Smith, 1964).

Why yeast fails to synthesize ubiquinone in the absence of air cannot be answered yet. The isoprenoid side chain is probably synthesized without oxygen from mevalonic acid in the way that squalene or the colorless carotenoid compounds are (cf. Ciba Foundation, 1959a). How the benzoquinone moiety of ubiquinone is made in yeast is not known. Rudney and Sugimura (1961) could not get incorporation of labeled phenylalanine into UQ_6 of yeast. Phenylalanine is, however, thought to be the precursor of the ring structure of UQ in animal tissues, this being an O_2-requiring reaction (Olson *et al.*, 1963).

XI. MITOCHONDRIAL LIPIDS

Recent studies (Marinetti *et al.*, 1958; Basford, 1959; Fleischer *et al.* 1961; Redfearn, 1961; Getz *et al.*, 1962) on mammalian mitochondria showed that they contained about 30% lipids by dry weight. Of these lipids, 90–95% were phospholipids. These, in turn, were composed of about equal amounts (35–40% each) of lecithin (phosphatidylcholine) and cephalins (phosphatidylethanolamine and phosphatidylserine), as well as lesser amounts (10–15% each) of phosphatidylinositol and of cardiolipin (polyglycerol phosphatides). According to Brierley and Merola (1962), it was the cardiolipin-containing fraction of phospholipids which was most active in restoring enzymatic activity to the various preparations isolated from mitochondria, but this might be due to a detergent effect. The polyunsaturated fatty acid linoleic acid was the main fatty acid in the cardiolipin of rat liver preparations. Mitochondrial lipids also were shown to contain 1–2% of steroids, some carotenoids, and about 0.5% ubiquinone.

In stimulating reviews by Bloch and his co-workers (Goldfine and Bloch, 1963; Erwin and Bloch, 1964) the following relationship was pointed out: all organisms that possess mitochondria also seem to contain sterols and polyunsaturated fatty acids. The converse of this generaliza-

on does not hold, however, because there are some organisms that con-
ain polyunsaturated fatty acids but no mitochondria (the blue-green algae,
i their chromatophores), and there are also organisms which have sterols
ut apparently no mitochondria (the red algae). The polyunsaturated
atty acids of yeast and other fungi, except *Phycomyces blakesleeanus,*
re primarily linoleic and α-linolenic acids; which compounds they share
ith the blue-green, red, and green algae, as well as with all of the higher
lants. The animals, and the ciliated and amoeboid protozoa, on the other
and, share the γ-linolenic acid derivatives, which have also been found
i *P. blakesleeanus* (Bernhard and Albrecht, 1948). Some unusual poly-
nsaturated fatty acids have been found in *Dictyostelium discoideum*
Davidoff and Korn, 1963), the synthesis of which does not correspond
o either the α- or the γ-linolenic acid pathways.

The formation of polyunsaturated fatty acids from monounsaturated
ones always requires molecular oxygen, while the formation of mono-
insaturated fatty acids from saturated ones takes place by oxidative de-
aturation only in the fungi, animals, and in some of the algae and bac-
eria. Other bacteria are able to accomplish the latter transformation by
naerobic means, while the green plants seem to have a third mechanism
till, requiring oxygen (Erwin and Bloch, 1964). The biosynthesis of
terols also requires molecular oxygen (Goldfine and Bloch, 1963).

The inability of bakers' yeast to grow under prolonged anaerobiosis,
nless ergosterol and oleic acid (or other monounsaturated fatty acids)
re added to the usual growth factors, was demonstrated by Andreasen
nd Stier (1953, 1954, 1956) and Harris (1956). These findings helped
eventually to elucidate the sterol and unsaturated fatty acid synthetic path-
ways. Apparently, polyunsaturated fatty acids are not required for the
anaerobic growth of yeast.

The only other case of steroid growth factor requirement among fungi
is that shown by Vishniac (1955) for *Labyrinthula vitellina,* which re-
quires cholesterol, but not ergosterol.

Finally, as an indirect indication of a relationship between sterols and
the respiratory system, the induction of respiration-deficient mutants in
yeast by elevated temperatures [according to the method of Sherman
(1959)] is counteracted to some extent by ergosterol (Parks and Starr,
1963).

We know that the various phospholipids mentioned above and the
sterols are present in fungi, in some cases in large quantities (cf. Eddy,
1958). Various monounsaturated and polyunsaturated fatty acids occur in
fungi, sometimes representing the major portion of the total fatty acids
(Klein, 1957, 1960; Corwin *et al.,* 1957; Deinema, 1961; Leegwater and
Craig, 1962; Hartman *et al.,* 1962). Increased accumulation of free linoleic

and linolenic acids in the *poky* mutants of *N. crassa,* in comparison to the wild type, was reported by Hardesty and Mitchell (1963).

Lester (1963) found that cardiolipin comprised approximately 10% of the phospholipids of yeast mitochondria and that the cardiolipin content of yeast varied in direct proportion to its cytochrome content. Respiratory particles (mitochondrial fragments) isolated from *Claviceps purpurea* had the following phospholipid composition (Anderson *et al.,* 1964): 28% phosphatidyl inositol, 27% phosphatidyl choline, 13% phosphatidyl serine, 18% phosphatidyl ethanolamine, 7% polyglycerol phosphatides, and 7% residue; no plasmalogens.

XII. ELECTRON TRANSPORT

The enzymatic activity of cytochromes first became evident when the spectral change accompanying their reduction was observed on the addition of glucose to starved yeast cells (Keilin, 1925); and when the oxidation of succinate was linked to similar spectral changes in muscle homogenates (Keilin, 1930). The respiratory importance of Warburg's oxygen-transferring enzyme (cytochrome a_3) was, of course, established at about the same time (see Section II). The preparation of cytochrome c from yeast cells made it possible to study separately two parts of the "succinoxidase" system: these would be called today the succinate:cytochrome oxidoreductase system and the cytochrome c:O_2 oxidoreductase. It was soon found that the oxidation of NADH can similarly be achieved with the simultaneous reduction of oxidized cytochrome c, and subsequently separate preparations were obtained for each of these three activities.

The oxidation of added succinate or NADH by cell-free preparations may be measured (1) by observing the rate of oxygen uptake manometrically or polarographically; (2) by measuring the half-time of the reduction of the cytochrome system at known initial oxygen concentration spectrophotometrically; or (3) by measuring the rate of disappearance of NADH or the rate of fumarate appearance, also spectrophotometrically. Similarly the enzyme activities in which cytochrome c serves as electron acceptor or donor may be measured by the rate of increase or decrease of the absorption intensity at 550 mμ.

The enzymatic oxidation of reduced cytochrome c with oxygen is a first order reaction with respect to the cytochrome c concentration, and this is true even at high cytochrome c concentrations, although the first-order velocity constant may have lower values (Smith, 1954a; Smith and Conrad, 1956). The enzymatic reduction of cytochrome c with succinate or NADH, on the other hand, follows Michaelis-Menten kinetics, giving initially a straight line for the appearance of reduced cytochrome c. This is illustrat

Fig. 10, where first the oxidation by yeast extract of added cytochrome c shown, followed by inhibition by cyanide and the slow reduction of cytochrome c with endogenous substrates, then its rapid reduction with added ıccinate.

Since the cytochrome c oxidizing portion of the respiratory system ɔntains all the A-type cytochromes, and the cytochrome c reducing porɔn contains the B-type component, the cytochrome sequence, sub-

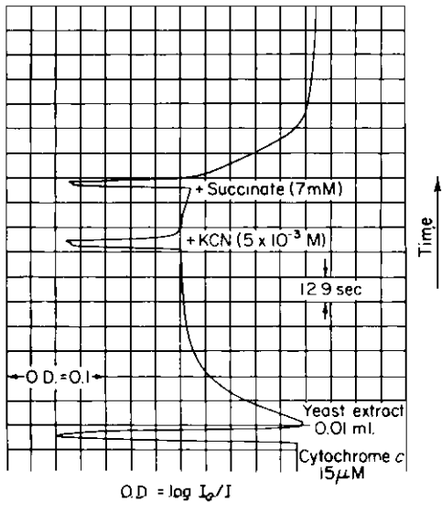


Fig. 10. Oxidation of reduced cytochrome c by cytochrome c oxidase of yeast ıtract, followed by reduction of the cytochrome c by the yeast reductase in the esence of endogenous substrate, then after addition of succinate. Cytochrome c ɔncentration in test was 15 μM; buffer was 0.015 M phosphate, pH 7.0; final conɔntration of succinate was 7 mM. Yeast extract was prepared from starved comercial bakers' yeast. Sharp peaks in the trace result from the introduction of irring rod and mark the time of addition of reagents. Reproduced, hy permission, om Smith (1954a).

rate—B—C—A—O_2 has been postulated at an early stage and still apcars to be valid. For a while doubts were cast on the role cytochrome c layed in the system, and it has even been called an artifact, but there seems ɔ be general agreement now concerning its participation. The above seuence was borne out by the redox potentials of the various cytochromes Falk and Perrin, 1961), which are increasingly positive in the postulated rder. Kinetic studies, concerning the time sequence at which the different ytochromes become oxidized on admission of oxygen, or reduced on addion of substrate, corroborate this order (Chance and Williams, 1956).

Inhibitors have also contributed to our understanding of the system. Besides cyanide, CO, and azide, two other inhibitors have been very useful; BAL (2,3-dimercaptopropanol) and antimycin A (for its structure, see Strong *et al.*, 1960). In the presence of these two inhibitors, cytochrome b remains reduced whereas the others are oxidized. The BAL-sensitive locus (also known as the Slater factor) was first thought to be cytochrome b itself, then cytochrome c_1; now it is assumed to be between these two, but its nature is not known. The antimycin A-sensitive locus may or may not be identical with the BAL-locus.

After the discovery of the ubiquinones as components of the electron transport system, a role similar to that of cytochrome c was claimed for them. Thus, succinate:UQ, NADH:UQ, UQ:cytochrome c, and UQ:O_2 oxidoreductase activities have been described from mammalian mitochondrial preparations. But, in spite of these manifest activities, the same problem arises with respect to these compounds as did before with cytochrome c: are they real or artificial electron carriers? The evidence was based at first entirely on extraction and reconstitution studies (cf. Crane, 1962). Recently, spectrophotometric evidence has also been obtained for the reversible oxidation and reduction of UQ at rapid enough rates to justify the inclusion of UQ as an essential component of the electron transport system of mammalian mitochondria (cf. Hatefi, 1963).

The present concept of the electron transport system is as follows: oxidized flavoproteins react with the substrates succinate and lactate, or with the reduced coenzyme NADH. What reacts with the consequently reduced flavine enzymes, how ubiquinone and cytochromes b and c_1 are arranged with respect to each other (whether in series or parallel), and which of these actually are active in native mitochondria, are questions still largely unresolved. Once the electrons are transferred past this area, it is agreed that they then pass through cytochrome c. After this, they go to the cytochrome a + a_3 complex, on which an oxygen molecule is bound, so that the electrons can finally enter into orbit around oxygen nuclei.

The main difficulty with this concept is, as has often been pointed out, that we are trying to describe a solid-state process in terms of reactions between soluble components. Unfortunately, this is still the only framework we have. It should be clear, in any case, that the phrase "electron transport chain" may be quite misleading, since it indicates a linear array of components along which the electrons are transmitted, the linearity of which, at the present time, is not certain.

Cell-free preparations of bakers' yeast have been studied with regard to these enzymatic activities by Chantrenne (1944), Slonimski (1953, 1956), Nossal *et al.* (1956, 1957), Vanderwinkel *et al.* (1958), Hebb and Slebod-

nik (1958), Chaix (1961), Linnane and Still (1955a,b), Vitols and Linnane (1961), and Mackler *et al.* (1962), in addition to those previously mentioned. Mahler *et al.* (1964a) estimated that the electron transport system of yeast is capable of carrying 10^4 (reducing equivalents per heme) per minute at 29°. In most cases the particulate preparations exhibited tricarboxylic acid cycle activities as well, and there is no doubt today that yeast mitochondria contain a functional tricarboxylic acid cycle (Krebs and Lowenstein, 1960) (see also Chapter 11 of this volume).

Cytochrome oxidase determinations were carried out on *Aspergillus niger* (Martin, 1954), and on *A. oryzae* (Imamoto *et al.*, 1959; Iwasa *et al.*, 1959; Iwasa, 1960), as well as on *Neurospora crassa* (Shepherd, 1951; Nicholas *et al.*, 1954; Tissières and Mitchell, 1954; Tissières, 1954; Turian, 1960), on *N. sitophila* conidia (Owens, 1955), on *N. tetrasperma* (Cheng, 1954; Holton, 1960), and on *Glomerella cingulata* (Sussman and Markert, 1953). Respiratory enzymes were shown not to be directly involved in the activation process of dormant ascospores of *Neurospora* (Sussman, 1961).

Among the aquatic phycomycetes, cytochrome oxidase and "succinoxidase" preparations were obtained from *Allomyces* by B. A. Bonner and Machlis (1957) and by Turian (1960), the latter of whom also pointed out a connection between a deficiency in cytochrome oxidase and the accumulation of carotenoid compounds in the male gametophytes, an interesting finding in view of the common pathway of carotene, ubiquinone, and sterol biosynthesis (Ciba Foundation, 1959a; Grant, 1962). Cytochrome oxidase was shown by Cantino and Hyatt (1953) to be present also in *Rhizophlyctis rosea* (a chytrid) and in *Blastocladiella emersonii*, but it was not found in the resistant sporangia of the latter (cf. Cantino and Turian, 1959). Ward (1958) showed cytochrome oxidase activity in the slime mold *Physarum polycephalum*.

Among the Basidiomycetes, the respiratory system was thoroughly investigated and found to be similar to that of yeast in *Schizophyllum commune* by Niederpruem and Hackett (1961) and in the urediospores of *Puccinia graminis* by White and Ledingham (1960). Boulter and Burges (1955) demonstrated a normal cytochrome oxidase in *Polystictus versicolor;* a somewhat abnormal cytochrome oxidase was indicated in *Ustilago sphaerogena* by Grimm and Allen (1954). Finally, *Fusarium lini* was shown by Kikuchi and Barron (1959) to have the same kind of electron transport system.

In the studies cited, mammalian cytochrome c was used as electron donor in most, if not all, cases. Only recently have any attempts been made to compare activities with the organism's native cytochrome c (Yamanaka *et al.*, 1962; Armstrong *et al.*, 1961).

XIII. OXIDATIVE PHOSPHORYLATION

Having seen the central role that fungi, primarily yeast, have played in the discovery and elucidation of the mechanism of cellular respiration, it comes as a surprise to find that practically all the recent work on oxidative phosphorylation has been concentrated on animal tissues and bacteria. It seems that research has taken this turn mainly because of the difficulties encountered in breaking yeast cells or mold mycelium. There are drastic methods for breaking the cells, such as treatment with acetone or ammonium hydroxide, or drying and autolyzing, which might be suitable for the preparation of certain enzymes but are out of question for phosphorylating systems. Better methods have been designed, including rapid shaking (Nossal, 1953; Utter *et al.*, 1958) or blending (Vitols and Linnane, 1961), which can yield actively phosphorylating particles from yeast. Dissolving fungal cell walls with snail enzyme (Eddy and Williamson, 1957; Chaix, 1961; Aguirre and Villanueva, 1962) seems to be a promising approach as well. For methods of isolating mitochondria, see also Allfrey (1959).

The phosphorylating yeast particles prepared by Vitols and Linnane and by Nossal were, as a rule, spun down at 20,000–25,000 *g* for 15–30 minutes, after the cell debris had been removed. Vitols and Linnane (1961) used Sucrose-Tris-Versene medium for the isolation of the particles. By further fractionation a lighter phosphorylating fraction and a heavier fraction containing tricarboxylic acid cycle activity were obtained. The best P:O ratios achieved in succinate oxidizing yeast particles thus far has been about 1.6, in comparison to 2 in liver mitochondria. The P:O ratio during NADH oxidation was not any higher than for succinate in yeast particles, although in mammalian ones values approaching 3 have been obtained. Mackler *et al.* (1962) also prepared yeast particles, but homogenized them further and obtained a fraction of much smaller "electron transport particles" lacking phosphorylating activity.

Among the other fungi, oxidative phosphorylation has been observed only in isolated mitochondria from *Aspergillus oryzae*, the Oriental counterpart of bakers' yeast, by Iwasa (1960) with P:O ratios up to 1.6, and in mitochondria from *Allomyces macrogynus* by B. A. Bonner and Machlis (1957) with very low P:O values. The abundance and large size of *Allomyces* mitochondria has been pointed out by Ritchie and Hazeltine (1953).

Inhibitor studies on oxidative phosphorylation have been pursued ever since the "uncoupling" effect of 2,4-dinitrophenol, arsenate (cf. Simon, 1953; Lardy, 1956; Laties, 1957) and dicoumarol (cf. Slater *et al.*, 1961) was observed. In the presence of these chemicals, electron transport

may proceed, usually at an accelerated rate, without a concomitant production of adenosine triphosphate from adenosine diphosphate.

Related studies concerning the phosphorus turnover in cells were aimed at explaining the Pasteur effect, that is, the suppression of fermentation by respiration. The explanation of this phenomenon is generally based now on a competition between the various phosphorylating systems for adenosine diphosphate (ADP), and in certain cases for inorganic phosphate [cf. Dickens (1959); Ciba Foundation (1959b); Cold Spring Harbor (1961) Symposium on Cellular Regulatory Mechanisms; Society of General Physiologists Symposia on Control Mechanisms: D. M. Bonner, 1961; and Wright, 1963].

Another approach to oxidative phosphorylation was used by Chance and collaborators, who measured the oxidation-reduction levels of the various electron transport components spectrophotometrically after the exhaustion of ADP in the system. Since in a well-coupled mitochondrion the lack of ADP slows down the electron flow, the exhaustion of available ADP causes the electrons to accumulate temporarily on the substrate side of a phosphorylation site, and to be depleted on the oxygen side of the same site. Thus the respiratory component on the substrate side will become more reduced, and the component on the oxygen side will become more oxidized, with respect to their conditions previous to the exhaustion of ADP. This opposite change in neighboring components has been called the "crossover phenomenon" (cf. Chance and Williams, 1956; Chance, 1961a). Three phosphorylating sites have been suggested on this basis in liver mitochondria during the oxidation of NAD(P)H, including sites between NAD(P)H and flavoprotein, between cytochromes b and c_1, and between cytochromes c and a. The same three crossover points were shown in intact yeast cells oxidizing ethyl alcohol (Chance, 1959a). The evidence for distinguishing between cytochromes c and c_1 is based on low-temperature "frozen steady-state" spectra (Chance and Spencer, 1959). From the amount of adenine nucleotides in yeast cells, and from the amount of O_2 needed to exhaust the ADP supply when glucose is added to starved cells, Chance (1959b) estimated the effective P:O ratio in this case to be about 1.

The reversibility of electron transport and oxidative phosphorylation has received a great deal of attention recently (Klingenberg, 1961; Chance, 1961b). This property of the respiratory system has been invoked to explain the strange oxidation-reduction behavior of cytochrome b in yeast (Chance and Maitra, 1963; Slater and Colpa-Boonstra, 1961).

The various theoretical mechanisms proposed for oxidative phosphorylation are reviewed by Racker (1961) and by Lehninger and Wadkins (1962); and for phosphohistidine as the main intermediate in the synthesis of high-energy phosphate bonds, see the review by Boyer (1963).

XIV. STRUCTURE OF FUNGAL MITOCHONDRIA

The relationship between the various enzymatically active particles obtained from mitochondria and the actual macromolecular and microscopic structure of the mitochondria still remains to be established. Probably none of the particulate fractions of yeast mentioned in the previous section correspond to intact mitochondria, but it is difficult to tell exactly which portions of the mitochondria they do represent.

Mitochondrial structure has been carefully studied in recent years in animal tissues (cf. Palade, 1956; Sjöstrand, 1960, 1963) and has generally been described in terms of multiple layered membranes of characteristic thickness, ca. 180 Å, which surround the entire structure externally and subdivide it internally. The internal "membrane elements" are the cristae mitochondriales, and they seem to be continuous, in certain regions, with the inner half of the external membrane elements (which is why they are named 'cristae,' i.e., "ridges" or "infoldings"). Two layers of the membrane elements consist of lipid materials, separated by a central protein layer. It has recently been shown (*cf.* Blair *et al.*, 1963; Parsons, 1963) with negatively stained electron microscope preparations that the surface of the cristae are occupied by projecting units with stems and spherical heads (75–80 Å in diameter). The "elementary particle" fraction of Green and co-workers (see also Green and Fleischer, 1962) consists of particles of approximately the same size as the spherical heads of these projections. The difficulty is, however, that the size of these particles is too small to accommodate more than one or two hemoprotein or flavorprotein molecules; the whole electron transport system could certainly not fit into them. Furthermore, when these particles are separated from the mitochondrial membranes by sonic oscillation, practically the total amount of cytochromes (except for some b and c_1) stays with the membrane (Chance *et al.*, 1964). The only fungus in which these particles were shown to occur so far is *Neurospora crassa* (Stoeckenius, 1963).

After long debates on the existence of yeast mitochondria and their identification by means of cytological stains (Janus green B, Altmann's method, tetrazolium compounds; see McClary, 1964), thin-section electron micrographic observations have finally decided the issue. Crystate mitochondria have been observed in various yeasts by several investigators (Agar and Douglas, 1957; Hagedorn, 1957; Yotsuyanagi, 1959; Thyagarajan *et al.*, 1961; Vitols *et al.*, 1961; Marquardt, 1962), and even in the ascospores of the yeast *Nadsonia fulvescens* (Kawakami, 1961). The nonidentity of yeast mitochondria and the refractile granules was acknowledged by Hirano and Lindegren (1961) in agreement with Yotsuyanagi (1955). Mitochondria

were similarly observed in *Allomyces* by Turian and Kellenberger (1956) and in *Neurospora* by Zalokar (1961). The zoospore of *Blastocladiella emersonii* was shown (Cantino *et al.*, 1963) to contain a single mitochondrion at the base of the flagellum.

Moore and McAlear (1962) by electron microscopy surveyed fungus specimens from over fifty genera for mitochondria and reported them to be ubiquitous and typical. Their general shape is globose to cylindric. Cristae may be sparse to numerous, and in all except one case they appeared to be lamellate. In the exception they seemed to be tubular; in no instance were they labyrinthine (as in protozoa). Nicklowitz (1957) and Heitz (1959) both emphasized the presence of tubular, rather than lamellate, crystae in the mitochondria of the slime mold *Badhamia utricularis* and of the basidiomycetes *Coprinus disseminatus* and *Lycoperdon gemmatum*. Tubular cristae were also reported for *Thraustotheca roseum*, while concentrically arranged internal membranes were seen in the mitochondria of *T. aureum* (Goldstein *et al.*, 1964).

XV. ORIGIN OF FUNGAL MITOCHONDRIA

The question how new mitochondria originate in a growing or dividing cell has often been asked, but our knowledge of this process is still very limited. In recent papers, Luck (1963a,b) considers three possibilities for the origin of mitochondria: (1) by division of the existing mitochondria, (2) by *de novo* synthesis, and (3) by rearrangement of already present nonmitochondrial membranous structures. The first two alternatives have, of course, been long debated. Luck attempted to verify one of these three hypotheses by using a cholineless mutant of *Neurospora crassa* and supplying it with C^{14}- or H^3-labeled choline, which is a constituent of lecithin, which, in turn, is a major component of the mitochondrial phospholipids, as we have seen in Section XI. Thus he specifically labeled the mitochondria, and he could follow by autoradiography of isolated fractions the subsequent distribution of the H^3 label among the mitochondrial population that arises in a nonlabeled choline-containing medium. If mitochondria arise *de novo*, then the label should be distributed nonrandomly in subsequent generations, that is, the label in the original mitochondria should not be diluted appreciably. He found, in fact, that the label was distributed at random after three cycles of doubling of the cell mass, and this randomization could not be attributed to the fractionation procedure. This finding excludes *de novo* synthesis and does not favor synthesis from structural precursors, leaving as the most likely possibility that of divisions. This conclusion implies, of course, the continuity of mitochondrial descent, at least during the vegetative growth of *Neurospora*.

A controversy has been developing on a question that has bearing on the continuity of mitochondrial descent in yeast, namely whether anaerobically grown yeast has or has not any mitochondria. Using thin-section electron microscopy, Linnane *et al.* (1962), Wallace and Linnane (1964), and Polakis *et al.* (1964) reported the complete lack of mitochondrial structures; while Morpurgo *et al.* (1964) found mitochondria to be present when the cells were grown anaerobically with ergosterol, and no mitochondria when grown without ergosterol (in this case growth stops in 5–7 generations, as the cells run out of endogenous ergosterol). Ergosterol was supplied to the cells in the other studies, as well, except for that by Wallace and Linnane (1964), so that its presence or absence may not be the sole reason for the discrepant findings. That the number of mitochondria is very much diminished in exponential phase aerobically grown yeast has been shown by Ephrussi *et al.* (1956) and Yotsuyanagi (1962a), but their presence in these cells has never been doubted. Similarly, mitochondria were demonstrated in aerobically grown *petite* mutants of yeast, although their structure was somewhat abnormal and they lacked respiratory function (Ephrussi and Slonimski, 1955; Yotsuyanagi, 1962b).

REFERENCES

Abrams, R., A. M. Altschul, and T. R. Hogness. (1940). *J. Biol. Chem.* **136**:777–794.

Abrams, R., A. M. Altschul, and T. R. Hogness. (1942). *J. Biol. Chem.* **142**:303–316.

Agar, H. D., and H. C. Douglas. (1957). *J. Bacteriol.* **73**:365–375.

Aguirre, M. J. R., and J. R. Villanueva (1962). *Nature* **196**:693–694.

Allen, P. J., and W. H. Price. (1950). *Am. J. Botany* **37**:393–402.

Allfrey, V. (1959). *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 1, pp. 193–290. Academic Press, New York.

Anderson, J. A., F. K. Sun, J. K. McDonald, and V. H. Cheldelin. (1964). *Arch. Biochem. Biophys.* **107**:37-50.

Andreasen, A. A., and T. J. B. Stier. (1953). *J. Cellular Comp. Physiol.* **41**:23–36.

Andreasen, A. A., and T. J. B. Stier. (1954). *J. Cellular Comp. Physiol.* **43**:271–281.

Andreasen, A. A., and T. J. B. Stier. (1956). *J. Cellular Comp. Physiol.* **48**:317–328.

Armstrong, J. McD., J. H. Coates, and R. K. Morton. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 385–388. Pergamon Press, New York.

Armstrong, J. McD., J. H. Coates, and R. K. Morton. (1963). *Biochem. J.* **86**:136–145.

Ball, E. G., and O. Cooper. (1952). *J. Biol. Chem.* **198**:629–638.

Ball, E. G., and O. Cooper. (1957). *J. Biol. Chem.* **226**:755–762.

Ball, E. G., C. F. Strittmatter, and O. Cooper. (1951). *J. Biol. Chem.* **193**:635–647.

Barrett, J. (1961). *Biochim. Biophys Acta* **54**:580–582.

Basford, R. E. (1959). *Biochim. Biophys. Acta* **33**:195–201.

Beinert, H. (1960). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 2, part A, pp. 339–416. Academic Press, New York.

Beinert, H., D. E. Griffiths, D. C. Wharton, and R. H. Sands. (1962). *J. Biol. Chem.* **237**:2337–2346.
Bernhard, K., and H. Albrecht. (1948). *Helv. Chim. Acta* **31**:977–988.
Bernstein, E. H., and W. W. Wainio. (1960). *Arch. Biochem. Biophys.* **91**:138–143.
Bhatnagar, G. M., and P. S. Krishnan. (1960). *Arch. Mikrobiol.* **36**:131–138.
Bhuvaneswaran, C., and A. Sreenivasan. (1961). *Enzymologia* **23**:194–201.
Bishop, N. I. (1961). *In* "Quinones in Electron Transport," Ciba Foundation Symposium pp. 385–404. Little, Brown, Boston, Massachusetts.
Blair, P. V., T. Oda, D. E. Green, and H. Fernández-Morán. (1963). *Biochemistry* **2**:756–764.
Boeri, E., I. Smith, and A. Lindenmayer. (1956). Unpublished experiments.
Bomstein, R., R. Goldberger, and H. Tisdale. (1961). *Biochim. Biophys. Acta* **50**:527–543.
Bonner, B. A., and L. Machlis. (1957). *Plant Physiol.* **32**:291–301.
Bonner, D. M., ed. (1961). "Control Mechanisms in Cellular Processes." Symposium of Soc. Gen. Physiologists. 248 pp. Ronald Press, New York.
Borei, H., and A. Sjödén. (1943). *Naturwissenschaften* **31**:324–325.
Boulter, D. (1957). *J. Gen. Microbiol.* **16**:305–316.
Boulter, D., and A. Burges. (1955). *Experientia* **11**:188–189.
Boulter, D., and E. Derbyshire. (1957). *J. Exptl. Botany* **8**:313–318.
Boulter, D., and H. M. Hurst. (1960). *In* "The Ecology of Soil Fungi" (D. Parkinson and J. S. Waid, eds.), pp. 277–285 and 310. Liverpool Univ. Press, Liverpool, England.
Boyer, P. D. (1960). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 3, Part. B, pp. 353–368. Academic Press, New York.
Boyer, P. D. (1963). *Science* **141**:1147–1153.
Brierley, G. P., and A. J. Merola. (1962). *Biochim. Biophys. Acta* **64**:205–217.
Brown, G. L. (1953). *Acta Chem. Scand.* **7**:435–440.
Cantino, E. C., and M. T. Hyatt. (1953). *J. Bacteriol.* **66**:712–720.
Cantino, E. C., and G. Turian. (1959). *Ann. Rev. Microbiol.* **13**:97–124.
Cantino, E. C., J. S. Lovett, L. V. Leak, and J. Lythgoe. (1963). *J. Gen. Microbiol.* **31**:393–404.
Casida, L. E., Jr., and S. G. Knight. (1954). *J. Bacteriol.* **67**:171–175.
Castor, LaR. N., and B. Chance. (1955). *J. Biol. Chem.* **217**:453–465.
Chaix, P. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 225–233. Pergamon Press, New York.
Chance, B. (1952). *J. Biol. Chem.* **197**:567–576.
Chance, B. (1953a). *J. Biol. Chem.* **202**:383–396
Chance, B. (1953b). *J. Biol. Chem.* **202**:397–406.
Chance, B. (1959a). *J. Biol. Chem.* **234**:3036–3040.
Chance, B. (1959b). *J. Biol. Chem.* **234**:3041–3043.
Chance, B. (1961a). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 597–622. Pergamon Press, New York.
Chance, B. (1961b). *In* "Biological Structure and Function" (T. W. Goodwin and O. Lindberg, eds.), Vol. 2, pp. 119–138. Academic Press, New York.
Chance, B., and P. K. Maitra. (1963). *In* "Control Mechanisms in Respiration and Fermentation" (B. Wright, ed.), pp. 307–332. Ronald Press, New York.
Chance, B., and F. L. Spencer, Jr. (1959). *Discussions Faraday Soc.* **27**:200–205.
Chance, B., and G. R. Williams. (1956). *Advan. Enzymol.* **17**:65–134.
Chance, B., M. Klingenberg, and E. Boeri. (1956). *Federation Proc.* **15**:231.

Chance, B., D. F. Parsons, and G. R. Williams. (1964). *Science* **143**:136–139.
Chantrenne, H. (1944). *Enzymologia* **11**:213–221.
Chantrenne, H. (1955a). *Biochim. Biophys. Acta* **18**:58–62.
Chantrenne, H. (1955b). *Biochim. Biophys. Acta* **16**:410–417.
Chantrenne, H., and C. Courtois. (1954). *Biochim. Biophys. Acta* **14**:397–400.
Chantrenne, H., and S. Devreux. (1959). *Biochim. Biophys. Acta* **31**:134–141.
Chauvet, J. (1943). *Enzymologia* **11**:57–69.
Cheng, S.-C. (1954). *Plant Physiol.* **29**:458–467.
Chin, C. H. (1950). *Nature* **165**:926–927.
Ciba Foundation. (1959a). "Symposium on the Biosynthesis of Terpenes and Sterols," 311 pp. Little, Brown, Boston, Massachusetts.
Ciba Foundation. (1959b). "Symposium on the Regulation of Cell Metabolism," 387 pp. Little, Brown, Boston, Massachusetts.
Ciba Foundation. (1961). "Symposium on Quinones in Electron Transport," 453 pp. Little, Brown, Boston, Massachusetts.
Clavilier, L., G. Péré, P. P. Slonimski, and M. Somlo. (1964). *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, pp. 673–674.
Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.
Commission on Enzymes of the International Union of Biochemistry. (1961). "Report," 159 pp. Pergamon Press, New York.
Conn, E. E. (1960). *In* "Comparative Biochemistry" (M. Florkin and H. S. Mason, eds.), Vol. 1. pp. 441–485. Academic Press, New York.
Corwin, L. M., L. J. Schroeder, and W. G. McCullough. (1957). *Arch. Biochem. Biophys.* **72**:112–118.
Crane, F. L. (1961). *In* "Quinones in Electron Transport," Ciba Foundation Symposium, pp. 36–75. Little, Brown, Boston, Massachusetts.
Crane, F. L. (1962). *Biochemistry* **1**:510–517.
Crewther, W. G., and F. G. Lennox. (1953). *Australian J. Biol. Sci.* **6**:410–427.
Dam, H., and E. Søndergaard. (1960). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 3, Part B, pp. 329–352. Academic Press, New York.
Darby, R. T., and D. R. Goddard. (1950). *Physiol. Plantarum* **3**:435–446.
Davidoff, F., and E. D. Korn. (1963). *J. Biol. Chem.* **238**:3199–3215.
Deinema, M. H. (1961). *Mededel. Landbouwhogeschool Wageningen* **61**:1–54.
Dickens, F. (1959). *Australian J. Exptl. Biol. Med. Sci.* Suppl. to Vol. 37: 11 pp.
Diplock, A. T., J. Green, E. E. Edwin, and J. Bunyan. (1961). *Nature* **189**:749–750.
Dixon, M., and E. C. Webb. (1958). "Enzymes," 782 pp. Academic Press, New York.
Doeg, K. A., S. Krueger, and D. M. Ziegler. (1960). *Biochim. Biophys. Acta* **41**:491–497.
Dolin, M. I. (1961). *In* "The Bacteria" (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. 2, pp. 319–363. Academic Press, New York.
Eddy, A. A. (1958). *In* "The Chemistry and Biology of Yeasts" (A. H. Cook, ed.), pp. 157–249. Academic Press, New York.
Eddy, A. A., and D. H. Williamson. (1957). *Nature* **179**:1252–1253.
Edwin, E. E., J. Green, A. T. Diplock, and J. Bunyan. (1961). *Nature* **189**:750–751.
Ehrenberg, A., and T. Yonetani. (1961). *Acta Chem. Scand.* **15**:1071–1080.
Elkind, M. M., and H. Sutton. (1957). *Arch. Biochem. Biophys.* **72**:96–111.
Elvehjem, C. A. (1931). *J. Biol. Chem.* **90**:111–132.
Ephrussi, B. (1953). "Nucleo-Cytoplasmic Relations in Micro-Organisms," 127 pp. Oxford Univ. Press (Clarendon), London and New York.

Ephrussi, B. (1956). *Naturwissenschaften* **43**:505–511.
Ephrussi, B., and P. P. Slonimski. (1950). *Biochim. Biophys. Acta* **6**:256–267.
Ephrussi, B., and P. P. Slonimski. (1955). *Nature* **176**:1207–1208.
Ephrussi, B., P. P. Slonimski, Y. Yotsuyanagi, and J. Tavlitzki. (1956). *Compt. Rend. Trav. Lab. Carlsberg* **26**:87–102.
Erickson, R. E., K. S. Brown, Jr., D. E. Wolf, and K. Folkers. (1960). *Arch. Biochem. Biophys.* **90**:314–317.
Ernster, L. (1961). *In* "Biological Structure and Function" (T. W. Goodwin and O. Lindberg, eds.), Vol. 2, pp. 139–168. Academic Press, New York.
Erwin, J., and K. Bloch. (1964). *Science* **143**:1006–1012.
Estabrook, R. W. (1956). *J. Biol. Chem.* **223**:781–794.
Estabrook, R. W. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 436–457. Pergamon Press, New York.
Euler, H. von, and R. Blix. (1919). *Z. Physiol. Chem.* **105**:83–114.
Euler, H. von, H. Fink, and H. Hellström. (1927). *Z. Physiol. Chem.* **169**:10–51.
Euler, H. von, H. Hellström, and G. Günther. (1939). *Z. Physiol. Chem.* **258**:47–56.
Falk, J. E., and D. D. Perrin. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 56–71. Pergamon Press, New York.
Falk, J. E., R. Lemberg, and R. K. Morton, eds. (1961). "Haematin Enzymes," 666 pp. Pergamon Press, New York.
Fink, H. (1932). *Z. Physiol. Chem.* **210**:197–219.
Fink, H., and E. Berwald. (1933). *Biochem. Z.* **258**:141–146.
Fischer, H., and J. Hilger. (1924). *Z. Physiol. Chem.* **138**:288–306.
Fischer, H., and K. Schneller. (1924). *Z. Physiol. Chem.* **135**:253–293.
Fleischer, S., H. Klouwen, and G. Brierley. (1961). *J. Biol. Chem.* **236**:2936–2941.
Galzy, P., and P. P. Slonimski. (1957a). *Compt. Rend.* **245**:2423–2426.
Galzy, P., and P. P. Slonimski. (1957b). *Compt. Rend.* **245**:2556–2558.
George, P., and J. S. Griffith. (1959). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 1, pp. 347–389. Academic Press, New York.
Getz, G. S., W. Bartley, W. F. Stirpe, B. M. Notton, and A. Renshaw. (1962). *Biochem. J.* **83**:181–191.
Gloor, U., O. Isler, R. A. Morton, R. Rüegg, and O. Wiss. (1958). *Helv. Chim. Acta* **41**:2357–2362.
Goldfine, H., and K. Bloch. (1963). *In* "Control Mechanisms in Respiration and Fermentation" (B. Wright, ed.), pp. 81–103. Ronald Press, New York.
Goldstein, S., L. Moriber, and B. Hershenov. (1964). *Am. J. Botany* **51**:679.
Granick, S., and D. Mauzerall. (1961). *In* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. 2, pp. 525–616. Academic Press, New York.
Grant, J. K. (1962). *In* "Comparative Biochemistry" (M. Florkin and H. S. Mason, eds.), Vol. 3, pp. 163–203. Academic Press, New York.
Green, D. E. (1959). *Advan. Enzymol.* **21**:73–129.
Green, D. E., and S. Fleischer. (1962). *In* "Horizons in Biochemistry" (M. Kasha and B. Pullman, eds.), pp. 381–420. Academic Press, New York.
Gregolin, C., and A. D'Alberton. (1964). *Biochem. Biophys. Res. Commun.* **14**:103.
Gregolin, C., and A. Ghiretti-Magaldi. (1961). *Biochim. Biophys. Acta* **54**:62–66.
Gregolin, C., and T. P. Singer. (1961). *Biochem. Biophys. Res. Commun.* **4**:189.
Gregolin, C., and T. P. Singer. (1963). *Biochim. Biophys. Acta* **67**:201–218.
Grimm, P. W., and P. J. Allen. (1954). *Plant Physiol.* **29**:369–377.
Haas, F. (1955). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, pp. 699–703. Academic Press, New York.

Hagedorn, H. (1957). *Naturwissenchaften* **44**:641–642.
Hagihara, B., T. Horio, J. Yamashita, M. Nozaki, and K. Okunuki. (1956a). *Nature* **178**:629–630.
Hagihara, B., T. Horio, M. Nozaki, I. Sekuzu, J. Yamashita, and K. Okunuki. (1956b). *Nature* **178**:631–632.
Hardesty, B. A., and H. K. Mitchell. (1963). *Arch. Biochem. Biophys.* **100**:330–334.
Harris, M. (1956). *J. Cellular Comp. Physiol.* **48**:95–112.
Hartman, L., I. M. Morice, and F. B. Shorland. (1962). *Biochem. J.* **82**:76–80.
Hartree, E. F. (1955). *In* "Moderne Methoden der Pflanzenanalyse" (K. Paech and M. V. Tracey, eds.), Vol. IV, pp. 197–245. Springer, Berlin.
Hasegawa, H. (1962a). *J. Biochem.* (*Tokyo*) **52**:5–11.
Hasegawa, H. (1962b). *J. Biochem.* (*Tokyo*) **52**:12–15.
Hatefi, Y. (1963). *Advan. Enzymol.* **25**:275–328.
Hebb, C. Raut, and J. Slebodnik. (1958). *Exptl. Cell Res.* **14**:286–294.
Hebb, C. Raut, J. Slebodnik, T. P. Singer, and P. Bernath. (1959). *Arch. Biochem. Biophys.* **83**: 10–16.
Heitz, E. (1959). *Z. Naturforsch.* **14b**:179–180.
Heyman-Blanchet, T., and P. Chaix. (1959). *Biochim. Biophys. Acta* **35**:85–93.
Heyman-Blanchet, T., F. Zajdela, and P. Chaix. (1959). *Biochim. Biophys. Acta* **36**:569–570.
Hinkson, J. W., and H. R. Mabler. (1963). *Biochemistry* **2**:209–216.
Hirano, T., and C. C. Lindegren. (1961). *J. Ultrastruct. Res.* **5**:321–327.
Hockenhull, D. J. D., M. Herbert, A. D. Walker, G. D. Wilkin, and F. G. Winder. (1954). *Biochem. J.* **56**:73–82.
Holton, R. W. (1960). *Plant Physiol.* **35**:757–766.
Hübscher, G., M. Kiese, and R. Nicolas. (1954). *Biochem. Z.* **325**:223–238.
Huennekens, F. M. (1956). *Experientia* **12**:1–6.
Huennekens, F. M., S. P. Felton, N. Appaji Rao, and B. Mackler. (1961). *J. Biol. Chem.* **236**:PC57–PC59.
Imamoto, F., K. Iwasa, and K. Okunuki. (1959). *J. Biochem.* (*Tokyo*) **46**:141–150.
Iwasa, K. (1960). *J. Biochem.* (*Tokyo*) **47**:445–453, 484–491.
Iwasa, K., F. Imamoto, and K. Okunuki. (1959). *J. Biochem.* (*Tokyo*) **46**:113–120.
Iwatsubo, M., and F. Labeyrie. (1962). *Biochim. Biophys. Acta* **59**:614–623.
Kaplan, J. G. (1962). *Nature* **196**:950–952.
Kattermann, R., and P. P. Slonimski. (1960). *Compt. Rend.* **250**:220–221.
Kawakami, N. (1961). *Exptl. Cell Res.* **25**:179–181.
Kearney, E. B. (1960). *J. Biol. Chem.* **235**:865–877.
Keilin, D. (1925). *Proc. Roy. Soc.* **B98**:312–339.
Keilin, D. (1930). *Proc. Roy. Soc.* **B106**:418–444.
Keilin, D. (1953). *Nature* **172**:390–392.
Keilin, D., and C. H. Harpley. (1941). *Biochem. J.* **35**:688–692.
Keilin, D., and E. F. Hartree. (1939). *Proc. Roy. Soc.* **B127**:167–191.
Keilin, D, and E. F. Hartree. (1949). *Nature* **164**:254–259.
Keilin, D., and E. F. Hartree. (1951). *Biochem. J.* **49**:88–104.
Keilin, D., and E. F. Hartree. (1955). *Nature* **176**:200–206.
Keilin, D., and A. Tissières. (1953). *Nature* **172**:393–394.
Keilin, D., and A. Tissières. (1954). *Biochem. J.* **57**:xxix.
Kench, J. E., and J. W. Wilkinson. (1945). *Nature* **155**:579–580.
Kidder, G. W., III. (1961). Ph.D. dissertation, Univ. of Pennsylvania, Philadelphia, Pennsylvania.

Kikuchi, G., and E. S. G. Barron. (1959). *Arch. Biochem. Biophys.* **84**:96–105.
King, T. E., R. L. Howard, D. F. Wilson, and J. C. R. Li. (1962). *J. Biol. Chem.* **237**:2941–2946.
Klein, H. P. (1957). *J. Bacteriol.* **73**:530–537.
Klein, H. P. (1960). *J. Bacteriol.* **80**:665–672.
Klingenberg, M. (1961). *In* "Biological Structure and Function" (T. W. Goodwin and O. Lindberg, eds.), Vol. 2, pp. 227–240. Academic Press, New York.
Krebs, H. A., and J. M. Lowenstein. (1960). *In* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. 1, pp. 129–203. Academic Press, New York.
Labeyrie, F., P. P. Slonimski, and L. Naslin. (1959). *Biochim. Biophys. Acta* **34**:262–265.
Lardy, H. (1956). *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* pp. 287–294. Academic Press, New York.
Laties, G. G. (1957). *In* "Survey of Biological Progress" (B. Glass, ed.), Vol. 3, pp. 215–299. Academic Press, New York.
Lavate, W. V., J. R. Dyer, C. M. Springer, and R. Bentley. (1962). *J. Biol. Chem.* **237**:PC2715–PC2716.
Leegwater, D. C., and B. M. Craig. (1962). *Can. J. Biochem. Physiol.* **40**:847–855.
Lehninger, A. L., and C. L. Wadkins. (1962). *Ann. Rev. Biochem.* **31**:47–78.
Lemberg, R. (1961). *Advan. Enzymol.* **23**:265–322.
Lemberg, R., and J. W. Legge. (1949). "Hematin Compounds and Bile Pigments," 748 pp. Wiley (Interscience), New York.
Lemberg, R., P. S. Clezy, and J. Barrett. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 344–357. Pergamon Press, New York.
Lenhoff, H. M., and N. O. Kaplan. (1956). *J. Biol. Chem.* **220**:967–982.
Lester, R. L. (1963). *Federation Proc.* **22**:415.
Lester, R. L., and F. L. Crane. (1959). *J. Biol. Chem.* **234**:2169–2175.
Lester, R. L., F. L. Crane, and Y. Hatefi. (1958). *J. Am. Chem. Soc.* **80**:4751–4752.
Lester, R. L., Y. Hatefi, C. Widmer, and F. L. Crane. (1959). *Biochim. Biophys. Acta* **33**:169–185.
Lindegren, C. C., and S. Hino. (1957). *Exptl. Cell Res.* **12**:163–168.
Lindenmayer, A. (1959). *J. Cellular Comp. Physiol.* **53**:93–118.
Lindenmayer, A., and R. W. Estabrook. (1958). *Arch. Biochem. Biophys.* **78**:66–82.
Lindenmayer, A., and L. Smith. (1957). *Federation Proc.* **16**:212.
Lindenmayer, A., and L. Smith. (1964). *Biochim. Biophys. Acta* **93**:445–461.
Linnane, A. W., and J. L. Still. (1955a). *Arch. Biochem. Biophys.* **56**:264–265.
Linnane, A. W., and J. L. Still. (1955b). *Arch. Biochem. Biophys.* **59**:383–392.
Linnane, A. W., E. Vitols, and P. G. Nowland. (1962). *J. Cell Biol.* **13**:345–349.
Luck, D. J. L. (1963a). *Proc. Natl. Acad. Sci. U.S.* **49**:233–240.
Luck, D. J. L. (1963b). *J. Cell. Biol.* **16**:483–499.
Ludwig, G., and S. A Kuby. (1955). *Federation Proc.* **14**:247.
Lyr, H.(1955). *Planta* **46**:408–413.
Lyr, H. (1956). *Planta* **48**:239–265.
McClary, D. O. (1964). *Botan. Rev.* **30**:167–225.
McDonald, J. K., J. A. Anderson, V. H. Cheldelin, and T. E. King. (1963). *Biochim. Biophys. Acta* **73**:533–549.
McHale, D., J. Green, and A. T. Diplock. (1962). *Nature* **196**:1293–1294.
McHargue, J. S., and R. K. Calfee. (1931). *Plant Physiol.* **6**:559–566.
Mackler, B. (1961). *Biochim. Biophys. Acta* **50**:141–146.

Mackler, B., P. J. Collipp, H. M. Duncan, N. Appaji Rao, and F. M. Huennekens. (1962). *J. Biol. Chem.* **237**:2968–2974.
Mahler, H. R. (1961). *In* "Mineral Metabolism" (C. L. Comar and F. Bronner, eds.), Vol. 1, Part 1B, pp. 743–879. Academic Press, New York.
Mahler, H. R., and A. de S. Pereira. (1962). J. Mol. Biol. **5**:325–347.
Mahler, H. R., B. Mackler, S. Grandchamp, and P. P. Slonimski. (1964a) *Biochemistry* **3**:668–677.
Mahler, H. R., G. Neiss, P. P. Slonimski, and B. Mackler. (1964b). Biochemistry **3**:893–895.
Mann, T. (1944). *Biochem. J.* **38**:339–345.
Margoliash, E. (1962). *J. Biol. Chem.* **237**:2161–2174.
Marinetti, G. V., J. Erbland, J Kochen, and E. Stotz. (1958). *J. Biol. Chem.* **233**:740–742.
Marquardt, H. (1962). *Z. Naturforsch.* **17b**:42–48.
Martin, S. M. (1954). *Can. J. Microbiol.* **1**:6–11.
Mason, H. S. (1957). *Advan. Enzymol.* **19**:79–233.
Melnick, J. L. (1941). *J. Biol. Chem.* **141**:269–281.
Mickelson, M. N. (1950). *J. Bacteriol.* **59**:659–666.
Moore, R. T., and J. H. McAlear. (1962). *Abstr. 8th Intern. Congr. Microbiol., Montreal, 1961* p. 31 (*Biol. Abstr.* **41**:8724).
Morpurgo, G., G. Serlupi-Crescenzi, G. Tecce, F. Valente, and D. Venetacci. (1964). *Nature* **201**:897–899.
Morton, R. A. (1961). *Vitamins Hormones* **19**:1–42.
Morton, R. K. (1961). *Nature* **192**:727–731.
Morton, R. K., J. McD. Armstrong, and C. A. Appleby. (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 501–523 Pergamon Press, New York.
Nagai, S., N. Yanagishima, and H. Nagai. (1961). *Bacteriol. Rev.* **25**:404–426.
Neilands, J. B. (1952). *J. Biol. Chem.* **197**:701–708.
Neilands, J. B. (1957). *Bacteriol. Rev.* **21**:101–111.
Nicholas, D. J. D. (1956). *Nature* **178**:148–149.
Nicholas, D. J. D., A. Nason, and W. D. McElroy. (1954). *J. Biol. Chem.* **207**:341–351.
Nicklowitz, W. (1957). *Exptl. Cell Res.* **13**:591–595.
Niederpruem, D. J., and D. P. Hackett. (1961). *Plant Physiol.* **36**:79–84.
Nosoh, Y. (1964). *Arch. Biochem. Biophys.* **105**:439–445.
Nossal, P. M. (1953). *Australian J. Exptl. Biol. Med. Sci.* **31**:583–589.
Nossal, P. M., D. B. Keech, and D. J. Morton. (1956). *Biochim. Biophys. Ac* **22**:412–421.
Nossal, P. M., I. A. Hansen, and J. N. Ladd. (1957). *Biochim. Biophys. Ac* **23**:103–115.
Nygaard, A. P. (1961a). *J. Biol. Chem.* **236**:920–925.
Nygaard, A. P. (1961b). *J. Biol. Chem.* **236**:1585–1588.
Nygaard, A. P. (1963). *In* "Control Mechanisms in Respiration and Fermentatio (B. Wright, ed.), pp. 27–46. Ronald Press, New York.
Ohta, J. (1954). *J. Biochem. (Tokyo)* **41**:489–497.
Okunuki, K. (1961). *Advn. Enzymol.* **23**:29–82.
Okunuki, K., B. Hagihara, I. Sekuzu, and T. Horio. (1958). *Proc. Intern. Syn Enzyme Chem., Tokyo Kyoto, 1957* pp. 264–272. Maruzen, Tokyo.

Olson, R. F., R. Bentley, A. S. Aiyar, G. H. Dialameh, P. H. Gold, V. G. Ramsey, and C. M. Springer. (1963). *J. Biol. Chem.* **238**:PC 3146–3148.
Omura, T., and R. Sato. (1964). *J. Biol. Chem.* **239**:2370–2378, 2379–2385.
Orii, Y., I. Sekuzu, and K. Okunuki. (1962). *J. Biochem. (Tokyo)* **51**:204–215.
Owens, R. G. (1955). *Contrib. Boyce Thompson Inst.* **18**:145–152.
Packter, N. M., and J. Glover. (1960). *Nature* **187**:413–414.
Palade, G. E. (1956). *In* "Enzymes: Units of Biological Structure and Function" (O. H. Gaebler, ed.), pp. 185–215. Academic Press, New York.
Paléus, S., and H. Tuppy (1961). *In* "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 363–369. Pergamon Press, New York.
Parker, J. (1959). *Biochim. Biophys. Acta* **35**:496–509.
Parks, L. W., and P. R. Starr. (1963). *J. Cellular Comp. Physiol.* **61**:61–65.
Parsons, D. F. (1963). *Science* **140**:985–987.
Paul, K.-G. (1951). *In* "The Enzymes" (J. B. Sumner and K. Myrbäck, eds.), Vol. 2, Part 1, pp. 357–396. Academic Press, New York.
Paul, K.-G. (1960). *In* "The Enzymes" (P. D Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 3, Part B, pp. 277–328. Academic Press, New York.
Pett, L. B. (1936). *Biochem. J.* **30**:1438–1145.
Pittenger, T. H. (1956). *Proc. Natl. Acad. Sci. U.S.* **42**:747–752.
Polakis, E. S., W. Bartley, and G. A. Meek. (1964). *Biochem. J.* **90**:369–374.
Racker, E. (1961). *Advan. Enzymol.* **23**:323–399.
Raut, C. (1953). *Exptl. Cell Res.* **4**:295–305.
Redfearn, E. R. (1961). *In* "Biological Structure and Function" (T. W. Goodwin and O. Lindberg, eds.), Vol. 2, pp. 181–192. Academic Press, New York.
Redfearn, F. R., and J. Friend. (1961). *Nature* **191**:806–807.
Ringler, R. L., S. Minakami, and T. P. Singer. (1963). J. Biol. Chem. **238**:801–810.
Ritchie, D., and P. Hazeltine. (1953). *Exptl. Cell Res.* **5**:261–274.
Rudney, H., and T. Sugimura. (1961). *In* "Quinones in Electron Transport," Ciba Foundation Symposium, pp. 211–227. Little, Brown, Boston, Massachusetts.
Sano, S., and S. Granick. (1961). *J. Biol. Chem.* **236**:1173–1180.
Schade, A. L., and K. V. Thimann. (1940). *Am. J. Botany* **27**:659–670.
Schaeffer, P. (1952). *Biochim. Biophys. Acta* **9**:261–270, 362–368.
Sels, A. A. (1958a). *Compt. Rend. Soc. Biol.* **152**:1204–1205.
Sels, A. A. (1958b). *Arch. Intern. Physiol. Biochim.* **66**:127.
Shavlovskii, G. M. (1959). *Tr. Inst. Mikrobiol., Akad. Nauk SSSR* **6**:157–174 (*Biol. Abstr.* **36**:61513).
Shavlovskii, G. M., and A. M. Bogatchuk. (1960) *Biokhimiya* **25**:1043–1045 (*Biol. Abstr.* **36**:82067)
Shepherd, C. J. (1951). *Biochem. J.* **48**:483–486.
Sherman, F. (1959). *J. Cellular Comp. Physiol.* **54**:37–52.
Shoup, C. S., and F. T. Wolf. (1946). *J. Cellular Comp. Physiol.* **28**:365–379.
Sih, C. J., P. B. Hamilton, and S. G. Knight. (1958). *J. Bacteriol.* **75**:623–628.
Simon, E. W. (1953). *Biol. Rev. Cambridge Phil. Soc.* **28**:453–479.
Singer, T. P. (1961). *In* "Biological Structure and Function" (T. W. Goodwin and O. Lindberg, eds.), Vol. 2, pp. 103–118. Academic Press, New York.
Singer, T. P., E. B. Kearney, and V. Massey. (1957a). *Advan. Enzymol.* **18**:65–111.
Singer, T. P., V. Massey, and E. B. Kearney. (1957b). *Arch. Biochem. Biophys.* **69**:405–421.
Singer, T. P., E. B. Kearney, C. Gregolin, E. Boeri, and M. Rippa. (1961). *Biochim. Biophys. Acta* **54**:52–61.

Singer, T. P., C. Gregolin, and T. Cremona. (1963). *In* "Control Mechanisms Respiration and Fermentation" (B. Wright, ed.), pp. 47–79. Ronald Press, Ne York.
Sjöstrand, F. S. (1960). *Radiation Res.* Suppl. 2, pp. 349–383.
Sjöstrand, F. S. (1963). *J. Ultrastruct. Res.* **9**:340–361.
Slater, E. C. (1958a). *Advan. Enzymol.* **20**:147–199.
Slater, E. C. (1958b). *Biochem. Soc. Symp. (Cambridge, Engl.)* **15**:76–102.
Slater, E. C. (1962). *Vitamins Hormones* **20**:521–525.
Slater, E. C., and J. P. Colpa-Boonstra. (1961). *In* "Haematin Enzymes" (J. Falk, R. Lemberg, and R. K. Morton, eds.), pp. 575–592. Pergamon Press, Ne York.
Slater, E. C., J. P. Colpa-Boonstra, and J. Links (1961). "Quinones in Electr Transport," Ciba Foundation Symposium, pp. 161–185. Little, Brown, Bosto Massachusetts.
Slonimski, P. P. (1952). *Compt. Rend.* **235**:1064–1066.
Slonimski, P. P. (1953). *Actualites Biochim.* **17**:203.
Slonimski, P. P. (1956). *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955*, pp. 24 252. Academic Press, New York.
Slonimski, P. P., and W. Tysarowski. (1958). *Compt. Rend.* **246**:1111–1114.
Smith, L. (1954a). *Arch. Biochem. Biophys.* **50**:285–298.
Smith, L. (1954b). *Arch. Biochem. Biophys.* **50**:299–314.
Smith, L. (1954c). *Arch. Biochem. Biophys.* **50**:315–321.
Smith, L. (1954d). *Bacteriol. Rev.* **18**:106–130.
Smith, L. (1955). *J. Biol. Chem.* **215**:833–846.
Smith, L. (1961). *In* "The Bacteria" (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. pp. 365–396. Academic Press, New York.
Smith, L., and H. Conrad. (1956). *Arch. Biochem. Biophys.* **63**:403–413.
Somlo, M. (1962). *Biochim. Biophys. Acta* **65**:333–346.
Stevenson, I., P. J. Hayward, F. W. Hemming, and R. A. Morton. (1962). *Natu* **196**:1291–1293.
Stier, T. J. B., and J. G. B. Castor. (1941). *J. Gen. Physiol.* **25**:229–233.
Stoeckenius, W. (1963). *J. Cell Biol.* **17**:443–454.
Stotz, E. H., M. Morrison, and G. Marinetti. (1956). *In* "Enzymes: Units of Biolo ical Structure and Function" (O. H. Gaebler, ed.), pp. 401–416. Academic Pre New York.
Strong, F. M., J. P. Dickie, M. E. Loomans, E. E. van Tamelen, and R. S. Dewe (1960). *J. Am. Chem. Soc.* **82**:1513–1514.
Sugimura, T., and H. Rudney. (1960). *Biochim. Biophys. Acta* **37**:560–561.
Sugimura, T., K. Okabe, and H. Rudney. (1964). *Biochim. Biophys. Acta* **82**:35 354.
Sussman, A. S. (1961). *Quart. Rev. Biol.* **36**:109–116.
Sussman, A. S., and C. L. Markert. (1953). *Arch. Biochem. Biophys.* **45**:31–40.
Takemori, S., and T. E. King. (1962). *Biochim. Biophys. Acta* **64**:192–194.
Tamiya, H. (1928). *Acta Phytochim. (Japan)* **4**:215–218.
Tamiya, H. (1942). *Advan. Enzymol.* **2**:183–238.
Tamiya, H. (1958). *Proc. Intern. Symp. Enzyme Chem., Tokyo Kyoto, 1957* 21–24. Maruzen, Tokyo.
Theorell, H. (1951). *In* "The Enzymes" (J. B. Sumner and K. Myrbäck, eds.), Vo Part 1, pp. 397–427. Academic Press, New York.

Thyagarajan, T. R., S. F. Conti, and H. B. Naylor, (1961). *Exptl. Cell Res.* **25**:216–218.
Tissières, A. (1954). *Biochem. J.* **58**:142–146.
Tissières, A., and H. K. Mitchell. (1954). *J. Biol. Chem.* **208**:241–249.
Tissières, A., H. K. Mitchell, and F. A. Haskins. (1953). *J. Biol. Chem.* **205**:423–433.
Townsley, P. M., and J. B. Neilands. (1956). *J. Biol. Chem.* **224**:695–705.
Turian, G. (1960). *Pathol. Microbiol.* **23**:687–699.
Turian, G., and K. Kellenberger. (1956). *Exptl. Cell Res.* **11**:417–422.
Utter, M. F., D. B. Keech, and P. M. Nossal. (1958). *Biochem. J.* **68**:431–440.
Vanderwinkel, E., S. Bourgeois, and J. M. Wiame. (1958). *Arch. Intern. Physiol. Biochim.* **66**:129–130.
Vishniac, H. S. (1955). *J. Gen. Microbiol.* **12**:464–472.
Vitols, E., and A. W. Linnane. (1961). *J. Biophys. Biochem. Cytol.* **9**:701–710.
Vitols, E., R. J. North, and A. W. Linnane. (1961). *J. Biophys. Biochem. Cytol.* **9**:689–699.
Wainio, W. W., and S. J. Cooperstein. (1956). *Advan. Enzymol.* **17**:329–392.
Wallace, P. G., and A. W. Linnane. (1964). *Nature* **201**:1191–1194.
Warburg, O. (1949). "Heavy Metal Prosthetic Groups and Enzyme Action." 230 pp. Oxford Univ. Press (Clarendon), London and New York.
Warburg, O., and E. Haas. (1934). *Naturwissenschaften* **22**:207.
Warburg, O., and E. Negelein. (1929). *Biochem. Z.* **214**:64–100.
Warburg, O., and E. Negelein. (1934). *Naturwissenschaften* **22**:206–207.
Ward, J. M (1958). *Science* **127**:596.
Ward, J. M., and W. J. Nickerson. (1958). *J. Gen. Physiol.* **41**:703–724.
White, G. A., and G. A. Ledingham. (1960). *Plant Physiol.* **35**:xi.
Wolf, R. T. (1947). *Arch. Biochem.* **13**:83–92.
Wright, B. E., ed. (1963). "Control Mechanisms in Respiration and Fermentation," Symposium of Soc. Gen. Physiologists, 357 pp. Ronald Press, New York.
Yamanaka, T., T. Horio, and K. Okunuki. (1960). *Biochim. Biophys. Acta* **40**:349–351.
Yamanaka, T., H Nakajima, and K. Okunuki. (1962). *Biochim. Biophys Acta* **63**:510–512.
Yčas, M. (1956). *Exptl. Cell Res.* **11**:1–6.
Yčas, M., and L. L. Drabkin. (1957). *J. Biol. Chem.* **224**:921–933.
Yčas, M., and T. J. Starr. (1953). *J. Bacteriol.* **65**:83–88.
Yonetani, T. (1960a). *J. Biol. Chem.* **235**:845–852.
Yonetani, T. (1960b). *J. Biol. Chem.* **235**:3138–3143.
Yonetani, T., and G. W. Kidder, III. (1963). *J. Biol. Chem.* **238**:386–388.
Yoshikawa, H. (1937). *J. Biochem. (Tokyo)* **25**:627–655.
Yotsuyanagi, Y. (1955). *Nature* **176**:1208–1209.
Yotsuyanagi, Y. (1959). *Compt. Rend.* **248**:274–277.
Yotsuyanagi, Y. (1962a). *J. Ultrastruct. Res.* **7**:121–140.
Yotsuyanagi, Y. (1962b). *J. Ultrastruct. Res.* **7**:141–158.
Zalokar, M. (1961). *J. Biophys. Biochem. Cytol.* **9**:609–614.

ADDENDUM TO SECTION VII

Further extensive work on the various yeast mutants unable to utilize nonfermentable carbon sources was reported by F. Sherman [*Genetics* 48:375–385 (1963); 49: 39–48 (1964)], and by F. Sherman and P. P. Slonimski [*Biochim. Biophys. Acta* 90: 1–15 (1964)]. In anaerobically grown yeast, T. Heyman-Blanchet, L. Ohaniance, and P. Chaix [*Biochim. Biophys. Acta* 81:462–472 (1964)] failed to find any cytochrome 590, instead they reported a pigment with low-temperature absorption at 582.5, 545, and 422 mμ, and considered it to be a Zn-protoporphyrin compound. They confirmed the presence of the pigment in these cells with the main absorption band at 503 mμ (cf. Fig. 7), and of cytochrome 556, which was also extracted in soluble form [P. Chaix and P. Labbé, *Compt. Rend.* 258:1645–1647 (1964)].

ADDENDUM TO SECTION XV

The hypothesis that mitochondria arise only from other mitochondria or "promitochondria" and carry their own hereditary DNA, has been discussed in detail by A. Gibor and S. Granick [*Science* 145:890–897 (1964)]. For yeast, evidence in favor of mitochondrial DNA and precursor particles was produced by G. Schatz [*Biochem. Biophys. Res. Commun.* 12:448–451 (1963)], and by G. Schatz, E. Haslbrunner, and H. Tuppy [*Biochem. Biophys. Res. Commun.* 15:127–132 (1964)].

CHAPTER 13

Utilization of Inorganic Nitrogen Compounds and Amino Acids by Fungi

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I. INTRODUCTION

Fungi usually utilize simple inorganic nitrogen compounds. Thus ammonium salts or nitrates are suitable sources of nitrogen for most fungi although with nitrate there may be a lag in growth before the necessary enzymes concerned in nitrate reduction are produced.

In considering the metabolism of inorganic nitrogen compounds by fungi, a useful comparison may be made with the bacterial systems, which have received more attention. Thus a variety of bacteria and fungi use nitrate in one of two ways: (1) *assimilation* into cell nitrogen, or (2) *dissimilation* or *nitrate respiration*, where nitrate acts as an alternative hydrogen acceptor to oxygen. There is no definite evidence, however, that fungi convert nitrate to a mixture of nitrogen gas and its oxides by a process of denitrification as do some bacteria.

The oxidation of ammonia to nitrite and nitrate is carried out by chemoautotrophic nitrifying bacteria. Certain heterotrophic fungi also oxidize ammonia to nitrate, but at much slower rates.

Even in the specialized field of nitrogen fixation there is a parallel between bacteria and fungi since the yeasts *Rhodotorula* and *Pullularia* fix atmospheric nitrogen.

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The metabolism of amino acids and protein synthesis in fungi have many features in common with bacteria.

II. PHYSIOLOGICAL STUDIES

A. Nitrate

Most groups of fungi utilize nitrate nitrogen although some do not, e.g., some members of the Saprolegniaceae, Blastocladiales, and some higher basidiomycetes. Most actinomycetes use nitrate as a sole source of nitrogen, but even here there are notable exceptions. It is difficult to make a proper appraisal of this type of information since most of the data are based on spore inoculation tests with media containing nitrate, and relatively few tests have been made with mycelia pregrown with other nitrogen sources. Where this has been done the nitrate-reducing enzymes have been induced in the preformed mycelium. Another difficulty is that the oxygen pressure in the culture medium is seldom defined. It is now known that some fungi grown with restricted air supply dissimilate nitrate rapidly as an alternative hydrogen acceptor to oxygen, a process sometimes resulting in the accumulation of toxic amounts of free nitrite (10^{-3} to $10^{-2}M$) in the medium (Walker and Nicholas, 1961b). The effects of nitrite on the growth of preformed mycelium of *Neurospora* reported in Table I show that it is inhibited at 5×10^{-3} *M* nitrite or above.

TABLE I

TOXIC EFFECTS OF ADDED NITRITE TO MYCELIUM OF *Neurospora crassa* GROWN IN CULTURE SOLUTIONS WITH AMMONIUM AS SOLE NITROGEN SOURCE[a]

Molarity of $NaNO_2$ added	Increase in dry weight between 24 and 48 hours	Per cent reduction in yield of compared with controls	Nitrite in water extracts (mμmoles/mg protein)
0	1.20	0	0
1×10^{-4}	1.20	0	0
1×10^{-3}	1.05	0	30
5×10^{-3}	0.75	35	48
1×10^{-2}	0.48	60	85
5×10^{-2}	0.30	75	90

[a] The fungus was grown for 24 hours at 25°C in a KNO_3 medium when various concentrations of $NaNO_2$ were added aseptically *in vivo* and the pH of the medium was adjusted to 8.0 with *N* NaOH. After a further 24 hours of incubation the mycelium was harvested, dried, and weighed. Nitrite and protein were determined in crude homogenates of fresh mycelia.

B. Nitrite

Fungi are usually more tolerant of nitrous acid than are bacteria, presumably because they are better able to grow at a lower pH. Nitrite is used readily by some fungi, e.g., *Fusarium niveum, Coprinus* sp., *Phymatotrichum omnivorum, Scopulariopsis brevicaulis*, and *Rhizophlyctis rosea*. Failure to utilize nitrite by fungi may in part result from a pH effect since growth is best in alkaline medium, indicating that the nonionized acid rather than the nitrite ion is toxic. In some fungi, nitrite toxicity results in the accumulation of pyruvic acid (Nord and Mull, 1945). Since nitrite does not usually accumulate in the medium under aerobic conditions, it must be assimilated at about the same rate as nitrate. Thus *S. brevicaulis* takes up nitrite at 1.2 mg N per gram dry weight per hour, which compares closely with the uptake of nitrate or ammonia under similar conditions. This fungus grows well on potassium nitrite, but its growth response is quite distinct from that on ammonium nitrite. There is no drop in pH during growth with the latter compound, an indication that ammonia and nitrite are taken up simultaneously at comparable rates. A similar relation between nitrite and ammonia uptake was found when ammonia was added to

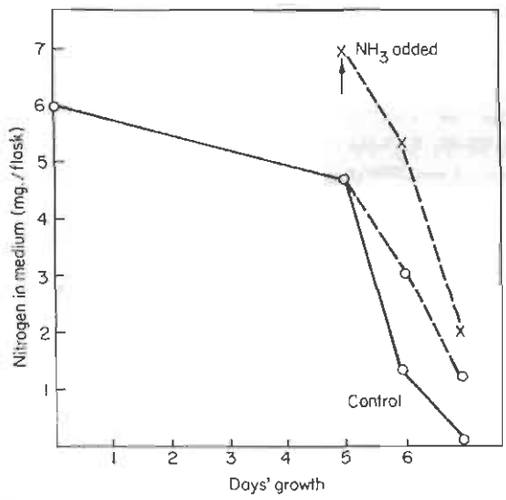


FIG. 1. *Scopulariopsis brevicaulis*. Effect of addition of ammonia on the uptake of nitrite from the medium. O, Nitrite nitrogen; X, ammonia nitrogen; —, culture in nitrite only; – – –, culture in nitrite and ammonia. From Morton and MacMillan (1954).

a rapidly growing culture of *S. brevicaulis* in a nitrite medium. Ammonium sulfate, added when the mycelium was 5 days old, was taken up immediately, and nitrite uptake continued in the presence of ammonia at only a slightly reduced rate (Fig. 1). In the reverse experiment the addition of nitrite to a culture growing in an ammonia medium gave different results. Nitrite at concentrations equivalent to ammonia in a standard medium was somewhat toxic to the fungus unless it had acquired a tolerance by previous growth on high levels of nitrite. The high levels of nitrite inhibited the assimilation of ammonia for about 2 days when the fungus began again to assimilate both forms simultaneously. The inhibitory effects of nitrite on the growth of preformed mycelia of *Neurospora crassa* are shown in Table I.

C. Ammonia

Ammonia, a weak electrolyte, enters cells of many organisms by passive diffusion of its undissociated molecule (Davson and Danielli, 1952). The entry of ammonia into *Valonia* cells is dependent on the concentration difference of the molecule between the cell sap and the external solution and on the supply of cell constituents that combine with ammonia (Osterhout, 1935; Jacques, 1939). Yeast cells are very permeable to the ammonia molecule (Äyräpää, 1950) and, in general, the penetration of undissociated molecules of weak electrolytes is dependent on concentration gradients (Simon and Beevers, 1952). When *Scopulariopsis brevicaulis*, grown in a medium without ammonium, was supplied with an ammonium salt, it entered the cell more rapidly than it was removed by assimilation until an equilibrium level of ammonia was reached which was not related to metabolism but was dependent on the external concentration over a wide range (MacMillan, 1956). The cells were also shown to be permeable to ammonia in the outward direction and the rate of entry or loss was dependent on the difference between the internal and external environment. These results support the view that ammonia enters the cells by the free diffusion of the undissociated molecule. The uptake of ammonia by *Penicillium griseofulvum* and by *Fusarium graminearum* was very similar (see Fig. 2). The ammonia uptake, which was rapid at alkaline pH values, reached an equilibrium level after 1 hour. With *F. graminearum* there was a sharp drop in the ammonia uptake at pH 8.5, presumably because of the death of the cells. It was not established whether the high pH per se, or the high internal amounts of ammonia, or both, produced the toxic effects. The equilibrium levels of ammonia in this fungus are higher than in *Scopulariopsis* under the same conditions; this may mean that the internal pH of the cells is lower in *Fusarium* and the fungus is less sensitive to a low external

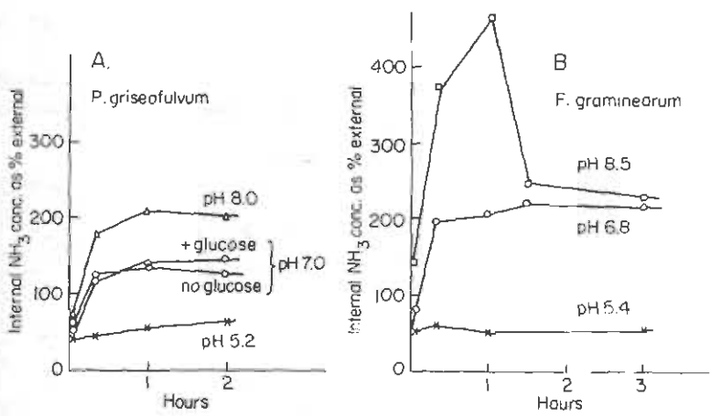


FIG. 2. Ammonia in mycelium of (A) *Penicillium griseofulvum*) and (B) *Fusarium graminearum* shaken in buffers containing 0.4 mg of ammonia nitrogen per milliliter. From MacMillan (1956).

pH. In this respect *Aspergillus niger* resembles *Fusarium*, although here there is a small effect of pH on the internal concentration of ammonia.

D. Ammonium Nitrate

When ammonium nitrate is supplied there is presumptive evidence that ammonia is used first because of a sharp drop in pH during early growth, but no experiments with N^{15}-labeled ammonium or nitrate radicals have been done. Brian *et al.* (1947) reported an unusual pattern in *Myrothecium verrucaria*: when glucose was supplied, it grew readily on ammonium tartrate but scarcely at all on ammonium sulfate. Normal growth occurred when neutral salts of organic acids were added. Growth was as poor on ammonium nitrate as on ammonium sulfate although the fungus grew well on potassium nitrate. Growth on ammonium nitrate was promoted by adding organic acids. Several other species including a strain of *Scopulariopsis brevicaulis* showed the same pattern. Brian *et al.* concluded that in these fungi the assimilation of nitrate is somehow blocked by ammonia, which was therefore used preferentially, and that for the continued assimilation of ammonia one of a number of organic acids is required. The subsequent results of Morton and MacMillan (1954) showed clearly that the primary effect of organic acids on the assimilation of ammonia in fungi was in controlling the pH drift of the culture solution since the fungi used were very sensitive to the adverse effects of low pH. The internal concentration of organic acids in fungi was maintained at levels

that did not limit the uptake of ammonia as long as glucose was being metabolized.

The growth of *S. brevicaulis* on different nitrogen sources is shown in Table II. About 15–20% ammonia was assimilated from ammonium ni-

TABLE II

GROWTH OF *Scopulariopsis brevicaulis* ON DIFFERENT NITROGEN SOURCES (WITH GLUCOSE)

Parameter	Nitrogen source				
	KNO_3	NH_4NO_3	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + tartrate	$(NH_4)_2SO$ + malate
Mean dry matter (mg flask)	200	18	12	160	225
Nitrogen assimilated (% of initial)	100	15 20	15 20	100	100
Range of final pH	8.0 8.1	3.0 3.9	2.7 3.8	3.4-4.4	7.5 8.6

[a] After Morton and MacMillan (1954).

trate or sulfate in the first 4 days of growth, but no more was utilized, even after 40 days or more. The addition of a potassium or a sodium salt of an organic acid permitted complete assimilation of ammonia, although the final dry weight varied with the type of organic acid used. On ammonium nitrate there was an initial uptake of ammonia after which no more nitrogen was absorbed. This effect resulted from a low pH since the uptake of either ammonia or nitrate at pH 3–4 is low (see Fig. 3).

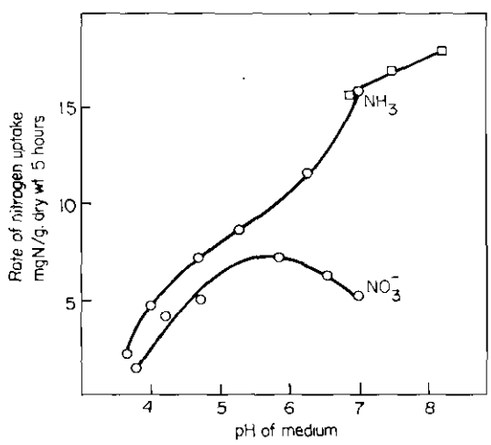


FIG. 3. *Scopulariopsis brevicaulis*. Rate of uptake of ammonia and nitrate in relation to pH of medium. ○—○, 0.1 *M* citrate phosphate buffers; □—□, 0.1 *M* pyrophosphate buffer (separate experiments). From Morton and MacMillan (1954).

The suppression of nitrate assimilation by ammonia appears to be a general phenomenon in fungi and is illustrated for *Scopulariopsis brevicaulis* in Fig. 4 (*Left*). The fungus was grown first in nitrate, then ammonium sulfate was added on the fourth day. The mycelium assimilated the added am-

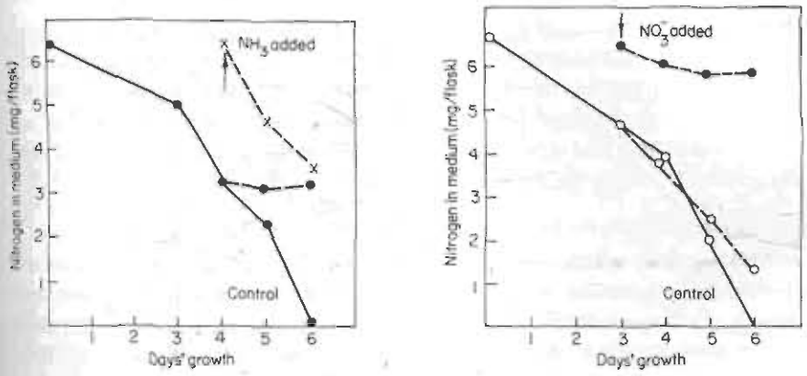


Fig. 4. *Scopulariopsis brevicaulis*. (*Left*) Effect of addition of ammonia on the uptake of nitrate from the medium. —, Culture in nitrate only; – – –, culture in nitrate and ammonia; •, nitrate N; X, ammonia N. (*Right*) Effect of addition of nitrate on the uptake of ammonia from the medium. —, Culture in ammonia; – – –, culture in ammonia and nitrate; •, nitrate N; ⊙, ammonia N. From Morton and MacMillan (1954).

monia immediately at a high rate without a lag whereas the assimilation of nitrate was stopped within 24 hours of adding ammonia. In another experiment, nitrate added to 3-day cultures of the fungus growing in an ammonia medium with succinate had no effect on the rate of ammonia assimilation, and practically no nitrate was assimilated while ammonia was still present (Fig. 4 (*Right*). Similar results have been obtained with *Alternaria solani, Aspergillus repens, Botrytis allii, Cladosporium herbarum, Diplodia natalensis, Mucor ramannianus, Myrothecium verrucaria,* and *Penicillium chrysogenum.*

When nitrate-grown mycelia of *A. niger, B. alii, P. chrysogenum,* and *Trichoderma viride* was transferred to nitrate, ammonium nitrate, and ammonium media, the rate of nitrate uptake was reduced by 42–82% in the presence of ammonia whereas the rate of ammonia uptake was reduced by only 6–11% in the presence of nitrate in the first 5 hours after transfer. There are, however, some species, e.g., *A. niger, A. oryzae, F. graminearum, P. griseofulvum,* that use nitrate simultaneously with ammonia although relatively more slowly.

III. ENZYMATIC STUDIES

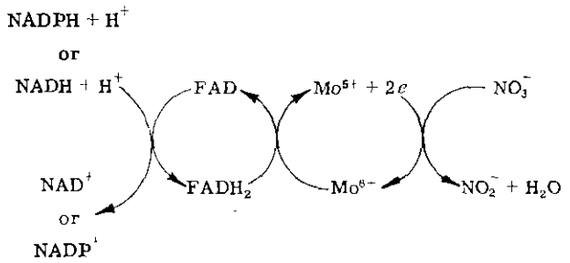
A. Nitrate Reductase

1. Assimilatory Enzyme

The pioneer work of Steinberg (1937), who showed that *Aspergillus niger* required molybdenum when grown with nitrate as the sole nitrogen source, was confirmed by Nicholas and Fielding (1951). Nason and Evans (1953) partially purified an assimilatory nitrate reductase from *Neurospora crassa* which reduced nitrate to nitrite with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the hydrogen donor:

$$NO_3^- + NADPH + H^+ \rightarrow NO_2^- + NADP + H_2O$$

The enzyme, which was shown to contain flavine adenine dinucleotide (FAD), was inhibited by cyanide and various metal-chelating agents. Although a metal requirement was inferred, the authors obtained no response with several metals including molybdenum. Subsequently it was established that molybdenum is a functional constituent of nitrate reductase (Nicholas *et al.*, 1954; Nicholas and Nason, 1954a,b; Nicholas, 1959a,b; Nicholas and Stevens, 1955). The metal, which concentrated in purified fractions of the enzyme to the exclusion of others, was removed by dialyzing the enzyme against cyanide and glutathione. The apoenzyme was reactivated specifically by molybdenum. The enzyme freed of the micronutrient transferred electrons from NADPH to FAD and thence to suitable redox dyes, but not to nitrate. When molybdenum was returned to the apoenzyme, nitrate was reduced. Thus the metal links the flavine component to nitrate. Molybdate reduced with sodium hydrosulfite mediated the enzymatic reduction of nitrate, which was unaffected by flavine. The most reduced state of the hydrosulfite-treated molybdate was valence 5+, and this was an effective electron carrier in the system after flavine (Nicholas and Stevens, 1955). It is likely that two molybdenum atoms are required to effect the two-electron transfer to nitrate:



Since the enzyme has a phosphate requirement at the molybdenum site which can be replaced by tellurate, arsenate, or selenate, it is likely that these complex with molybdate in the enzyme (Nicholas and Scawin, 1956; Kinsky and McElroy, 1958). The phosphomolybdate complex may facilitate the oxidation-reduction between flavine and nitrate. It is known that the chemical reduction of these anion–molybdate complexes is more readily accomplished than is that in hexavalent molybdate solutions alone. It has been suggested that the phosphomolybdic complex prevents "over-reduction" of molybdenum in the enzyme. Further evidence that molybdenum is involved in electron transfer in the enzyme has come from recent studies with electron paramagnetic resonance studies (EPR) with the purified enzyme from *Neurospora*. An enzyme with similar properties has been prepared from *Hansenula anomala* by Silver (1957).

Kinsky and McElroy (1958) observed that NADPH:cytochrome c reductase activity closely paralleled that of nitrate reductase during purification (see Fig. 5). They suggest that one enzyme system is involved in nitrate and cytochrome c reduction. A flavine reductase is the first rate-limiting step for both systems and a molybdoprotein is essential for nitrate

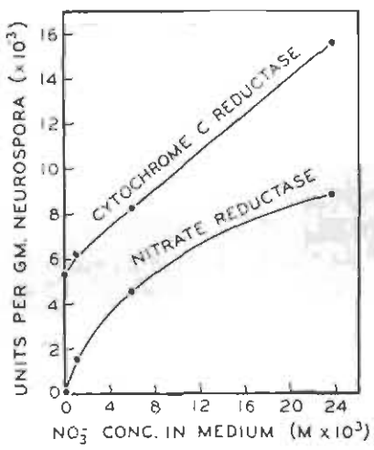


FIG. 5. Effect of nitrate concentration on the adaptive formation of nitrate and cytochrome c reductase activity. *Neurospora* was grown at room temperature without forced aeration in basal growth medium containing the following (grams per liter): NH_4Cl, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; NaCl, 0.2; $CaCl_2$, 0.2; sucrose, 20.0; sodium tartrate, 5.0; biotin, 5 μg; trace element solution, 5 ml. Fifty milliliters of medium was dispensed in 250-ml Erlenmeyer flasks, and various amounts of sodium nitrate were added as indicated on the abscissa. After 3 days' growth, mycelia were harvested and crude extracts were prepared and assayed for enzyme activity. From Kinsky and McElroy (1958).

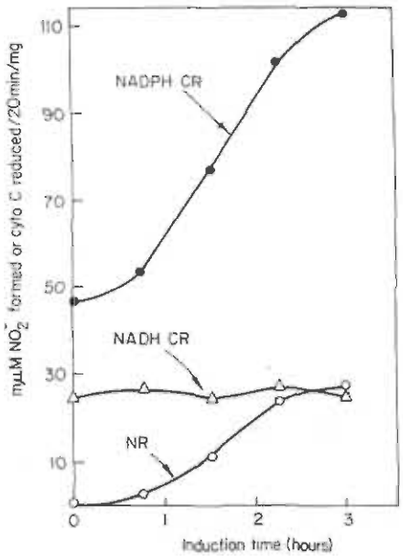


FIG. 6. Time course of enzyme induction in *Neurospora*. Mycelial sections wer incubated for various times in the complete induction medium containing nitrate. Sec tions were then harvested and dried; extracts were prepared and assayed for nitrat reductase (NR), NADPH-cytochrome c reductase (NADPH CR), and NADH-cytc chrome c reductase (NADH CR). From Kinsky (1961).

reduction using reduced FMN as the hydrogen donor. Kinsky (1961 showed that NADPH cytochrome c reductase and nitrate reductase wer induced in parallel fashion in *Neurospora*, as illustrated in Fig. 6.

Sorger (1963) found that sucrose density gradient preparations of crud extracts of *Neurospora* had nearly identical activity profiles for the tw activities. From genetic studies with mutants of *Neurospora* and *Aspe*

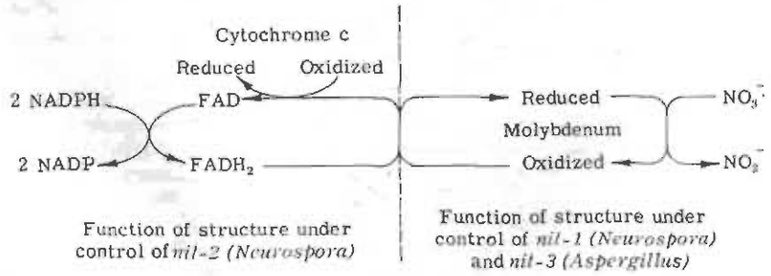


FIG. 7. Functional model of nitrate reductase in *Neurospora* and *Aspergillus*.

gillus, it was concluded that there was one genetic locus for NADPH to flavine and another one for the reduced flavine to the molybdenum protein (Fig. 7).

2. Dissimilatory Enzymes

A dissimilatory nitrate reductase is also present in fungi grown under semi-anaerobic conditions when the oxygen supply is limiting (Walker and Nicholas, 1961b). As in the comparable bacterial system, the enzyme requires iron as well as molybdenum for its activity (Fewson and Nicholas, 1961b). It was found that reduced methyl or benzyl viologen is the most effective hydrogen donor, and under these conditions there is no flavine requirement. Although NADPH is an effective donor for the assimilatory enzyme, it does not function for the purified dissimilatory system.

It is now clear that the assimilatory and dissimilatory nitrate reductase systems in fungi differ only in the penultimate electron transfer sequence to the terminal nitrate reductase which is a molybdenum-containing protein (Fig. 8).

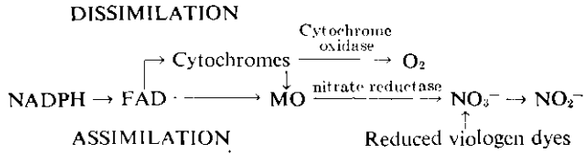


FIG. 8. Alternative pathways of electron transfer to nitrate and oxygen in fungi.

It is of interest that a purified nitrate reductase from *Neurospora*, using reduced benzyl viologen as the hydrogen donor, does not contain NADPH: cytochrome c reductase activity and is composed of a molybdenum-containing protein only. Thus the penultimate electron transfer sequence to nitrate has been removed from the terminal molybdenum protein. The latter functions as the nitrate reductase with reduced benzyl viologen as the donor (Nicholas and Wilson, 1964).

3. Enzyme Induction

Nitrate reductases so far examined in fungi are induced by their substrate, nitrate. The presence of ammonia in the nutrient often reduces enzyme activity in the mycelium. Thus in *Scopulariopsis brevicaulis* the enzyme is suppressed by ammonia even when nitrate is provided (Morton, 1956). This effect occurs *in vivo*, but not *in vitro*. Kinsky (1961) showed that ammonia, the end product of nitrate reduction, decreased the formation of nitrate reductase in *Neurospora*. This is an example of a "feedback inhibition," i.e., inhibition by the end product of a reaction, and is anala-

gous to similar effects reported for various metabolic pathways in bacteria. It probably provides a physiological control of nitrate utilization in fungi.

B. Nitrite Reduction

The immediate reduction product of nitrate in fungi is nitrite. The assimilatory pathway from nitrite to ammonia is not as clearly defined as is the conversion of nitrite to nitric oxide and nitrogen gas which occurs during denitrification in bacteria.

A nitrite reductase isolated from *Neurospora* by Nason *et al.* (1954) reduced nitrite to ammonia when NADH was the hydrogen donor, but NADPH was ineffective. The enzyme, which was stimulated by FAD, was inhibited by metal-binding agents such as cyanide, 8-hydroxyquinoline, and salicylaldoxime. After being purified fiftyfold by Nicholas *et al.* (1960), the enzyme contained FAD, copper, and iron. They proposed that copper is required for the terminal step, coupling the electron transfer sequence to nitrite. During this reaction univalent copper reduces nitrite nonenzymatically and the divalent copper is then enzymatically reduced by the penultimate donor system:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{cytochrome} \rightarrow Cu^{+2} \rightarrow NO_2^-.$$

This enzyme has strikingly similar properties to the nitrite reductases from denitrifying bacteria, e.g., *Pseudomonas stutzeri* (Chung and Najjar, 1956a; *P. aeruginosa* (Walker and Nicholas, 1961c). It is of interest that the purified nitrite reductases have cytochrome oxidase activity.

C. Nitric Oxide Uptake

Nitric oxide has been identified as a product of nitrite reductase in de nitrifying bacteria (Chung and Najjar, 1956a, b; Fewson and Nicholas 1960), and there is evidence that the purified enzyme from *Neurospor* also yields nitric oxide (Walker and Nicholas, 1961c). It has also bee shown that under strict anaerobic conditions, nitric oxide is readily take up by a range of microorganisms including fungi. Iron deficiency depresse the uptake of nitric oxide by these organisms. Although it is clear th nitric oxide is an intermediate in bacterial denitrification, there is insuf cient evidence as yet that nitric oxide, or a compound with which it equili rates, is en route to ammonia during nitrate assimilation in fungi.

D. Hyponitrite Reductase

An enzyme system has been found in *Neurospora* which catalyzes t reduction of hyponitrite to ammonia. This enzyme, which requires NAD

is distinct from nitrite reductase since the latter does not utilize hyponitrite (Medina and Nicholas, 1957). Since hyponitrite is unstable they followed enzyme activity by changes in extinction at 245 mμ for hyponitrite: this occurred rapidly when NADH was added with the enzyme. A pyridine nucleotide-linked hyponitrite reductase has also been found in bacteria (McNall and Atkinson, 1957) and in higher plants (Vaidyanathan and Street, 1959). Nitroxyl, nitrous oxide, nitramide, and dihydroxyammonia, which are at the same oxidation level as hyponitrite (+1 charge for the N atom), all have been suggested as possible intermediates in nitrite reduction, but experimental support for them is scanty. It has been suggested that a compound like nitroxyl (NOH) containing one nitrogen atom is an intermediate in nitrate reduction and that this is in equilibrium with hyponitrite, which can decompose to give nitrous oxide. This scheme would be similar for the assimilation and dissimilation of nitrate (Fig. 9).

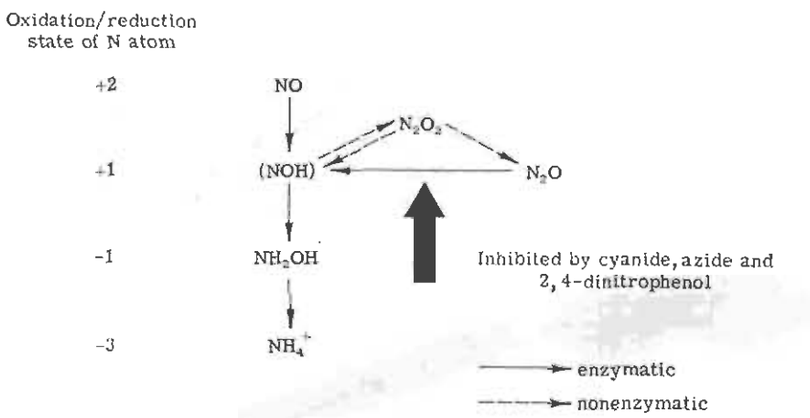


FIG. 9. Scheme for the nitrate reduction with nitroxyl (NOH) as an intermediate.

The following lines of evidence support this scheme: (1) Nitrous oxide has frequently been reported as a product of denitrification, and it is likely to be formed nonenzymatically when some intermediate at the nitroxyl or hyponitrite level accumulates. Thus it is possible that under some conditions the intermediate is formed at a greater rate than it can be reduced. The compound would then dimerize to give hyponitrite with subsequent decomposition to nitrous oxide and water. (2) The existence in solution of hyponitrite or nitrous acid also explains the nitrogen "losses" observed by several investigators, who measured only nitrate, nitrite, and nitrogenous gases, ammonia, and cell protein during denitrification. (3) This scheme explains the utilization of hyponitrite (Medina and Nicholas, 1957a,b;

McNall and Atkinson, 1957). Thus hyponitrite would exist in equilibrium with the intermediate and would also produce nitrous oxide; either of these might then be used enzymatically. (4) The true intermediate in nitrate assimilation is likely to contain only one N atom, as is the case for the likely intermediates, e.g., nitrite, nitric oxide, hydroxylamine, and ammonia. An intermediate such as hyponitrite or nitrous oxide would involve the consecutive formation and breakage of a N-N bond, which, although possible, seems to be unlikely. It is apparent that should nitroxyl, or a similar compound at the same oxidation level, be an intermediate in nitrate assimilation and in denitrification, then there must be two pathways for its reduction; the first to give hydroxylamine in the assimilatory sequence and the other to produce nitrogen gas. At present it is not known whether these reductions take place (Fewson and Nicholas, 1961a).

E. Hydroxylamine Reductase

The assimilatory reduction of hydroxylamine to ammonia has been demonstrated in a wide range of microorganisms (Nicholas, 1959a,b; Walker and Nicholas, 1961a). Hydroxylamine reductase is usually less active in fungi grown without nitrate. Thus in *Neurospora* a NADH-hydroxylamine reductase has been identified which has the following stoichiometry: $NH_2OH + NADH + H^+ \rightarrow NH_3 + NAD + H_2O$. The enzyme is a flavoprotein inhibited by metal-chelating agents. Enzyme activity is much

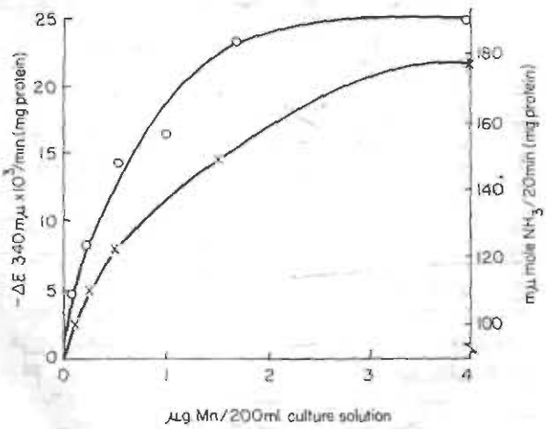
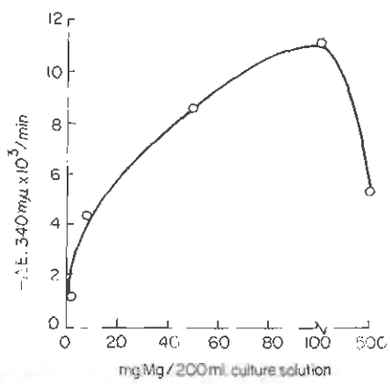


FIG. 10. Effect of manganese content of culture solution on hydroxylamine reductase in cell-free extracts of *Neurospora* measured by two independent methods: NADH oxidation at 340μ, O—O; and NH_3 production in Conway Units, X—X. From Nicholas (1959b).

ıced in extracts of mycelium deficient in manganese or in magnesium as vn in Figs. 10 and 11. The effects of metal deficiencies on nitrite, hy-



IG. 11. Effect on magnesium content of culture solution on hydroxylamine re-.ase in cell-free extracts of *Neurospora* measured by NADH oxidation at 340

ıitrite, and hydroxylamine reductases in *Neurospora* based on ammonia ıduction are shown in Table III.

TABLE III

.FFECTS OF METAL DEFICIENCIES ON NITRITE, HYPONITRITE, AND HYDROXYLAMINE REDUCTASES IN *Neurospora crassa* (BASED ON AMMONIA PRODUCTION)[a,b]

luctase enzymes	Omit Fe	Omit Cu	Omit Mn	Omit Mo	Omit Zn
rite	22	36	53	100	68
ponitrite	51	53	60	100	100
droxylamine	100	100	57	100	95
ight of mycelium	32	43	41	40	59

[a] After Medina and Nicholas (1957b).
[b] Values as percentages of those in normal mycelium.

In considering these effects it is clear that any metal deficiency affecting ɜ hydroxylamine or hyponitrite systems would also inhibit nitrite re-.ctase, provided the reductive pathway to ammonia involved these inter-ɜdiates. Thus a deficiency of manganese that reduced hydroxylamine ductase also depressed nitrite and hyponitrite reductases. A deficiency of agnesium was also found to restrict hydroxylamine reductase (Nicholas,)59a,b). Although manganese and magnesium were necessary for the rmation of the enzyme, neither metal accumulated in purified fractions

of the enzyme, nor did their addition stimulate the enzyme. It is likely that the two metals are required for the formation of the enzyme rather than for its action. The enzyme requires phosphate for maximal activity, but this effect can be produced also with arsenate or pyrophosphate.

F. Ammonia Utilization

The production of ammonia from hydroxylamine has been shown to occur in several microorganisms, and large amounts of ammonia have been found in the culture medium during dissimilatory nitrate reduction by spore-forming bacteria (Klaeser, 1914; Verhoeven, 1952). Ammonia has been considered by the Wisconsin school (Wilson, 1958) as the "key intermediate" in nitrogen fixation, i.e., the final inorganic product of fixation before incorporation into organic compounds. It has generally been considered to play a similar role in assimilatory nitrate reduction (Verhoeven, 1956; Nicholas, 1959a,b). Glutamic dehydrogenase prepared from *Neurospora* was shown to aminate α-ketoglutaric acid with ammonium ions forming glutamic acid. Zinc was required for enzyme formation (Nicholas and Mabey, 1960) and is thus similar to the enzyme from animal sources (Vallee, 1955; Adelstein and Vallee, 1958). There is no doubt that this is a key enzyme in the production of organic nitrogen compounds in fungi. Silver and McElroy (1954), who found that *Neurospora* mutant 2003 lacked glutamic dehydrogenase activity, suggest that ammonia enters organic substances through unspecified reactions since its growth on ammonia appeared to be unimpaired.

G. Reduction of Organic Nitro Compounds

There have been several suggestions in the literature that nitrate and/or nitrite are bound in organic complexes before reduction (Burström, 1946; de la Haba, 1950). Biochemical mutants of *Neurospora crassa* blocked at various points in nitrate reduction were studied by Silver and McElroy (1954). Cell-free extracts of four mutants showed that the genetic block affected the apoenzyme of nitrate reductase either directly or indirectly. In two strains no nitrate reductase was found; another had detectable enzyme only in mycelium grown at pH 6 or greater, and the fourth strain produced a heat-stable inhibitor of the enzyme prepared from the wild-type strain. Five different types of nitrite mutants were examined. Two accumulated hydroxylamine and nitrite in the culture medium, another was pH sensitive and required pyridoxine for growth. When the vitamin was limiting, nitrite accumulated and nitrite reductase activity was reduced. Silver and McElroy postulated a scheme (Fig. 12) to explain their results.

They suggested that pyridoxine operates directly or indirectly in nitrite reduction by the condensing of free hydroxylamine with pyridoxal phosphate to form oxime. The oxime could then be reduced to the amine, which would then undergo transamination reactions to regenerate pyridoxal phosphate. Attempts to reduce the oxime of pyridoxal phosphate, however, have not been successful with extracts of *Neurospora*. The oximes of pyru-

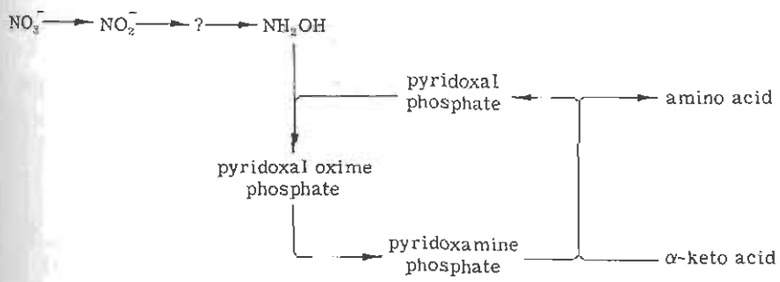
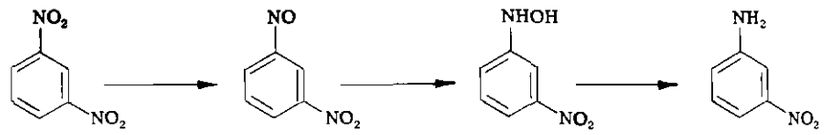


FIG. 12. Scheme of nitrate reduction postulated by Silver and McElroy (1954).

vic acid, α-ketoglutaric acid, and pyridoxal phosphate labeled with C^{14}-carboxyl have been prepared, but there is no convincing evidence that any of them are reduced to the corresponding amino acid since all the C^{14} was recovered in the corresponding α-keto acid, apparently after release of hydroxylamine which is then converted to ammonia (Nicholas, 1959a,b). Another pyridoxine-requiring mutant showed no increase in its requirement for pyridoxine whether grown on nitrate, nitrite, or ammonia. Pyridoxine deficiency resulted in a marked accumulation of ammonia in the medium, presumably because of the suppression of the pyridoxine-dependent transamination processes (Nicholas, 1959b). Thus it would appear that ammonia is an obligatory intermediate in the reductive sequence and that the oxime formation may provide a detoxication mechanism only since there is no evidence that oximes are reduced directly to amino acids in fungi.

It is of interest that ferredoxin has been implicated in nitrite and hydroxylamine reductases in anaerobic bacteria, e.g., *Clostridium pasteurianum* (Mortenson *et al.*, 1962; Valentine *et al.*, 1963) and in higher plants (Losada *et al.*, 1963), but ferredoxin has not been found in fungi.

Reports of the reduction of aromatic nitro compounds in animal tissues (Lipschitz, 1920) and in bacteria (Saz and Slie, 1954a,b) were followed by those for *Neurospora* (Zucker and Nason, 1955). In *Neurospora* an enzyme system has been described that reduced *m*-dinitrobenzene to *m*-nitroaniline via *m*-nitrosonitrobenzene:



There may be three enzymes involved here, and they appear to be metal-dependent flavoproteins, but the metals have not been identified. The enzymes have a broad specificity for various nitroaryl compounds although chloramphenicol and furacin are not very reactive as substrates. These enzymes, unlike nitrate, nitrite and hydroxylamine reductases, are present in mycelium grown with ammonium salts. These enzymes do not appear to function in the assimilation of nitrate since there is no evidence that any of these compounds are incorporated into amino acids. Thus these compounds may serve as suitable electron acceptors for enzymes in *Neurospora* in a nonspecific way. Westerfield *et al.* (1957) found that *Aspergillus niger* reduced *p*-nitrobenzene sulfonamide to *p*-aminobenzene sulfonamide and implicated an iron-dependent flavoprotein enzyme.

The lack of success in finding an organic reductive pathway for nitrite does not rule out the possibility that it exists. The evidence from nutrition experiments including trace metal deficiency studies, isolated enzymes, biochemical mutants, and comparative biochemical studies strongly suggests that the inorganic reductive pathway (Fig. 13) is a physiologically important route in fungi.

There is no convincing evidence that the organic reductive sequence in-

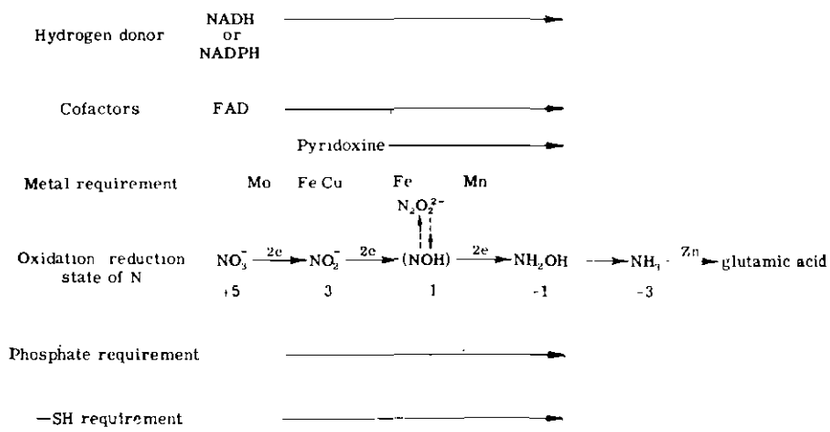


FIG. 13. Inorganic reductive pathway for nitrate.

volving either the conversion of oximes to amino acids, or the reduction of aromatic nitro compounds, has assimilatory significance in fungi. The formation of oximes may be regarded only as a detoxication mechanism for removing hydroxylamine, which inhibits growth; the oximes are hydrolyzed and the hydroxylamine formed is reduced to ammonia by hydroxylamine reductase.

IV. HETEROTROPHIC OXIDATION OF INORGANIC NITROGEN COMPOUNDS

The oxidation of inorganic substrates by chemoautotrophic bacteria, e.g., ammonia and hydroxylamine by *Nitrosomonas* and nitrite by *Nitrobacter,* are well known. Some heterotrophic fungi also oxidize ammonium ions to nitrite and nitrate. Thus *Aspergillus aureus* and *A. batatae,* as well as strains of *Penicillum* form nitrate from nitrite during growth in a peptone medium, and *A. wentii* and *A. flavus* convert ammonium ions to nitrite and thence to nitrate under similar conditions. Eylar and Schmidt (1959) have made a comprehensive survey of nearly a thousand heterotrophic fungi and found that thirteen strains of *A. flavus* oxidize ammonia to nitrite and nitrate. The fungus was shown to produce bound hydroxylamine, nitrite and nitrate when grown in a medium containing peptone, amino acids, or ammonium salts. It is claimed that β-nitropropionic acid was present in the culture filtrates. Treatment of the culture medium, containing an ammonium salt, with alumina prior to inoculation with the fungus, inhibited nitrite or nitrate formation, but not that of bound hydroxylamine. The activity was restored by returning cerium chloride ($10^{-3}M$) to the medium. The fungus contained an active peroxidase capable of producing nitrite from β-nitropropionic acid. A role for peroxidase in the oxidation of ammonia to nitrite has also been suggested for some heterotrophic bacteria by Kuznetsov (1950). Recent work with cell-free extracts of *A. wentii* has shown that the oxidation of ammonia to nitrite and nitrate (see Fig. 14) is stimulated on the addition of a suitable electron carrier such as cytochrome c (Aleem *et al.,* 1964). They established that the oxidation of hydroxylamine to nitrite and of nitrite to nitrate is mediated by their respective cytochrome c reductases. The addition of NADP also stimulated the oxidation of ammonia, hydroxylamine, or nitrite to yield nitrate. Although β-nitropropionic acid has been suggested as a possible intermediate in heterotrophic nitrification, results with cell-free extracts of *A. flavus, A. wentii,* and *P. aterovenatum* suggest that an inorganic route involving hydroxylamine, similar to the one in nitrifying bacteria, is the main pathway for ammonia oxidation in these heterotrophic fungi.

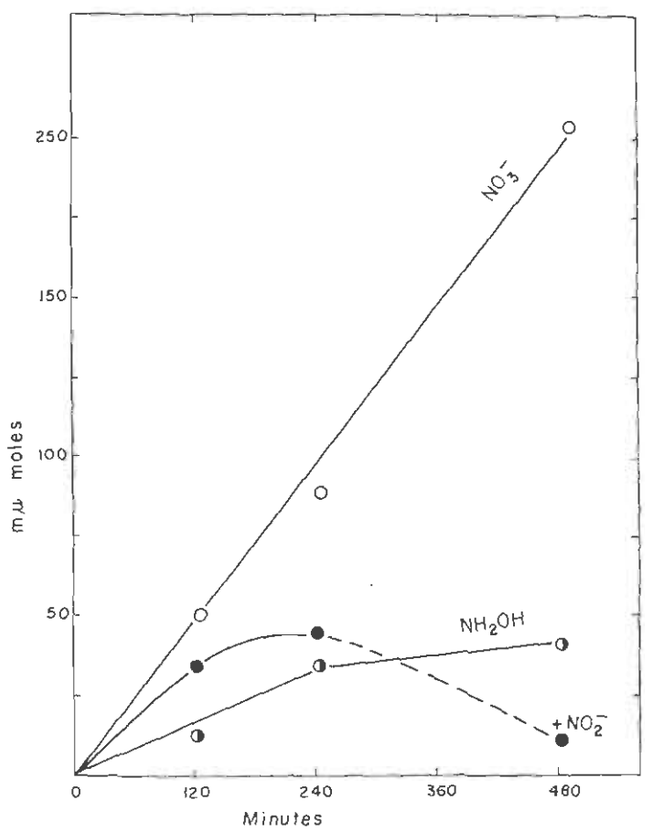


FIG. 14. The pattern of ammonium oxidation by cell-free extracts of *Asperg wentii*. Reaction mixtures contained 2.0 ml of cell-free extracts, 0.2 ml of 2% c chrome c, 0.1 ml of NADPH (3 mg/ml), 10 μmoles of $(NH_4)_2HPO_4$, and 0.1 *M* buffer, pH 8.0 to make a total volume of 10 ml. Samples of 0.5 ml were taken every minutes and tested for the formation of NH_2OH (◑), NO_2^- (●), and NO_3^- (○). F Aleem *et al.* (1964).

V. AMINO ACID METABOLISM

The uptake of amino acids by fungi has not been extensively stu Mathieson and Catcheside (1955) found that the growth of a histidin quiring mutant of *Neurospora crassa* was inhibited by certain other a acids in combination with lysine and arginine. The addition of the acid derivative of the required amino acid was found to overcome th hibition by certain other amino acids in *Neurospora* (Brockman *e*

1959). They suggest that the keto acid does not compete with the inhibitory amino acids for a permease. Zalokar (1960) showed that the initial rate of uptake of C^{14}-labeled amino acids was linear with time and was higher than the rate for their incorporation into proteins. Jones (1963) followed the rate of uptake of C^{14}-labeled amino acids in *Botrytis fabae* from aqueous solutions. He found that uptake varied with age of the fungus, pH of culture medium, and the concentration of the amino acid (Fig. 15). Both L- and D-isomers were accumulated, but there appeared to

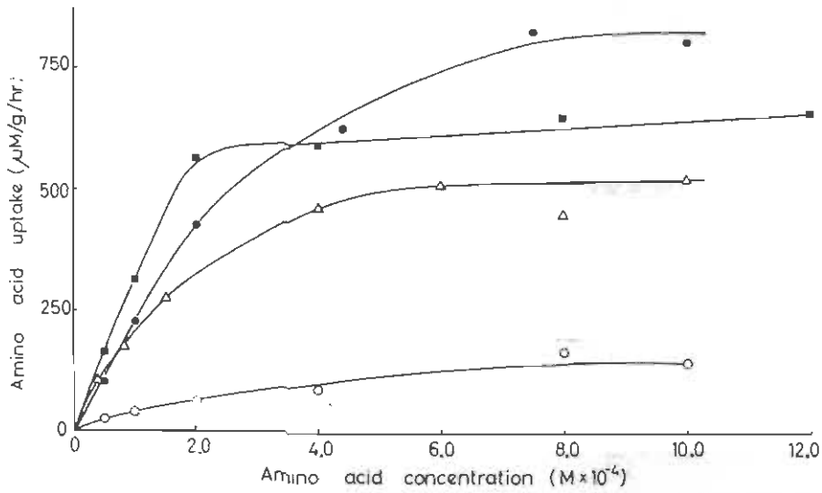


FIG. 15. The effect of amino acid concentration on rate of uptake by *Botrytis fabae*. Mycelial pellets were suspended in amino acid solutions of the indicated strengths, and the rate of uptake was measured. Δ, Glycine; •, L-valine; ■, L-lysine; ○, L-tryptophan. From Jones (1963).

be a greater affinity for the L-isomer. Unsubstituted $—NH_2$ and —COOH groups were necessary for the process, which was constitutive and was inhibited by uncouplers of oxidative phosphorylation, as shown in Table IV. Competition experiments with several amino acids suggested that the process of concentrating the amino acids in the mycelium was common to all types. Although both peptides and amines were accumulated, they did not compete effectively with the uptake of amino acids. The lack of specificity in the uptake of various amino acids in fungi is in marked contrast to bacteria (G. N. Cohen and Monod, 1957) and resembles the results with animal tissues (Finch and Hird, 1960).

It is unnecessary to consider in detail the metabolism of amino acids in fungi since the pathways have so many features in common with the bac-

teria. P. P. Cohen (1954), Greenberg (1954), McElroy and Glass (1955), and Cochrane (1958) give excellent reviews of this topic. Brief mention will be made of some features of the utilization of amino acids in fungi.

Very few fungi are unable to utilize ammonium nitrogen and they depend on amino acids. Some phycomycetes, e.g., *Blastocladiella emersonii, Saprolegnia elongatus,* and *Leptomitus lacteus,* utilize amino acids but not ammonia. Some reports in the literature that ammonium nitrogen is not suitable for some fungi may be unreliable since the pH of the culture medium may not have been adequately buffered during growth.

TABLE IV

THE EFFECT OF INHIBITORS ON THE UPTAKE OF 10^{-4} *M* L-HISTIDINE BY *Botrytis fabae*

Inhibitor	Concentration	Inhibition (%)
2,4-Dinitrophenol	10^{-5} *M*	30
2,4-Dinitrophenol	10^{-4} *M*	96
Na azide	10^{-3} *M*	72
Chloramphenicol	5×10^{-4} *M*	0
8-OH quinoline	2×10^{-4} *M*	15
Streptomycin	5×10^{-4} *M*	60
Tetramethylthiuramdisulfide	Satd. solution	68
Zn-dimethyldithiocarbamate	Satd. solution	34
Fe-dimethyldithiocarbamate	Satd. solution	44
2,4-Dichlorphenoxyacetic acid	4×10^{-5} *M*	61

[a] From Jones (1963).

Amino acids are utilized directly by most fungi, and the evidence ava able suggests that they are incorporated directly into protein and are r degraded first to ammonia. Free amino acids are readily detected in fun Glycine, asparagine, glutamic acid, and aspartic acid in well-buffered c ture media usually support good growth of a variety of fungi although th are not equally effective. Leucine, although utilized by *Trichophyt persicolor,* is not a generally good source of nitrogen; neither is tryptoph which supports a partial growth of the actinomycete *Streptomyces coelicol*

In many fungi growth is often best on a mixture of amino acids, a others grow well when amides are supplied. Thus asparagine serves be than aspartate for *Tricholoma imbricatum, Pyricularia oryzae, Le graphium* sp., and *Phycomyces blakesleeanus.* Whether this results fr the extra nitrogen in the amide or from better buffering capacity of medium in its presence is not known. There is evidence that some fu utilize the amide nitrogen of asparagine or glutamine more readily than amino nitrogen. Glutamine is readily utilized by *Tricholoma gambos*

and by other basidiomycetes. Asparagine has been shown to favor perithecial development in *Diaporthe phaseolorum*.

Specific amino acid requirements may be induced by mutation in many fungi but are uncommon in nature, and when they occur naturally they are more often partial rather than absolute requirements. Thus *Mycena rubromarginata* responds to the aromatic amino acids tyrosine or phenylalanine but grows without either, *Cenococcum graniforme* has a partial requirement for histidine, and *Eremothecium ashbyii* requires several amino acids only when grown in acid medium. Increased growth with cysteine and methionine is often related to a sulfur rather than a nitrogen requirement. When D-isomers of amino acids are utilized it is usual to find D-amino acid oxidase activity resulting in the release of ammonia, which is then incorporated into L-amino acids. Racemases which interconvert D- and L-forms are known in bacteria but not in fungi, although there has been no comprehensive search for them. The D- and L-amino acid oxidases in animal tissues are flavoproteins that mediate the following reactions:

$$\underset{\text{amino acid}}{R\text{–}CH(NH_2)COOH} + \tfrac{1}{2}O_2 \rightarrow \underset{\alpha\text{-keto acid}}{R.CO.COOH} + NH_3$$

The keto acid is usually decarboxylated, especially when catalase is absent:

$$R\text{–}CH(NH_2)COOH + O_2 \rightarrow R.COOH + NH_3 + CO_2$$

The effect of D-amino acid oxidase of *Neurospora crassa* on various amino acids is shown in Fig. 16 (Horowitz, 1944). The L-amino acid oxidase also found in *Neurospora* is thought to be a flavoprotein. This enzyme converts lysine to its keto analog, Δ'-dehydropipecolic acid, which may then be reduced to pipecolic acid.

Serine dehydrase present in many fungi deaminates nonoxidatively L-serine and L-threonine, forming pyruvate and α-keto butyrate, respectively. Neither D-serine nor D-threonine is used as a substrate by D-amino acid oxidase from *N. crassa*. Aspartase which converts aspartic acid to fumaric acid and ammonia is present in *N. crassa, Penicillium notatum,* and in many other fungi.

The well-known transamination processes in animal and bacterial cells which require pyridoxal phosphate, also occur in fungi:

$$\underset{\text{L-glutamic acid}}{HOOC.CH_2.CH_2(NH_2)COOH} + \underset{\alpha\text{-keto acid}}{R.CO.COOH} \rightarrow$$

$$\underset{\alpha\text{-ketoglutaric acid}}{HOOC.CH_2.CH_2.CO.COOH} + \underset{\alpha\text{-amino acid}}{R.CH.(NH_2).COOH}$$

Glutamic acid, which is probably the main donor of amino groups in transamination reactions in fungi is converted to α-ketoglutarate. Mutants

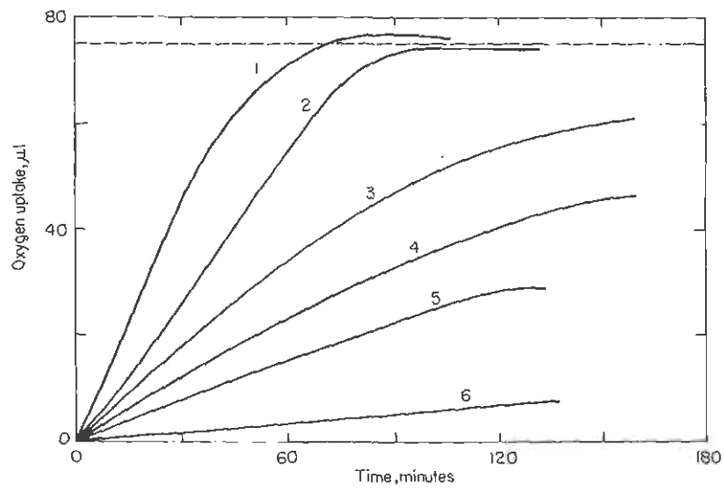


FIG. 16. The oxidation of some amino acids by the D-amino acid oxidase system of *Neurospora crassa*. Curves *1–6* represent, in sequence, the rate of oxidation of methionine, leucine, isoleucine, valine, lysine, and ornithine. The horizontal dashed line is drawn at the theoretical value for the complete oxidation of the D-isomer. Re drawn from Horowitz (1944).

of *N. crassa* which lack glutamic dehydrogenase have a requirement for any of several other amino acids, suggesting that these amino acids act as donors in transamination. Glutamic decarboxylase, which yields γ-amino butyric acid, has been found in many fungi including *Neurospora, Penicillium chrysogenum,* and *Endomycopsis vernalis*. It is known that in many strains of *Aspergillus, Neurospora,* and *Endomycopsis,* γ-aminobutyric acid donates its ω-amino group to α-ketoglutaric acid to form L-glutamic acid:

$$H_2N.CH_2.CH_2.CH_2.COOH + HOOC.CH_2.CH_2.CO.COOH \rightarrow HOOC.CH_2.CH_2.CH(NH_2).COOH + CH_3.CH_2.CH_2COC$$

L-glutamic acid — butyric acid

It is beyond the scope of this chapter to consider the biosynthesis amino acids so brilliantly exploited by the use of biochemical mutants fungi, e.g., *Neurospora* and *Aspergillus*. Adelberg (1953) has conclude however, that present-day techniques with isotopes, isolated enzymes, a biochemical mutants which are blocked at various points in a biosynthe pathway, cannot establish unequivocally that a given metabolite is an int mediate in a vital process.

Some of the interconversions studied by means of biochemical muta of *Neurospora crassa* and *Aspergillus* species may be mentioned brie

Glutamic acid is a starting point for ornithine synthesis; according to Vogel (1955), it proceeds via glutamic semialdehyde, but there is no evidence for acetylated intermediates as found in bacteria. Recent work suggests that the ornithine cycle operates in a number of fungi, resulting in arginine which is readily incorporated into protein. Proline and hydroxyproline are derived from glutamate via Δ'-pyrroline-5-carboxylic acid. Glycolic and glyoxylic acids are precursors of glycine, and glutamic acid serves as a suitable donor of the amino group. The sequence from cysteine to methionine involves cystathionine and homocysteine, and it is suggested that aspartic acid and homoserine are precursors of homocysteine and methionine as in yeast and bacteria (Black and Wright, 1955). The two dihydroxy acids considered to be precursors of isoleucine and valine are α,β-dihydroxy-β-ethylbutyric acid and α,β-dihydroxy-β-methylbutyric acid, respectively, which arise from pyruvic acid and threonine (Adelberg, 1955).

Aromatic amino acids are derived from glucose in fungi with shikimic and prephenic acids as possible precursors (Tatum *et al.*, 1954).

Much more work is required to elucidate biosynthetic pathways for amino acid synthesis in fungi by using other types of fungi in addition to the well-tried mutants of *Neurospora* and *Aspergillus*.

REFERENCES

Adelberg, E. A. (1953). The use of metabolically blocked organisms for the analysis of biosynthetic pathways. *Bacteriol. Rev.* **17**:253–267.

Adelberg, E. A. (1955). *In* "A Symposium on Amino Acid Metabolism" (W. D. McElroy and B. Glass, eds.), pp. 419–430. Johns Hopkins Press, Baltimore, Maryland.

Adelstein, S. J. and B. L. Vallee. (1958). Zinc in beef liver glutamic dehydrogenase. *J. Biol. Chem.* **233**:589–593.

Aleem, M. I. H., H. Lees, and R. Lyric. (1964). Biochemistry of heterotrophic nitrification. *Can. J. Biochem. Physiol.* (in press).

Äyräpää, T. (1950). On the base permeability of yeast. *Physiol. Plantarum* **3**:402–429.

Beck, S., and N. G. Wright. (1955). *In* "A Symposium on Amino Acid Metabolism" (W. D. McElroy and H. B. Glass, eds.), pp. 591–600. Johns Hopkins Press, Baltimore, Maryland.

Brian, P. W., P. J. Curtis, and H. G. Hemming. (1947). Glutinosin: a fungistatic metabolic product of the mould *Metarrhizium glutinosum* S. Pope. *Proc. Roy. Soc.* **B135**:106–119.

Brockman, H. E., A. G. de Busk, and R. P. Wagner. (1959). Mutant associated antagonisms in *Neurospora crassa*. *Arch. Biochem. Biophys.* **84**:455–461.

Burström, H. (1946). The nitrate nutrition of plants. A general survey of the occurrence and assimilation of nitrate. *Kgl. Lantbruks-Hogskol. Ann.* **13**:1–86.

Chung, C. W., and V. A. Najjar. (1956a). Cofactor requirements for enzymatic denitrification. I Nitrite reductase. *J. Biol. Chem.* **218**:617–626.

Cochrane, V. W. (1958). "Physiology of the Fungi," 524 pp. Wiley, New York.
Cohen, G. N., and J. Monod. (1957). Bacterial permeases. *Bacteriol. Rev.* **21**:169–194.
Cohen, P. P. (1954). *In* "Chemical Pathways of Metabolism" (D. M. Greenberg, ed.), Vol. 2, pp. 47–112. Academic Press, New York.
Davson, H., and J. F. Danielli. (1952). "The Permeability of Natural Membranes." 2nd ed., 365 pp. Cambridge Univ. Press, London and New York.
de la Haba, G. (1950). Studies on the mechanism of nitrate assimilation in *Neurospora*. *Science* **112**:203–204.
Eylar, O. R., and E. L. Schmidt. (1959). A survey of heterotrophic micro-organisms from soil for ability to form nitrite and nitrate. *J. Gen. Microbiol.* **20**:473–481.
Fewson, C. A., and D. J. D. Nicholas. (1960). The utilization of nitric oxide by micro-organisms and higher plants. *Nature* **188**:794–796.
Fewson, C. A., and D. J. D. Nicholas. (1961a). Utilization of nitrate by micro-organisms. *Nature* **190**:2–7.
Fewson, C. A., and D. J. D. Nicholas. (1961b). Nitrate reductase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* **49**:335–349.
Finch, L. R., and F. J. R. Hird. (1960). The uptake of amino acids by isolated segments of rat intestine. *Biochim. Biophys. Acta* **43**:278–287.
Greenberg, D. M. (1954). *In* "Chemical Pathways of Metabolism" (D. M. Greenberg, ed.), Vol. 2, pp. 47–112. Academic Press, New York.
Horowitz, N. H. (1944). The D-aminoacid oxidase of *Neurospora*. *J. Biol. Chem.* **154**:141–149.
Jacques, A. G. (1939). The kinetics of penetration XVI. The accumulation of ammonia in light and darkness. *J. Gen. Physiol.* **22**:501–520.
Jones, O. T. G. (1963). The accumulation of amino acids by fungi, with particular reference to the plant parasitic fungus *Botrytis fabae*. *J. Exptl. Botany* **14**:399–411.
Kinsky, S. C. (1961). Induction and repression of nitrate reductase in *Neurospora crassa*. *J. Bacteriol.* **82**:898–904.
Kinsky, S. C., and W. D. McElroy. (1958). *Neurospora* nitrate reductase, the role of phosphate flavine and cytochrome c reductase. *Arch. Biochem. Biophys.* **73**:466–483.
Klaeser, M. (1914). Die Reduction von Nitrater zu nitriter und ammoniake durch Bakterier. *Zentr. Bakteriol., Parasitenk., Abt. II* **41**:365–430.
Kuznetsov, S. I. (1950). Microbiological studies of lakes of Kokchetou, Kurgan, and Tyumen regions. I. *Trudy Lab. Sapropelevykh Otlozhenii, Akad. Nauk S.S.S.R., Inst. Lesa* **4**:5–14. (In Russian.)
Lipschitz, W. (1920). Mechanism of the poisonous action of aromatic nitro compounds and respiration problems of animal and plant cells. *Z. Physiol. Chem.* **109**:189–258.
Losada, M., J. M. Paneque, M. Ramirez, and F. F. Del Compo. (1963). Mechanism of nitrite reduction in chloroplasts. *Biochem. Biophys. Res. Commun.* **10**:298–303.
McElroy, W. D., and B. Glass, eds. (1955). "A Symposium on Amino Acid Metabolism." Johns Hopkins Press, Baltimore, Maryland.
MacMillan, A. (1956). The entry of ammonia into fungal cells. *J. Exptl. Botany* **7**:113–126.
McNall, E. G., and D. E. Atkinson. (1957). Nitrate reduction. II. Utilization of possible intermediates as nitrogen source and as electron acceptors. *J. Bacteriol.* **74**:60–66.

Mathieson, M. J., and D. G. Catcheside. (1955). Inhibition of histidine uptake in *Neurospora crassa*. *J. Gen. Microbiol.* **13**:72–83.

Medina, A., and D. J. D. Nicholas. (1957a). Hyponitrite reductase in *Neurospora*. *Nature* **179**:533–534.

Medina, A., and D. J. D. Nicholas. (1957b). Metallo-enzymes in the reduction of nitrite to ammonia in *Neurospora*. *Biochim. Biophys. Acta* **25**:138–141.

Mortenson, L. E., R. C. Valentine, and J. E. Carnahan. (1962). An electron transport factor from *Clostridium pasteurianum*. *Biochem. Biophys. Res. Commun.* 448–452.

Morton, A. G. (1956). A study of nitrate reduction in mould fungi. *J. Exptl. Botany* **7**:97–112.

Morton, A. G., and A. MacMillan. (1954). The assimilation of nitrogen from ammonium salts and nitrate by fungi. *J. Exptl. Botany* **5**:232–252.

Nason, A., and H. J. Evans. (1953). Triphosphopyridine nucleotide nitrate reductase in *Neurospora*. *J. Biol. Chem.* **202**:655–73.

Nason, A., R. G. Abraham, and B. C. Averbach. (1954). Enzymatic reduction of nitrite to ammonia by reduced pyridine nucleotides. *Biochim. Biophys. Acta* **15**:159–161.

Nicholas, D. J. D. (1959a). *Symp. Soc. Exptl. Biol.* **13**:1–23.

Nicholas, D. J. D. (1959b). *4th Intern. Congr. Biochem., Vienna, 1958* XIII (Colloquia), pp. 307–331. Macmillan (Pergamon), New York.

Nicholas, D. J. D., and A. H. Fielding. (1951). The use of *Aspergillus niger* for determining magnesium, zinc, copper, and molybdenum available in soils to crop plants. *J. Hort. Sci.* **26**:125–147.

Nicholas, D. J. D., and G. L. Mabey. (1960). Some properties of glutamic dehydrogenase from *Neurospora crassa*. *J. Gen. Microbiol.* **22**:184–190.

Nicholas, D. J. D., and A. Nason. (1954a). Molybdenum and nitrate reductase II. Molybdenum as a constituent of nitrate reductase. *J. Biol. Chem.* **207**:352–60.

Nicholas, D. J. D., and A. Nason. (1954b). Mechanisms of action of nitrate reductase from *Neurospora*. *J. Biol. Chem.* **211**:183–97.

Nicholas, D. J. D., and J. H. Scawin. (1956). A phosphate requirement for nitrate reductase from *Neurospora crassa*. *Nature* **178**:1474–1475.

Nicholas, D. J. D., and H. M. Stevens. (1955). Valency changes of molybdenum during the enzymic reduction of nitrate in *Neurospora*. *Nature* **176**:1066–1068.

Nicholas, D. J. D., and P. W. Wilson. (1964). A dissimilatory nitrate reductase from *Neurospora crassa*. *Biochim. Biophys. Acta* **86**:466–476.

Nicholas, D. J. D., A. Nason, and W. D. McElroy. (1954). Molybdenum and nitrate reductase. 1. Effect of molybdenum deficiency on the *Neurospora* enzyme. *J. Biol. Chem.* **207**:341–351.

Nicholas, D. J. D., A. Medina, and O. T. G. Jones. (1960). A nitrite reductase from *Neurospora crassa*. *Biochim. Biophys. Acta* **37**:468–476.

Nord, F. F., and R. P. Mull. (1945). *Advan. Enzymol.* **5**:165–205.

Osterhout W. J. V. (1935). How do eletcrolytes enter the cell? *Proc. Natl. Acad. Sci. U.S.* **21**:125–137.

Saz, A. K., and R. B. Slie. (1954a), Reversal of aureomycin inhibition of hacterial cell-free nitro reductase by manganese. *J. Biol. Chem.* **210**:407–412.

Saz, A. K., and R. B. Slie. (1954b). The inhibition of organic nitro reductase by aureomycin in cell-free etxracts. II Cofactor requirements for nitro-reductase enzyme complex. *Arch. Biochem. Biophys.* **51**:5–16.

Silver, W. S. (1957). Pyridine nucleotide-nitrate reductase from *Hansenula anomala*, a nitrate reducing yeast. *J. Bacteriol.* 73:241–246.

Silver, W. S., and W. D. McElroy. (1954). Enzyme studies on nitrate and nitrite mutants of *Neurospora*. *Arch. Biochem. Biophys.* 51:379–394.

Simon, E. W., and H. Beevers. (1952). The effect of pH on the biological activities of weak acids and bases. 1. The most usual relationship between pH and activity. *New Phytologist.* 51:163–190.

Sorger, G. J. (1963). TPNH-cytochrome *c* reductase and nitrate reductase in mutant and wild type *Neurospora* and *Aspergillus*. *Biochem. Biophys. Res. Commun.* 12:395–401.

Steinberg, R. A. (1937). Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. *J. Agr. Res.* 55:891–902.

Tatum, E. L., S. R. Gross, G. Ehrensvand, and L. Garnjobst. (1954). Synthesis of aromatic compounds by *Neurospora*. *Proc. Natl. Acad. Sci. U.S.* 40:271–276.

Vaidyanathan, C. S., and H. E. Street. (1959). Nitrate reduction by aqueous extracts of excised tomato roots. *Nature* 184:531–533.

Valentine, R. C., W. J. Brill, and R. S. Wolfe. (1963). Activity of PPNR in ferredoxin-dependent reactions of *Clostridium pasteurianum*. *Biochem. Biophys. Res. Commun.* 10:73–78.

Vallee, B. L. (1955). Zinc and metalloenzymes. *Advanc. Protein Chem.* 10:317–384.

Verhoeven, W. (1952). Aerobic spore forming nitrate reducing bacteria. Thesis, Delft University, Netherlands.

Verhoeven, W. (1956). *In* "Inorganic Nitrogen Metabolism" (W. D. McElroy and B. Glass, eds.), pp. 61–86. Johns Hopkins Press, Baltimore, Maryland.

Vogel, H. J. (1955). *In* "A Symposium on Amino Acid Metabolism" (W. D. McElroy and B. Glass, eds.), pp. 335–346. Johns Hopkins Press, Baltimore, Maryland.

Walker, G. C., and D. J. D. Nicholas. (1961a). Hydroxylamine reductase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* 49:361–368.

Walker, G. C., and D. J. D. Nicholas. (1961b). An iron requirement for a dissimilatory nitrate reductase in *Neurospora crassa*. *Nature* 189:141–142.

Walker, G. C., and D. J. D. Nicholas. (1961c). Nitrite reductase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* 49:350–360.

Westerfield, W. W., D. A. Richert, and E. S. Higgins. (1957). The metabolic reduction of organic nitro groups. *J. Biol. Chem.* 227:379–391.

Wilson, P. W. (1958). *In* "Handbuch der Pflanzenphysiologie" (W. Ruhland, ed.), Vol. VIII, pp. 9–42. Springer, Berlin.

Zalokar, M. (1960). The kinetics of amino acid uptake and protein synthesis in *Neurospora*. *Biochim. Biophys. Acta* 46:423–432.

Zucker, M., and A. Nason. (1955). *In* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, pp. 406–411. Academic Press, New York.

CHAPTER 14

Integration of Cellular Metabolism

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I. INTRODUCTION

Most of the knowledge of cell biochemistry was obtained by studying cell functions separately under artificial, but controlled, conditions. The task of integrating this knowledge has the inherent danger of ending by the enumeration of so many facts. The best that one can hope to achieve is to assemble the facts in such an order that it will bring together cellular events which have been studied separately, illuminating their relationships and their meaning in cell function, growth, and differentiation.

The first task will be to unify morphological and chemical knowledge into a chemical morphology of a cell. In essence, observable structures should be explained by their molecular composition. The second task, will be to look at the structures as functional units, each taking a special part in cell metabolism. The third and most difficult task will be to see how the functions of all these cell components are correlated so that they enable the cell to exist, to grow, and to reproduce.

It is difficult to restrict such a discussion to fungi as much work pertinent to the problem was done either on bacteria or mammalian cells. In fungi, most experimental work has been done on a few species. Fungi are a heterogeneous group and what is true for one taxon may not apply to another. The growth habits of fungi and their cell forms are, also, quite diverse. Therefore, this discussion will be restricted to the vegetative, multinuclear (coenocytic) hypha, such as is found in phycomycetes or in many ascomycetes and to the yeast cell.

II. LOCALIZATION OF CHEMICAL CONSTITUENTS IN THE CELL

A. Methods

The two most important methods for localizing chemical constituents in the cell are centrifugal fractionation and cytochemistry. Neither of these methods is beyond criticism, because each one introduces some kind of injurious manipulation of the cell. Therefore, the results of all the methods should be compared and their relative merits assessed. If a certain enzyme is found associated with a certain particle by differential centrifugation, the result becomes more reliable if the same association is observed by cytochemical methods. If another enzyme is found in the soluble fraction of a homogenized cell preparation, but is localized by a cytochemical method, one must attach more importance to this latter result.

1. Centrifugal Fractionation

Centrifugal fractionation is applied to broken-cell preparations to isolate cell particles which presumably have their counterparts in living cells. The chemistry and function of these particles can then be studied and the results projected into an intact cell. There are several reviews describing the method and its results, mostly as it applies to mammalian cells (Allfrey, 1959; de Duve *et al.*, 1962). Table I indicates the fungi studied by this method and the organelles isolated.

Breakage of cells may introduce many artifacts not present in the living cell. The harsh process of grinding cells, or breakage in some other way, may fragment cellular structures, so that what is collected may be only fragments of a cell organelle. There can be no doubt that many mitochondria are so fragmented, since they can exist as filamentous structures in a living cell. Microsomes are fragments of a more or less extensive lamellar system of the cell, the ergastoplasm. The breakdown products of cell nuclei may be recovered in other cell fractions (page 393). Cell components often associate in centrifuged homogenates with fractions from which they are separated in the cell. In *Neurospora* homogenates, polyphosphates were isolated in cell-wall fraction, but this association was proved to be an artifact (Harold and Miller, 1961) (Fig. 1).

Moreover, cellular structures may lose some of their constituents which become solubilized during homogenization. This can be illustrated by yeast fumarase and aconitase, which pass from particles into supernatant after extended disintegration procedures (Nossal, 1954).

Breakage of cells of fungi presents special difficulties because they are

often enclosed in resistant cell walls. Many ingenious devices exist for breaking cells, but the old-fashioned grinding of the mycelium in a mortar with either sand or glass powder is still useful. Mitochondria and microsomes can be isolated readily from such preparations, but nuclei seem to be disrupted during the process. Other methods usually give a lower percentage of disrupted cells, and, if used too long, damage the cell contents excessively.

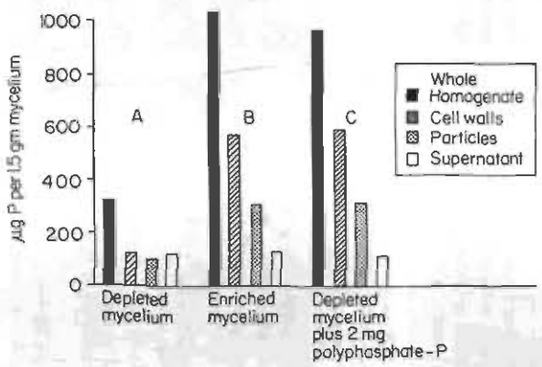


FIG. 1. Distribution of insoluble polyphosphate among centrifugal fractions of *Neurospora*. (A) mycelium depleted of polyphosphate by growing it in medium devoid of phosphate. (B) Mycelium enriched in polyphosphate by the addition of phosphate to the medium. (C) Depleted mycelium to which 2 mg polyphosphate-P were added just prior to fractionation, from Harold and Miller (1961).

In order to obtain cell organelles intact, particularly mitochondria, cells are usually disrupted in 0.25–0.88 *M* solutions of sucrose. Mannitol may be used for fungi which degrade sucrose (Tissières *et al.*, 1953). The medium should be properly buffered, especially for fungal cells or spores that are rich in organic acids. Some fungi may also liberate trace metals which could interfere seriously with enzyme function, unless they are chelated with EDTA or cysteine.

The protoplast is damaged whichever method is used to break cell walls. This was the reason for a search for gentler methods of disrupting cells. In bacteria, cell walls can be eliminated by lysozyme or penicillin, and the resulting "protoplasts" broken by osmotic shock (Weibull, 1958). In *Neurospora*, an osmotic mutant has been isolated (S. Emerson and Emerson, 1958), which, when treated with hemicellulase or snail enzymes grew as a "naked" protoplast without rigid cell walls and was highly sensitive to reduced osmotic pressure. Also, the cell wall can be removed in the wild-type by digestion with snail enzymes (Bachman and Bonner, 1959). However, the problem of obtaining many such cells for biochemical studies

TABLE I

ISOLATION OF CELL FRACTIONS IN FUNGI

Organism genus	Fractions isolated	Purpose of study	Method of breakage	References
Saccharomyces	Cell wall	Composition	Waring blendor, glass beads	Nickerson and Falcone, 1956a
	Cell membranes	Composition	Protoplast, osmotic shock	Bolton and Eddy, 1962
	Nuclei	DNA content	Protoplast, ultrasonics	Eddy, 1958
	Mitochondria ("particles")	Enzyme distribution	Nossal disintegrator	Nossal, 1954
	Mitochondria	Isolation	Nossal disintegrator	Linnane and Still, 1955
	Mitochondria	Definition	Ultrasonics	Vanderwinkel *et al.*, 1958
	Mitochondria	Oxidative phosphorylation	High speed blendor and glass beads	Vitols and Linnane, 1961
	Mitochondria	Lipid synthesis	Mechanical disintegration	Corwin *et al.*, 1957
	"Microsomes"	Lipid synthesis	Dry ice, glass beads, mortar	Klein, 1960
	Ribosomes	Composition	Carborundum, mortar	Chao and Schachman, 1956
	Ribosomes	Composition	Sand, mortar	Maeda, 1960
	Ribosomes	Protein synthesis	Carborundum, mortar	Webster, 1957
	Lipoprotein particles	Composition	Bacterial mill	Nyman and Chargaff, 1948
Candida	Mitochondria	Respiratory enzymes	- -	Takagi, 1959
Schizosaccharomyces	Ribosomes	Sedimentation coefficient	French press	Lederberg and Mitchison, 1962
Allomyces	Cell walls	Composition	Sonic oscillation	Aronson and Machlis, 1959
	Mitochondria	Respiration	Glass powder, mortar	B. A. Bonner and Machlis, 1957
	Chromospheres	Composition	Tissue homogenizer	Roren and Machlis, 1957
Mucor rouxii	Cell walls	Chemistry	Virtis homogenizer and ultrasonics with Ballotini glass beads	Bartnicki-Garcia and Nickerson, 1962

Neurospora	Cell walls	Polyphosphates	Glass beads, shaker	Harold and Miller, 1961
	Nuclei	Isolation	Sand, mortar	Reich and Tsuda, 1961
	Mitochondria	Respiratory enzymes	Sand, mortar	Tissières *et al.*, 1953
	Mitochondria	Succinic dehydrogenase	Quartz, mortar	Turian, 1962
	Mitochondria, microsomes	Inositol content	Sand, mortar	Shatkin and Tatum, 1961
	Microsomes	Chitin synthesis	Ten Broeck homogenizer	Glaser and Brown, 1957
	Microsomes	DNA–RNA complex	Hughes press	Schulman and Bonner, 1962
	Microsomes, supernatant	Protein synthesis	Glass, powder, mortar	Wainwright, 1959
	Ribosomes	Sedimentation coefficient	Alumina, mortar, glass bead, shaking	Storck, 1963
Aspergillus	Mitochondria	Oxidative phosphorylation	Sand, mortar	Iwasa *et al.*, 1959
Fusarium	Mitochondria	Electron transport	Glass powder, mortar	Kikuchi and Barron, 1959
	Mitochondria, microsomes	Enzymes	—	Maruyama and Alexander, 1962

remains. Similar experiments with *Saccharomyces* were more successful. There, large quantities of protoplasts were produced by digestion with snail juice by Nečas (1956) and later by Eddy and Williamson (1957). The problem of breaking fungal cells gently was probably the chief reason for the lag in the use of fungi in modern studies of the function of cell organelles.

Centrifugal separation of particles can be achieved either by using increasing centrifugal forces and separating precipitates at each step, or by a density gradient which distributes cell components of different specific gravity in layers in the centrifuge tube. A typical result of differential centrifugation can be demonstrated with *Neurospora*. Cell debris are sedimented at 1000 *g* in a few minutes. This contains unbroken hyphae, cell walls, and presumably nuclei. Mitochondria are brought down by 10,000 *g* for 15 minutes. Microsomes are sedimented only after prolonged centrifugation at 100,000 *g*. A layer of glycogen is deposited below microsomes. The supernatant still contains large amounts of ribonucleic acid (RNA), which may be either soluble RNA or degradation products of ribosomal RNA. Some of the lipid fraction rises to the surface after 10,000 *g*, and the rest after 100,000 *g*.

2. Cytochemistry

Cytochemical methods are used to detect materials in cells *in situ*. Substances are identified by their light absorption or, after an appropriate reaction, by the appearance of colored products. Several excellent books discuss cytochemical methods and describe them in detail (Danielli, 1958; Lison, 1960; Pearse, 1960; Glick, 1961). These methods were devised for the study of animal cells, but most can be adapted for use with plants (Jensen, 1962).

Few cytochemical observations can be made on living cells, but this should be easier in loosely growing hyphae of fungi than in many other organisms. Guilliermond (1941) and Dangeard (1956, 1958) took advantage of this fact to study mitochondria and vacuoles in *Saprolegnia* and other fungi by vital staining.

Usually, cells have to be fixed before being used for cytochemical reactions. Any fixative brings minor or major changes in the cytoplasm, which have to be taken into account in the study of localization of substances inside the cell. Thus, after osmic tetroxide and some other usual cytological fixatives glycogen was found unevenly distributed in bigger or smaller clumps in *Neurospora* hypha. When, however, freeze-substitution was used, glycogen appeared evenly distributed through the cytoplasm (Zalokar, 1961a). Freeze-substitution proves to be the best fixation method for small cells, such as fungal hyphae, because it avoids all fixation distortions of

gross cellular features and often preserves enzymatic systems better than other fixation methods.

In some fungal cells, the resolution of cytochemical reactions may not be optimal because of the small size and close association of cell organelles. Resolution can be improved by centrifuging living cells and performing cytochemical tests after the cell organelles are stratified. Fungal hyphae lend themselves particularly well to such a method, because the entire contents of a long cylindrical sector of mycelium can be stratified, as in an ultramicro test tube (Zalokar, 1960a). Inasmuch as it has been shown that centrifuged cells remain viable, this introduces fewer artifacts than centrifugation of disrupted cells. This may some day be combined with microsurgery by means of which each centrifuged fraction can be removed from the cell and analyzed separately.

It is beyond the scope of this review to describe individual cytochemical reactions. Instead, we summarize in Table II the work done on fungi with different cytochemical methods.

3. Autoradiography

Autoradiography can be considered a special branch of cytochemistry. In this procedure radioactive precursors of macromolecules are fed the cells, the cells are fixed, washed free of precursors, and the sites of radioactive decay, corresponding to the synthesized macromolecules, are detected with a photographic emulsion. The method has been used mainly to detect early formation sites of macromolecules. Thus, feeding an amino acid labeled with C^{14} or H^{3} can lead to the detection of proteins. If fixation occurs shortly after feeding, the newly synthesized protein can be found at the site of formation; if the experiment is run for a long time, all proteins of the cell become labeled and their distribution can be studied. Fungal hyphae prove to be excellent material for such research, since they can be easily fed under controlled conditions and moved quickly from one solution into another.

B. Metabolites

1. Inorganic Salts

Fungal cells often contain large quantities of inorganic substances. Some of them precipitate as crystals and are easy to locate, such as calcium oxalate and calcium carbonate (see Küster, 1956, pp. 492 and 505). Vacuoles contain polyphosphates which give metachromatic staining with toluidine blue and are responsible for vital staining of vacuoles with neutral red. Guilliermond (1941) and Dangeard (1956) observed that vital stains

TABLE II

CYTOCHEMISTRY OF FUNGI

Chemical (enzyme system) detected	Method used	Fungus	References
Iron	**Ferricyanide**	*Allomyces*	Ritchie, 1947
Polyphosphates	Lead, and benzidine, metachromatic stains	Various fungi of all systematic groups	Ebel *et al.*, 1958
	Neisser reagent	*Allomyces*	Turian, 1958
	Metachromatic stains	*Saccharomyces*	Wiame, 1947
Organic phosphorus	Molybdate of ammonia	*Allomyces*	Turian, 1958
Glycogen	Bauer's reaction	*Neurospora*	Zalokar, 1959b, 1960
Glycogen	Iodine	*Allomyces*	Turian, 1958
Polysaccharides	Period acid-Schiff (PAS)	*Allomyces*	Turian, 1958
	PAS	*Penicillium*	Stout and Koffler, 1
	PAS	*Saccharomyces*	Mundkur, 1960
	PAS	*Neurospora*	Zalokar, 1961b
Fat droplets	Sudan IV	*Saccharomyces*	Lindegren and Rafa 1950
Lipids	Sudan IV, OsO_4	*Neurospora*	Zalokar, 1960a
Proteins	Pauly, Sakaguchi, SH groups (Seligman)	*Neurospora*	Zalokar, 1959b, 196
—SH protein	Rapkine	*Allomyces*	Turian, 1956
Oxidative enzymes (mitochondria)	Janus green	*Rhodotorula*	Bautz, 1955
	Janus green	*Saprolegnia*	Guilliermond, 1941
	Janus green	*Neurospora*	Freese-Bautz, 1957
Succinic dehydrogenase	Tetrazolium	*Neurospora*	Zalokar, 1959b, 19(
	Triphenyl tetrazolium	*Candida*	Seyfarth, 1952; N son, 1954
	Triphenyl tetrazolium, Nadi, Janus Green	*Saccharomyces*	Mudd *et al.*, 1951
Cytochrome oxidase	Nadi	*Rhodotorula*	Marquardt and I 1954
	Nadi	*Saccharomyces*	Bautz and Marq 1953; Bautz, 19
	Nadi	*Allomyces*	Turian, 1958
	Nadi	*Blastocladiella*	Cantino and Hore 1956
	Nadi	*Neurospora*	Zalokar, 1959b, 1(
Peroxidase	Benzidine	*Neurospora*	Zalokar, 1959b, 1(
Alkaline phosphatase	Gomori	*Allomyces*	Turian, 1958
	Gomori; Meinhemer and Seligman	*Neurospora*	Zalokar, 1959b, 1

TABLE II (Continued)

Chemical (enzyme system) detected	Method used	Fungus	References
Acid phosphatase	Gomori	*Allomyces*	Turian, 1958
	Gomori; Rutenburg and Seligman	*Neurospora*	Zalokar, 1959b, 1960a
β-Galactosidase	6-Br-2 naphthyl-β-galactosidase and Diazo-Blue B	*Neurospora*	Zalokar, 1959b, 1960a
Volutin, RNA	Indophenol blue, RNase	*Saccharomyces*	Nečas, 1958
RNA	Methyl green–pyronine	*Allomyces*	Turian, 1957
RNA	Unna; acridine orange RNase	*Neurospora*	Zalokar, 1959b, 1960a
DNA and RNA (nucleus, nucleolus)	Acridine orange vital	*Saccharomyces*	Royan and Subramaniam, 1960
	Giemsa, DNase, RNase	*Lipomyces*	Ganesan and Roberts, 1959
	Giemsa, DNase, RNase	*Saccharomyces*	Ganesan, 1959
	Feulgen, Methyl green–pyronine	*Physarum*	Andersen and Pollock, 1952
DNA (nucleus)	Feulgen	*Saccharomyces*	Lindegren and Rafalko, 1950; DeLamater, 1950
	Fluorescent dyes	*Schizosaccharomyces*	Rustad, 1958
	Feulgen	*Allomyces*	Hatch, 1935; Ritchie, 1947; Jones, 1947; Turian, 1958
	Azure B SO_2	*Neurospora*	Huebschman, 1952; Zalokar, 1959b, 1960a

induce a precipitation of colored particles in vacuoles, indicating that, in the natural state, polyphosphates (which they called metachromatine) are colloidally dispersed. The so-called volutin granules (metachromatic granules) are highly concentrated deposits of polyphosphates (Wiame, 1947) and can exist in a cell next to vacuoles (Jordanov *et al.*, 1962).

The intracellular distribution of soluble ions has not been studied in fungi. It is possible that several, including potassium and sodium, are concentrated in vacuoles, thereby maintaining osmotic pressure. Studies on permeability of potassium and rubidium indicated that there must be special ion-binding sites in the cytoplasm (G. Lester and Hechter, 1959). Ritchie (1947) found iron in paranuclear bodies of *Allomyces*. Cell walls contain a large proportion of mineral components, such as those of *Mucor*, with 22% phosphates [as $(H_2PO_3)n$], 1% magnesium and calcium and traces of

silicon, iron, copper, chromium, aluminum, barium, cobalt, and manganese (Bartnicki-Garcia and Nickerson, 1962). According to Harold and Miller (1961), the presence of polyphosphates in the cell wall fraction of fungi is due to an artifact (see p. 378). Other inorganic ions frequently are associated with enzymes, and therefore are unevenly distributed in the cell.

2. Carbohydrates

Fungi usually show a high rate of endogenous respiration, which is due to an accumulation of reserve substances. The main reserve carbohydrate is glycogen; this can be located by many cytochemical reactions. In *Neurospora* hyphae (Zalokar, 1959b, 1961a) and in yeast (Mundkur, 1960), glycogen is distributed evenly throughout the cytoplasm in the form of submicroscopic granules about 200 A in diameter. The only regions devoid of glycogen are hyphal tips. Glycogen sediments easily in centrifuged living hyphae (Zalokar, 1960a) and can be obtained as a colorless pellet by centrifuging homogenized preparation at 100,000 g. Nothing is known about the localization of soluble carbohydrates inside the cell. In centrifuged cells, the periodic acid-Schiff reaction colors a bigger region than Bauer's reaction, an observation indicating that carbohydrates other than glycogen sediment over the glycogen layer.

Cell walls of fungi are made mainly of carbohydrates and their derivatives. These carbohydrates can be identified by cytochemical reactions, but better yet, they can be studied in isolated cell walls. More about this can be found in Section III and Chapter 3.

3. Lipids

Lipids and fatty substances are present in fat droplets called liposomes, sphaerosomes, or microsomes by different cytologists. These fat droplets can be identified by staining with Sudan III and IV and by their ability to reduce osmic tetroxide. They probably contain most of the fatty substances (triglycerides) that can be identified in fungi by chemical methods. Fat deposits become especially heavy in older cells or under abnormal growth conditions, a state similar to the fatty degeneration of animal cells.

Important constituents of the cytoplasm and cytoplasmic membranes are composed of lipids and lipoproteins. Membranes of mammalian cells contain cholesterol, phospholipids (lecithin, cephalin) and cerebrosides. Not much is known of the chemistry of lipids in fungal membranes. Various phospholipids and cerebrosides were found in fungi and are probably located on membranes (cf. Chapter 12). *Myo-inositol* was demonstrated to be a part of the cytoplasmic membranes in *Neurospora* (Shatkin and Tatum, 1961). Ergosterol is the main steroid found in fungi, where it

probably replaces cholesterol in the structure of cell membranes. The presence of ergosterol in cell membranes has been deduced from its binding of nystatin (Lampen *et al.*, 1962). Ergosterol and some other steroids are, however, not restricted to the cell membranes. Hexagonal crystals about 1 μ in diameter have been found in *Neurospora* and identified as ergosterol (Tsuda and Tatum, 1961).

Fat-soluble carotenoids are associated with fat droplets (Guilliermond, 1941), but in *Mutinus* (Phallales) they crystallize out as needles or platelets (Heim, 1946a,b). Carotenoids appear to accumulate in mitochondria during maturation of the mycelium, and this may indicate their site of formation (Heim, 1946a,b). The cytoplasm also is tinted by carotenoids, which are probably associated with various lipophilic structures. These include liposomes of submicroscopic size, which are scattered throughout the cell, but accumulate at the centripetal end in centrifuged living cells. In broken-cell preparations, however, a good proportion of the carotenoids remain in both microsomes and the supernatant after separation of mitochrondria and fat.

4. Other Metabolites and Metabolic Products

Measurements of ultraviolet absorption indicated that *S-adenosyl* phosphate, an important intermediate in metabolism, accumulates in vacuoles of *Candida* (Svihla and Schlenk, 1960). Thiamine was found to be localized mainly in mitochondria in yeast (Suomalainen and Rihtniemi, 1962). *Eremothecium* produces such amounts of riboflavin that it crystallizes in vacuoles (Guilliermond *et al.*, 1935).

Cells contain pools of amino acids that can be eliminated only with difficulty by starvation. The study of the kinetics of amino acid uptake in *Candida* (Cowie and McClure, 1956) and *Neurospora* (Zalokar, 1961b) showed that there are at least two pools of amino acids, one intermediate pool of relatively constant size from which amino acids are taken for protein synthesis, and an expansible pool into which excess amino acids are shunted to serve as a reserve. Nothing is known about the intracellular localization of these pools, but either the cisternae of the endoplasmic reticulum, or vacuoles, can be suggested as possible sites. Since old vacuolated cells are less active in amino acid uptake than young ones, the second possibility seems to be less probable.

C. Proteins and Enzymes

1. Proteins

There is no universal cytochemical reagent for proteins, but many cytochemical methods exist to detect proteins by certain reactive groups in their

molecule. An approximation of the amount of protein in different regions of hyphae can be obtained by using common cytological stains, many of which stain primarily proteins. Hyphae seem to be richest in protein near their tips; in older, distant regions, protein staining becomes weaker. This does not necessarily mean that the cytoplasm itself is more dilute, but

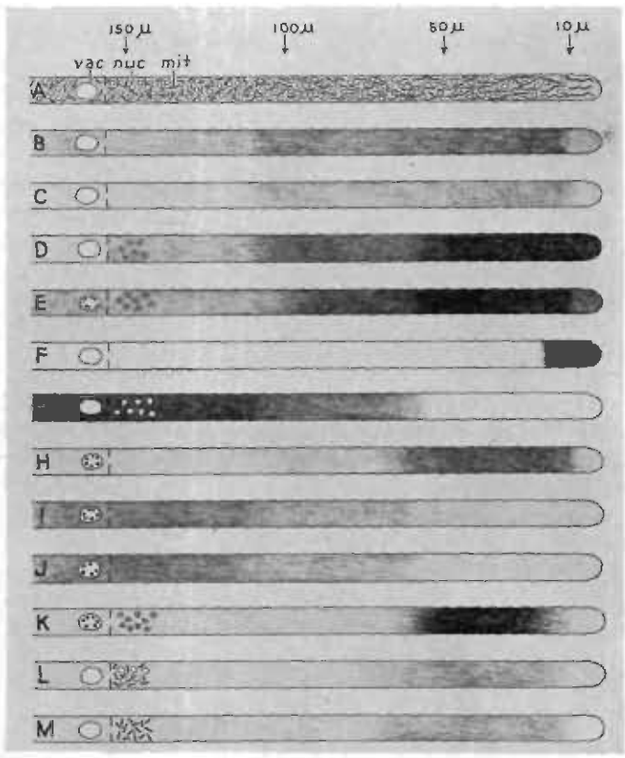
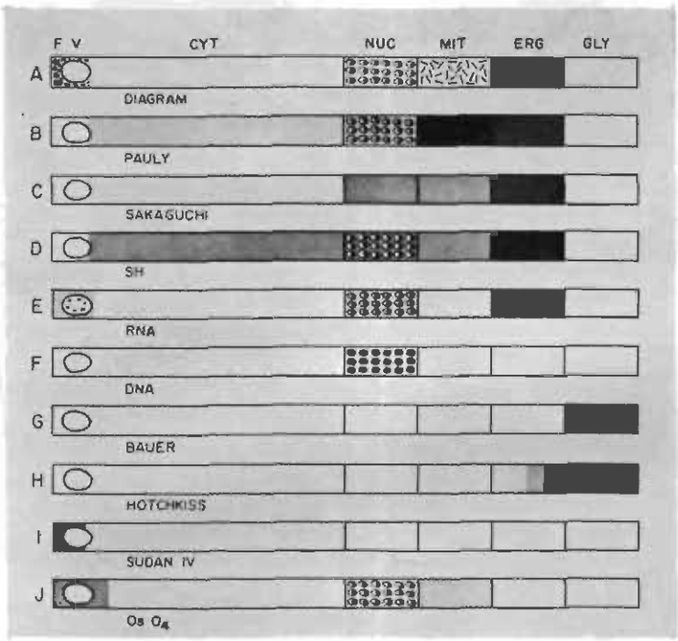


FIG. 2. Schematic presentation of the results of cytochemical tests on *Neurospora*. Shading indicates relative intensity of the reaction. Nuclei, mitochondria, and vacuolar inclusions are drawn only when they give a significant reaction. (A) Cytology of hyphal tips (first 100 μ) and young hypha (at the left): *vac*, vacuole; *nuc*, nucleus; *mit*, mitochondria. (B) Pauly's reaction for proteins. (C) Sakaguchi's reaction for proteins (arginine). (D) Barnett and Seligman's test for protein bound —SH groups. (E) Unna's stain for ribonucleic acid. (F) Unna's stain after ribonuclease. (G) Bauer's stain for glycogen. (H) Benzidine reaction for peroxidase. (I) Nadi reaction for cytochrome oxidase. (J) Tetrazolium reaction for succinic dehydrogenase. (K) Gomori's reaction for alkaline phosphatase. (L) Gomori's reaction for acid phosphatase. (M) β-Galactosidase by the method of Cohen *et al.*, from Zalokar (1959b).

rather that it contains more nonproteinaceous inclusions, such as vacuoles, fat droplets, or glycogen. Some fungi contain protein crystalloids in their cytoplasm (see Küster, 1956, p. 487).

Proteins containing a high proportion of basic amino acids are found in ribosomes, which stain strongly with basophilic stains and with the Sakaguchi reaction. The aromatic amino acids, as detected by the Pauly reaction (coupling with diazo compounds), appear more prominent in mitochondria. Reactions to detect protein bound —SH groups give more coloration on ribosomes. In growing hyphae, the —SH reaction is particu-



FIGS. 3 and 4. Cytochemistry of centrifuged hyphae of *Neurospora* (schematic). Shading indicates relative intensity of the reaction. Centrifugal direction to the right, from Zalokar (1960a).

FIG. 3. (A) Diagram of a centrifuged hypha indicating positions of sedimented cell components: *F*, fat; *V*, vacuole; *CYT*, "supernatant" cytoplasm; *NUC*, nuclei; *MIT*, mitochondria; *ERG*, ergastoplasm; *GLY*, glycogen. (B) Pauly's reaction for proteins. (C) Sakaguchi's reaction for proteins (arginine). (D) Barnett and Seligman's test for protein bound —SH groups. (E) Acridine orange stain for ribonucleic acid. (F) Huebschman's Azure A-SO_2 stain for nuclei (deoxyribonucleic acid). (G) Bauer's reaction for glycogen. (H) Hotchkiss reaction (periodate-Schiff) for carbohydrates. (I) Sudan IV stain for fats. (J) Osmium tetroxide staining for lipids.

larly prominent near the growing tips, which may indicate that proteins rich in —SH groups play a role in the growth processes. Strong coloration of nucleoli by all three methods indicates their high content of proteins.

Figure 2 recapitulates the results of cytochemical reactions applied to growing hyphae of *Neurospora;* and Figs. 3 and 4, as applied to centrifuged hyphae.

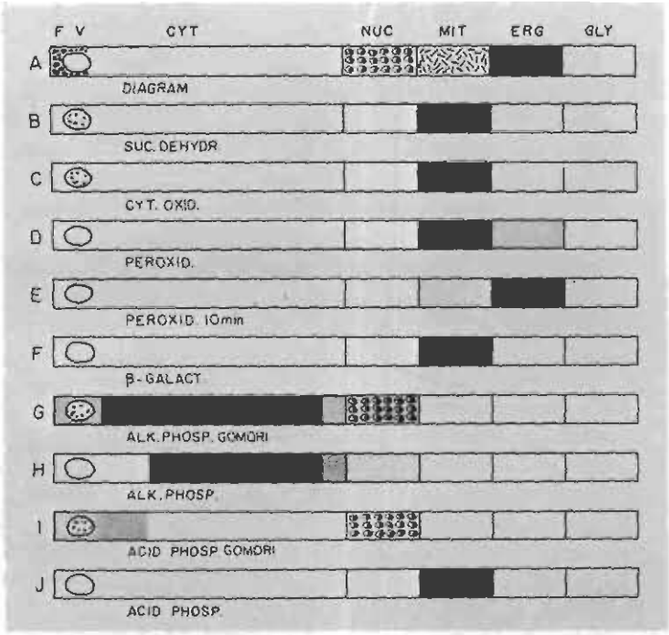


FIG. 4. (A) Same as Fig. 3A. (B) Succinic dehydrogenase (tetrazolium). (C) Cytochrome oxidase (Nadi). (D) Peroxidase (benzidine). (E) Peroxidase after 10 minutes. (F) *β-Galactosidase (o-nitronaphthyl galactoside).* (G) *Alkaline phosphatase* (Gomori, adenine 3-phosphate). (H) Alkaline phosphatase (β-naphthyl phosphate and Diazo-Blue B). (I) Acid phosphatase (Gomori, glycerophosphate). (J) Acid phosphatase (α-naphthyl phosphate and Diazo-Blue B).

2. *Enzymes*

It is most important for the understanding of the function of an intact cell to know the exact localization of the enzymes involved in metabolic reactions. In the following list, the localization of enzymes in different parts of fungal cells will be given, following the order in the review of de Duve

et al. (1962). Only enzymes whose distribution was followed in various cell fractions by centrifugation or cytochemistry will be considered.

Pyruvate oxidase—oxidizing pyruvate to acetyl CoA is found in mitochondria, while supernatant enzymes perform this oxidation via acetaldehyde and acetate (Holzer and Goedde, 1957).

Succinic dehydrogenase—mitochondria exclusively, by both centrifugation and cytochemical methods. Any differing reports could be traced to artifacts or uncritical application of the method.

Cytochrome oxidase—same as succinic dehydrogenase. Some reports show that not all mitochondria react positively, an indication of their heterogeneity (Bautz and Marquardt, 1953; Turian, 1958).

Peroxidase—in mitochondria (Zalokar, 1960a).

Polyphenol oxidase—in cell walls of sporangia (Cantino and Horenstein, 1956).

α-Glycerophosphatase—distributed between mitochondria and supernatant (Maruyama and Alexander, 1962).

Glucose 6-phosphatase—mainly in the supernatant (Maruyama and Alexander, 1962).

Disulfide reductase—in mitochondria of yeast, an enzyme was found which reduced disulfide linkages in cell-wall protein (Nickerson and Falcone, 1956a,b).

DPN-cytochrome reductase and *TPN-cytochrome reductase*—mainly in mitochondria, some in supernatant in *Fusarium* (Maruyama and Alexander, 1962). Presence in microsomes is dubious, since their preparation contained also succinic dehydrogenase and cytochrome oxidase, which are known to be restricted to mitochondria.

Alkaline phosphatase—Gomori reaction for alkaline phosphatase is positive in the cytoplasm and strongly stains mitochondria (Turian, 1958; Zalokar, 1959b). In centrifuged cells, a special region of the supernatant cytoplasm shows activity (Zalokar, 1960a). Mitochondria do not react, an indication that their reaction in the intact cell is an artifact. Alkaline phosphatase is also present in nucleoli, although this often was considered to be an artifact.

Acid phosphatase—Diffuse in cytoplasm, more in mitochondria, cell membrane, or cell wall. In centrifuged cells, mainly in the mitochondrial fraction (Zalokar, 1960a). De Duve *et al.* (1962) believe that lysosomes contain all cytoplasmic acid phosphatase. Since these granules have not yet been found in fungi, the localization in mitochondria may be accepted. Some of the apparently positive Gomori test in cell walls is probably due not to phosphatase, but to large amounts of phosphates composing the wall (Bartnicki-Garcia and Nickerson, 1962). In yeast, most of cellular acid phosphatase is located near the cell membrane (Schmidt *et al.*, 1963). Both phosphatases are absent in nuclear cap of *Allomyces* (Turian, 1958), which is considered to be an agglomeration of ribosomes; the phosphatases likewise are absent in the ribosomal layer of centrifuged living cells.

β-Galactosidase—in the mitochondrial fraction in centrifuged living cells (Zalokar, 1960a), but easily extracted in enzyme preparations (Landman and Bonner, 1952).

Invertase, cellobiase, trehalase, and *maltase*—located at the surface of fungal spores by acid inactivation (Mandels, 1953).

Invertase, melibiase—yeast cell surface (Friis and Ottolenghi, 1959a,b).

ATPase—in mitochondria, but the major part in the supernatant (Iwasa *et al.*, 1959); Bolton and Eddy (1962) found more than 80% bound to particles, presumably endoplasmic membranes.

Fumarase and *aconitase*—in yeast particles (mitochondria), passed into supernatant with extended disintegration procedure (Nossal, 1954).

Aldolase, aconitase, and *uricase*—predominantly in the supernatant in *Fusarium* (Maruyama and Alexander, 1962).

D. Nucleotides and Nucleic Acids

Fungi contain pools of free nucleotides (phosphates of adenine, uridine, and others) which are used in intermediary metabolism and for the synthesis of nucleic acids. As in the case of amino acids, at least two independent pools exist in the cell, as was shown in *Candida* and *Neurospora* (Cowie and Bolton, 1957; Zalokar, 1962). If the nucleotides remain in solution, the pools must be separated physically into two compartments. It has been shown that purines and pyrimidines, when fed to *Candida,* accumulate in the vacuoles, where they precipitate by crystallization (Svihla *et al.,* 1963). This indicates that the vacuole may be one of the two compartments, unless it constitutes another reserve pool.

DNA has been found only in nuclei. In *Saccharomyces* the identification of DNA was of particular value, since in early cytology it was a question whether the vacuolar structure or the adjacent granule was the actual nucleus. DNA can be localized by the very specific Feulgen reaction. In some fungi, better staining is obtained with a similar method, using sulfonated azure B instead of fuchsin (Huebschman, 1952). Other less specific stains were successfully used to identify nucleic acids. A modern method to demonstrate DNA uses radioactive thymidine, which is incorporated into DNA and is detected by autoradiography. This method failed entirely in *Neurospora* since thymidine was not incorporated directly (Fink and Fink, 1962) but was quickly degraded by the cell, so that radioactivity passed into many cellular substances.

RNA can be detected with basic dyes in combination with crystalline ribonuclease. However, one precaution is necessary: fungal cells contain a highly active phosphatase, so that what we may assume to be a specific digestion by added RNase, may be less specific digestion by internal enzymes that were not entirely inactivated by fixation. RNA usually is equally distributed throughout the cytoplasm and there are no excessively basophilic regions, as in pancreatic ergastoplasm. Centrifugation of living cells show that RNA is present mainly in ribosomes. According to electron micrography, the latter are mostly free inside the cytoplasm and not associated with the membranes of the endoplasmic reticulum (Zalokar, 1961a). Isolated *Neurospora* ribosomes have a sedimentation coefficient of 81 S and dissociate at lower concentrations of $MgCl_2$ into smaller particles, 19 S and 13 S (Storck, 1963). They contain 46.6% RNA and 53.4% protein. Ribosomes of yeast are similar, having a sedimentation coefficient of 80 S and 42%

RNA and 58% protein (Chao and Schachman, 1956). Yeast ribosomes also dissociate into smaller units with sedimentation coefficients of 60 S and 40 S (Chao, 1957) and 28 S, 19 S, and 5 S (Maeda, 1960). In *Schizosaccharomyces,* ribonucleoprotein particles of 120 S, 83 S, 60 S, and 38 S were isolated, the 83 S particle having 41% RNA and 59% protein (Lederberg and Mitchison, 1962).

RNA was found also in a mitochondrial fraction which later was proved to be contaminated with ribosomes, so that the presence of RNA in mitochondria is questionable. In the nucleus, RNA is prominent in nucleoli, as is common in cells of higher organisms. An interesting special case are spores of *Allomyces* where RNA is concentrated in chromospheres 3–4 μ in diameter (Roren and Machlis, 1957) and the so-called "nuclear cap" (Blondel and Turian, 1960).

Cytochemical methods do not permit a distinction to be made between different types of RNA. In centrifuged *Neurospora* cells, only traces of RNA remain in the "supernatant" cytoplasmic fraction—perhaps transfer RNA. On the other hand, it is possible that in the living cell, most of the transfer RNA is associated with ribosomes and sediments with them during centrifugation. A soluble factor which mediated the genetic control of protein synthesis was demonstrated in *Neurospora* by Wainwright (1960), who was able to obtain protein synthesis in a cell-free preparation in which both the ribosome fraction and the supernatant were required. The supernatant carried a gene-specific fraction, which must have been the equivalent of "messenger" RNA (Wainwright and McFarlane, 1962). Recently, Schulman and Bonner (1962) found in ribosome fractions a complex of DNA and RNA which is believed to contain RNA in the process of formation. Since DNA can be expected to occur in nuclei, it is highly probable that this fraction was liberated from nuclei during the disruption of the cell and sedimented with the microsomal fraction.

III. CELLULAR STRUCTURE AND FUNCTION

Section II extended cell morphology to the chemical level. Section III will carry the results of biochemical investigations of cell-free preparations to a morphological level. We have to consider a cell as a highly organized entity with special parts performing special functions. Some of these parts can be recognized as cell organelles. Some, such as nuclei or mitochondria, are well delimited and can be easily detected microscopically. Others, e.g., the endoplasmic reticulum, are only vaguely delimited regions of the cytoplasm with special properties. Yet others are submicroscopic units, such as ribosomes, recognizable only with the electron microscope. Finally, a large part of the cytoplasm does not consist of cell organelles, or a diffuse

unit, or an ultrastructure observable under the electron microscope, but is called the hyaloplasm or enchylema—everything that is left when visible structural elements have been removed. But even here, there may be a special structural organization which eludes us as yet.

A. Nucleocytoplasmic Relationships

In fungi, vegetative cells can contain one, two, or many nuclei. These nuclei are usually haploid, sometimes diploid, and in higher forms, may be paired in dikaryons. The function of nuclei is to carry genetic information from generation to generation and to pass it to the cytoplasm. Today we believe that DNA is the chemical basis of genes, and we express concisely the theory of gene function by the sequence DNA → RNA → protein.

1. Nuclear Replication

The first function of nuclei is the replication of DNA. Molecular events leading to this replication are discussed in many reviews (Cavalieri and Rosenberg, 1962; Grunberg-Manago, 1962). Briefly, it is believed that DNA is synthesized from triphosphates of deoxyribonucleosides which are linked together with the enzyme DNA-polymerase. DNA-polymerase has been demonstrated in various cells (Kornberg, 1960) and, strangely enough, was found in supernatants of broken cell preparations. The enzyme could, of course, have escaped from nuclei during preparation. In fungi, DNA-polymerase has not yet been isolated, but there is a report of an enzyme which synthesizes polynucleotides from ADP (Grunberg-Manago and Wisniewski, 1957) in yeast.

Nuclear reduplication could be studied better if cell divisions were synchronized. This can be done in bacteria, and has been achieved in yeast (Mitchison, 1957). In multinucleate slime molds this occurs naturally, and Nygaard *et al.* (1960) found that in *Physarum* DNA synthesis occurs 1–2 hours after nuclear division, followed by the rest of the interphase lasting 12–20 hours. RNA synthesis was continuous; it was not interrupted during DNA synthesis, but was reduced during mitosis. Continuous RNA synthesis was recorded also in yeast (Mitchison and Walker, 1959).

2. Production of Ribonucleic Acid

The second major function of nuclei is to transfer genetic information into cytoplasm, and this they do through RNA. Today we have ample evidence that RNA is produced in nuclei and then released to the cytoplasm. Early studies of Caspersson (1950) with yeast indicated an accumulation of RNA near nuclei. With the advent of radioactive tracers, RNA formation in nuclei could be demonstrated directly.

By removing or transplanting nuclei, and the use of autoradiography, at least a major part of RNA formation was shown to depend on nuclei in *Amoeba* and *Acetabularia* (Brachet, 1961). No fungus has yet been used for such operations. As the nuclei are very small in most fungal cells, and the resolution by autoradiography is limited, RNA formation by nuclei was demonstrated with the aid of centrifuged cells (Zalokar, 1960b). In *Neurospora,* when living cells are centrifuged, nuclei and RNA-rich ribosomes sediment in layers that are separated from each other by a layer of mitochondria. When C^{14}-adenine, H^3-uridine, or H^3-cytidine was fed to the cells immediately before centrifugation, newly labeled RNA appeared only in nuclei (in the first 4 minutes) and passed into the ribosomal layer later. Kinetic studies of precursor uptake and incorporation indicated that there could be no appreciable independent cytoplasmic synthesis of RNA.

Where are each of the three types of RNA formed—transfer RNA, ribosomal RNA, and messenger RNA? By definition messenger RNA should be formed in the nucleus. If RNA is made by DNA, it should reflect its base composition. It is known that cytoplasmic RNA has a base composition that differs significantly from that of DNA. Yčas and Vincent (1960) were able to demonstrate in yeast the existence of an RNA fraction, which became labeled a very short time after the addition of P^{32} and had a base composition more similar to DNA than to cytoplasmic RNA. According to Kitazume *et al.* (1962), this RNA is a precursor of other types of RNA in the cell. The finding of a DNA–RNA complex in *Neurospora* (Sebulman and Bonner, 1962) supports the hypothesis of production of RNA by DNA. Both experiments indicate strongly that in fungi, as in other cells, messenger RNA is made by DNA.

The origin of ribosomal RNA is more uncertain. Studies of nuclei and nucleoli of larger cells indicate that an RNA similar to ribosomal RNA accumulates in nucleoli and is presumably made by nucleolus-associated chromatin. In fungi, it has not as yet been possible to isolate free nucleoli, and the small size of nuclei has precluded autoradiographic studies of nucleolar functions. Transfer RNA also is probably made by nuclear DNA, since there are molecules specific for each amino acid which must have a specific sequence of nucleotides. In recent work, Yanofsky *et al* (1961), found that a suppressor mutation in certain cases may be due to a change in transfer RNA, thus supporting the idea of the genetic origin of this RNA.

3. Other Nuclear Functions

Besides nucleic acids, nuclei synthetize proteins, as can be shown by autoradiography. Some of these proteins may be enzymes needed in nucleic acid synthesis, whereas others should be proteins connected with the organization of genetic material, such as histones and other proteins or components

of chromosomes. A hypothesis has been proposed that the protein moiety of ribosomes is synthesized in nucleoli (J. Bonner, 1959).

An interesting finding is that nuclei contain active DPN-synthetase (Hogeboom and Schneider, 1952). If it could be shown that all cellular DPN is made in nuclei, this would have special significance for nuclear control of cellular processes. It should be noted that nuclei have a glycolytic system, probably involved in the generation of ATP (Mirsky and Osawa, 1961), but no enzymes involved in aerobic, energy-yielding reactions. Whatever other enzymatic processes were found in nuclei may have been due to cytoplasmic contamination and are not unambiguously established.

Nuclei control the cytoplasm through their genetic material, but cytoplasm controls also many phases of nuclear activity. In differentiated cells, or under particular physiological conditions, some genes become activated, or others are inactivated, as a result of metabolic processes in the cytoplasm. This can sometimes affect nuclear morphology: the size and appearance of nuclei differ in different cells of the same organism. All nuclei in a vegetative hypha seem to be equivalent and alike, but they change conspicuously in ascogenous hyphae. While vegetative cell division is difficult to follow and does not seem to conform to the classical mitosis (see Chapter 6), the mitotic divisions preceding and following meiotic division produce clearly observable chromosomes (Singleton, 1953).

An old hypothesis in cytology is that cell division starts when the nucleocytoplasmic ratio reaches a certain level during cell growth, but this has never been satisfactorily proved. Particularly in multinucleate fungal hyphae, where some nuclei are continuously dividing and others resting, a more complex mechanism should be sought. In the case of synchronous nuclear division in slime molds, special substances initiating mitosis may be involved.

4. Nuclear Cooperation

The fact that fungal cells contain several nuclei accounts for certain phenomena, not observable in uninucleate cells, as all the nuclei exert their effect on a common cytoplasm. In a normal hypha, some nuclei may be temporarily inactive because of division, but whether other nuclei are all equally active is open to question. Nuclear cooperation was best studied in heterokaryons where two genetically different sets of nuclei are brought together into a common cytoplasm. If one set is deficient in one biochemical reaction and the other in a different one, they can complement one another by releasing into the cytoplasm what the other nucleus lacks. In the case of two distinct genes, each nucleus probably furnishes the messenger RNA and, consequently, the enzyme which the deficient nucleus above cannot

produce. Since the study of heterokaryons is an important part of genetic studies in fungi, it will be discussed in more detail in Volume II.

A more complex situation exists in cases of interallelic complementation, where both nuclei contain the same deficient gene, but complementation still occurs in the cytoplasm. Several mechanisms could be involved: (1) one nucleus provides to the other a missing part for messenger RNA synthesis; (2) messenger RNA's complement each other in the cytoplasm; (3) messenger RNA's produce deficient polypeptides which then combine to give an active protein. The third mechanism was shown to be probable in a *Neurospora* mutant (Woodward *et al.*, 1958; Gross, 1962).

B. Protein Synthesis

RNA, which has its origin in nuclei, becomes active in the cytoplasm in the synthesis of proteins. It appears now that all three types of RNA collaborate in protein synthesis. Messenger RNA provides information on the sequence of amino acids, ribosomal RNA provides a "workbench" needed for the proper functioning of messenger RNA, and transfer RNA is used as an adapter, fitting individual amino acids into proper places on the messenger RNA. The chemistry of protein synthesis was discussed in Chapter 13 and has been covered by many recent reviews (Chantrenne, 1961; Novelli, 1960; Simpson, 1962).

In mammalian cells, there is a special region of the cytoplasm, called the ergastoplasm, which functions in protein synthesis. In cell-free preparations, the ergastoplasm breaks down and can be isolated as microsomes. It has been shown that these microsomes are able to incorporate amino acids into proteins. After special treatment, ribosomes can be liberated from microsomal membranes and used in protein-forming preparations.

In fungi, most of the ribosomes seem to be free in the cytoplasm, and cell membranes covered with ribosomes, as in the ergastoplasm, are rare. Therefore, ribosomes can be isolated directly from homogenized cells (Chao and Schachman, 1956; Storck, 1963). In *Neurospora,* it was shown that ribosomes are protein-synthesizing sites in living cells (Zalokar, 1960b). They were sedimented by centrifugation, and when cells were fed tritiated amino acids, newly formed proteins were detected in ribosomal layers by autoradiography. The protein remained associated with the ribosomes for a very short time, since after 1 minute all the proteinaceous cytoplasmic fractions became labeled. By kinetic studies of amino acid incorporation, the time required to assemble a protein molecule was estimated to be less than a few seconds (Zalokar, 1961b).

There is some indication that the three RNA's are not sufficient to main-

tain the normal rate of protein synthesis—all *in vitro* systems synthesize proteins at only a small fraction of the normal rate. In yeast, it was shown that ribosomes associated with membranes synthesize proteins faster than free ribosomes (Hauge and Halvorson, 1962). In centrifuged *Neurospora* cells, no radioactive amino acid uptake was noticed as long as the ribosome layer remained separated from the "supernatant" layer of the endoplasmic reticulum. As soon as the layers were remixed, shortly after the cessation of centrifugation, incorporation started again. Do the cytoplasmic membranes provide additional enzymes needed in protein synthesis, or do ribosomes find access through them to amino acid pools? If the intermediate pool of amino acids is in intracisternal spaces which, according to Palade (1956), are all interconnected and probably in contact with the outside of the cell, then it is conceivable that ribosomes have to be associated with membranes to have access to amino acids. An intriguing possibility is that cytoplasmic membranes orient or spread out ribosomes to activate them. This is based on the observation of Hanzon *et al.* (1959) that after freeze-drying, which seems to be the least drastic method of cell fixation, ribosomes were not visible in ergastoplasm of pancreas cells.

C. Organization of Enzymes into Functional Units

While a biochemist works most often with enzymes in homogenized preparations, the cell has its enzymes organized in various structures. The structural organization is needed for the coordination of enzyme function. Many enzymes behave differently when free in solution or when adsorbed on surfaces or associated with other colloidal systems (McLaren and Babcock, 1959; Oparin *et al.*, 1962). Proper arrangement of enzymes may lead to more efficient use of substrates, or structural confinement may prevent enzymes from attacking substrates where, or when, not desired.

1. Mitochondria

The most conspicuous organized enzyme systems are mitochondria, the energy-generating system of the cell. Energy is produced by coupling three main reactions, the tricarboxylic acid cycle, electron transport, and oxidative phosphorylation. Chapters 11 and 12 discuss the chemistry of these reactions. Fungi were one of the earliest organisms in which mitochondria were studied (Guilliermond, 1941), but we derive most of our present knowledge of mitochondrial chemistry and function from beef heart muscle (Green and Fleischer, 1962; Novikoff, 1961).

We are now beginning to understand the internal organization of enzymes in mitochondria. Primary dehydrogenases of the Krebs cycle are only loosely

bound, since they pass into solution in broken-cell preparations. More firmly bound is succinic dehydrogenase, part of the succinic oxidase system which is responsible for the typical cytochemical reaction of mitochondria with Janus green or neotetrazolium. Succinic acid is oxidized to fumaric acid by removal of two hydrogens, which are passed through an electron transport chain involving flavoprotein, a series of cytochromes, and ubiquinone to elementary oxygen. The components necessary for electron transport are probably arranged inside the so-called "electron transport" particles in a very specific configuration (Criddle *et al.*, 1962). DPN, the cofactor in electron transport, although a relatively small molecule, appears to be bound to mitochondria, but is liberated during their isolation (R. L. Lester and Hatefi, 1958). Yeast mitochondria, however, do not lose their DPN (Vitols and Linnane, 1961) during isolation.

The main task of mitochondria is to generate energy which is transferred in the form of ATP, a product of oxidative phosphorylation. How this ties up with the electron transport chain is still an open problem (see Chapter 12). The process requires intact mitochondrial structure and may reside in the cristae. In yeast cell-free preparations, a heavy fraction which contained typical mitochondria with cristae could oxidize succinate, pyruvate, and malate and had active oxidative phosphorylation, whereas a light fraction, composed of simple vesicles, oxidized succinate at a similar rate but did not possess the other activities (Vitols and Linnane, 1961).

The fine structure of mitochondria changes with their functional state. During anaerobic growth, yeast mitochondria appear as vesicles with only a few short cristae (Yotsuyanagi, 1962). Toward the end of growth, when cells show more aerobic respiration, mitochondria develop typical cristae. Particles isolated from anaerobic yeast were found to contain succinic and DPNH dehydrogenase activity, but no cytochromes (Linnane *et al.*, 1962). Therefore it is important, when effects of heredity are sought, to consider growth conditions in the study of mitochondrial structure. The particular appearance of mitochondria may be due only to metabolic changes that, in turn, are due to mutation.

The microscopic appearance of mitochondria also varies under different conditions. Swelling was observed when oxidative phosphorylation was uncoupled by chemicals (Lehninger and Wadkins, 1962). Under adverse growth conditions, hyphae contain long filamentous mitochondria. Growing tips of hypha contain small, rounded mitochondria which become filamentous farther back in the cell in *Saprolegnia* (Guilliermond, 1941), but the reverse occurs in *Neurospora* (Zalokar, 1959b). Observation of living cells shows mitochondria in constant movement, and they have been seen to fuse or break into smaller pieces. Intensive cytoplasmic streaming, and their

own movements, bring mitochondria into contact with all parts of the cell. This may make possible a direct transfer of certain substances between mitochondria and other organelles.

2. Glycolytic System

Pasteur believed that only an organized cellular structure was capable of fermentation. Büchner demonstrated that fermentation was possible after breaking yeast cells and thus opened the fruitful era of enzymology. Broken-cell preparations, however, ferment less actively than living cells, so that cellular organization may still be needed for normal activity of enzymes. All the glycolytic enzymes of yeast and, as far as is known, of other fungi, are found in the supernatant. However, it is not known whether they are as freely soluble in the cytoplasmic liquid phase, or whether they are organized on, or inside, particular structures. Such a structure may persist in acetone extracted dried yeast, which was capable of fermenting added glucose faster than soluble enzyme preparations, and did not depend upon added phosphate (Rothstein *et al.*, 1959).

Other biosynthetic functions are probably organized in particles. Lipid synthesis was shown to occur in microsome-like particles in yeast (Klein 1960) although, after gentle breaking of cells, particles sedimenting at lower centrifugal forces seem to possess this capacity (Corwin *et al.*, 1957). The latter finding agrees with the observation of Steiner and Heinemann (1954), and Tarwidowa (1938), that mitochondria (Nadi-positive granules) accumulate fat in various fungi.

Fungal cells seem to be devoid of Golgi apparatus and lysosomes, both of which are involved in the accumulation and excretion of proteins or enzymes. The search for them should be made in fungi accumulating proteinaceous reserves or actively excreting enzymes.

3. Cell Membrane

The cell surface exerts an important function in a free-living cell, which is in direct contact with the environment and dependent on it for all its food. The cell wall has mainly a protective function, whereas the function of the cytoplasmic membranes is to regulate intake and release of substances. Since both structures are contiguous, it is often difficult to distinguish between them and much work on cell permeability does not do so (see Chapter 1 Danielli, 1958).

A distinct cytoplasmic membrane is present in fungi. No special studies of its structure have been made, but it can be assumed that it is similar to membranes of other cells, or to the so-called "unit" membrane. Isolated membranes of yeast contain 40% lipid, 40% protein, 5% RNA, 0.8

DNA, and 4% hexose (Bolton and Eddy, 1962). The apparent function of lipids is to provide a structural basis for a membrane by constituting a double layer, with the hydrophobic parts end to end and the hydrophilic ones associated with a layer of protein on both sides of the membrane (Robertson, 1959).

The protein molecules may have more than structural function. By different methods, the presence of several enzymes has been ascertained for the membrane (or cell surface). These include phosphatases, involved in sugar uptake in yeast, as found by inactivation with uranyl ions (see Chapter 15), and acid phosphatase, as detected by Gomori's reaction. Phosphorylating enzymes may be present there as a part of the mechanism of active uptake of nucleosides (Zalokar, 1962). An inducible galactoside permease has been postulated in bacteria as an enzyme needed for the uptake of galactose, and similar situations exist in *Neurospora*. It is probable that such permeases, whether consitutive or inducible, are associated with cell membranes and function in the transport of different oligosaccharides.

Some recent work on other organisms points to the possibility that an enzyme system is responsible for transport of inorganic ions. In red blood cells (Post and Albright, 1961), and in nerve cells (Skou, 1961), a phosphatase was found to be activated by potassium and sodium ions which competed with each other in such a way as to indicate a possible role for the enzyme in establishing the K-Na balance inside those cells. In a slightly different way, a phospholipid phosphorylase may be involved in sodium excretion in salt glands of birds (Hokin and Hokin, 1960).

4. Surface and Extracellular Enzymes

Fungal cell surfaces contain another set of enzymes which may be involved in permeability but, as it appears now, are active mainly in degrading extracellular nutrients to make them available for absorption. The main characteristic of those enzymes is that they are present at the cell surface and can be partially washed off in water or other materials without damaging the cell.

Different carbohydrases, which are active in breaking down polysaccharides of the nutrient environment of the fungus, are associated with the cell surface. It appears that these enzymes are excreted through the spaces of the cell wall (Burger *et al.*, 1961; Friis and Ottolenghi, 1959a,b), from which they can be liberated by the action of snail gastric juice. Some of the enzymes are only loosely bound and can be obtained by simple washing of cells, e.g., *Neurospora* α-glucosidase (Eberhart, 1961). Conidiospores of *Neurospora* contain large quantities of diphosphopyridine nucleotidase (Zalokar and Cochrane, 1956). An eluate of conidia contains the enzyme

with a specific activity 200 times greater than that in an extract of vegetative mycelium. A possible function of this enzyme would be to inhibit fermentation which might be initiated outside the spores by other microorganisms.

Finally, fungi produce enzymes that are secreted into the environment and clearly have a function in making outside nutrients available to the cell. The discussion of these enzymes may be out of the context of cell structure, but they are important for the understanding of cell function. α-Amylase of *Aspergillus* is of prime importance economically in breaking down starch to give sugar for fermentation. Conditions of amylase production have been studied by many authors (e.g., Schröder, 1961).

Pathogenic fungi excrete pectinases and cellulases (Wood, 1960; Gascoigne and Gascoigne, 1960) which loosen cell walls of plants and facilitate penetration of the parasite into the plant. In *Fusarium,* it has been demonstrated that the amount of enzyme produced by mycelium is proportional to its infectivity (Paquin and Coulombe, 1962). In the same fungus, mutants unable to produce pectinases have been isolated and these were unable to infect their hosts. Similar enzymes excreted by soil molds and wood-destroying fungi aid in the decomposition of plant material.

It would be interesting to understand better the process of enzyme secretion by the fungal cell. According to current hypotheses, enzymes must be produced on ribosomes. What is their further fate? Do they accumulate in lysosome-like particles before liberation? If not, how are they secreted?

D. Function of Other Cell Structures

1. Cell Wall

Although the cell wall has been discussed previously (Chapter 3), we should like to consider it here as an integral part of a cell. In fungi, the wall has a double function, first, to protect and separate the cell from the environment; and second, to give a cell its shape. The protective function depends on the structural and chemical resistance of the wall. In higher plants, mechanical firmness is achieved with properly oriented microfibrils of cellulose, embedded in a pectin matrix and further reinforced by lignification (Frey-Wyssling, 1959; Setterfield and Bayley, 1961). Likewise, fungal cell walls are composed of fibrillar and amorphous material (Chapter 3).

In cases of sclerotization, a melanin-like substance is deposited in the cell walls (R. Emerson and Fox, 1940), and a polyphenol oxidase can be demonstrated in such walls (Cantino and Horenstein, 1955). As melanins are related to lignins chemically (all are derivatives of phenols or terpenes), it can be assumed that they provide a resistant binding element between microfibrils, both reinforcing the structure and increasing chemical resistance.

Many fungi produce aerial hyphae or fruiting bodies which must be protected from evaporation. Lipids make up part of the cell wall of hyphae, but only a small proportion is readily extracted with fat solvents, so that it is not probable that they make a continuous layer at the surface, but rather are bound to other constituents of the cell wall (Hurst, 1952). Many conidiospores are water repellent and must contain lipids at the surface.

On the other hand, cell walls of lichens and of fungi that absorb moisture (droplets) are freely permeable to water, which they can trap by colloidal swelling (Smith, 1962). Their cellular water does not need to be preserved since the cytoplasm is resistant to drying.

The second function of the cell wall is to give the cell form. In yeast and *Neurospora,* cell walls have been removed without injuring the cells: naked "protoplasts" rounded up into globules. Such "protoplasts" became highly sensitive to osmotic shock, indicating another function of the cell wall, that of making it possible for the cell to keep its turgor.

Recently, several interesting studies on the change of form in fungi have been made. *Candida* was found to grow, under appropriate conditions, either as a yeast or as a filamentous organism (Nickerson, 1954). In *Mucor rouxii,* which normally is filamentous, yeast-like growth was induced with CO_2 (Bartnicki-Garcia and Nickerson, 1962). *Neurospora* grows normally as a spreading mycelium, but produces a compact colony in the presence of sorbose or some other agents (de Terra and Tatum, 1961). Also, several mutants of this organism have been found which grow "colonially" on the usual growth media. These changes of form were correlated with changes in the chemical composition of the cell wall. However, we do not know yet whether this change in chemical composition really explains the phenomenon, or that it may be only another manifestation of a changing growth habit. Further discussion of this problem will be found in Chapter 26 and in Volume II.

2. Vacuole

One conspicuous aspect of plant cells including fungi, is the presence of vacuoles. In a growing mycelium, the hyphal tips are completely filled with cytoplasm and vacuoles appear only at a certain distance from the end. With aging of the hyphae, vacuoles increase in size and finally fill most of the space. The prime function of the vacuole seems to be to provide turgor which is needed for growth. Many cells need turgor to retain their shape; most aerial hyphae are able to remain rigid not because of the rigidity of their walls, but because of turgor.

Besides this osmotic function, vacuoles serve as a repository for reserve substances. The studies of Svihla *et al.* (1963) showed that purines and pyrimidines which are taken into the cell from the medium accumulate in

vacuoles. One of the two pools of amino acids, as well as nucleic acid precursors (see Sections II, B, 4 and II, D), may well be organized in the vacuole. Polyphosphates accumulate in vacuoles as well as in metachromatic granules (Wiame, 1947). Deposition is particularly active in phosphate-rich medium.

IV. CELLULAR ASPECTS OF GROWTH

A. Types of Growth

The essence of growth is replication of living material. Under favorable conditions the protoplast doubles in size such that its mass increases at a logarithmic rate. Actually, the protoplast is usually organized into units of relatively small volume—the cells—so that growth means replication of cells. In the so-called "yeast type" of growth, cells separate and become independent, but in the majority of fungi the protoplasm remains a continuum.

In yeast-type growth, the growing cytoplasm expands into a bud. When the bud reaches the size of the original cell, as a rule, it separates and reinitiates the cycle.

In plasmodial growth, the protoplast can replicate anywhere in the plasmodium, so that it can be assumed that each small portion reproduces itself. This may not be entirely true because all parts of the plasmodium are not exactly similar, e.g., peripheral hyaloplasm as compared with the internal cytoplasm, or the expanding "crawling end," which is rich in cytoplasm as compared with the "rear end," which has more inclusions and vacuoles.

Hyphae grow at their tips (apical growth) into which the cytoplasm streams. Reinhardt (1892) has likened it to a plasmodium building a cell wall along its path. This type of growth permits hyphae to penetrate continuously into new territory. The growing tips represent only the points of extension of the cell wall. New protoplasts continuously stream into this area, as had been noticed by earliest workers on fungi (Reinhardt, 1892). In *Neurospora,* a fast-growing hypha advances about 5 mm an hour so that a section of this length should have reproduced itself during this time. Since many branches are formed in this region, the rate of protoplasmic growth must be phenomenal to keep up with the advancing hyphal tip. Actually, the protoplast doubles, at its fastest, every 2 hours; hence its mass must increase in old hyphae, from which the excess streams into the advancing growing region. This can be demonstrated by following the incorporation of radioactive amino acids into proteins, whose synthesis is an indication of cytoplasmic growth. Incorporation varied only slightly

along a 5-mm interval behind the hyphal tip, and it occurred in every part of living hyphae (Zalokar, 1959b).

In connection with apical growth, hyphae are differentiated along their length. The growing tip is active in new cell wall synthesis, the median portion has most other growth activities, and the posterior portion is emptied of functioning cytoplasm, which streams forward, and becomes filled with vacuoles. The role of these vacuoles in growth and streaming will be discussed in more detail in Chapter 16.

The growing tip can be used to study the process of cell wall formation. New cell wall synthesis requires nutrients and energy; on the other hand, cell functions not connected with wall formation may be deleterious. It is possible to see that hyphal tips are rich in cytoplasm and contain long filamentous mitochondria, but are devoid of nuclei. Cytochemical study shows an increase in RNA and protein concentration as compared to more distal regions and an especially notable increase in alkaline phosphatase (Fig. 2). Even though it is not known which enzymes are involved in cell wall synthesis, the presence of alkaline phosphatase suggests that active phosphorylation is undoubtedly needed in energy transfer and in the building of polysaccharide bonds. Inasmuch as the wall is made of polysaccharide, a sugar should be amply supplied to this region. In view of the fact that hyphae often are aerial, they must obtain sugar either by diffusion or by active transport from distant parts of the hyphae (see Chapter 16). Cytochemical studies show that the protoplast is loaded with glycogen, which suddenly disappears near the tip. This phenomenon indicates that sugar is transported to the tip in the form of glycogen, which is hydrolyzed at the tip and used in cell wall synthesis. In *Candida*, mitochondria may be directly involved in cell wall synthesis (Nickerson and Falcone, 1956b). They contain a disulfide reductase which breaks S—S bonds in a sulfur-containing cell wall protein. This enzyme is absent in a divisionless mutant of *Candida* that grows in filamentous form.

B. Replication of Cell Organelles

Cell organelles replicate along with replication of the protoplast. If the multiplication rate of any organelle were less than that of the cell (protoplast), then it would disappear from the cytoplasm in a few generations. On the contrary, if the organelles were to multiply faster they would crowd the cytoplasm. In the discussion of cytoplasmic inheritance (Volume II, Chapter 7), the existence of such unstable organelles or particles will be discussed in connection with their significance in differentiation and heredity. At present we shall examine the mode of reproduction of normal cell constituents.

One of the big questions that has plagued cytologists for a long time is the continuity of cell organelles. Are they produced by growth and fission of their predecessors, or are they capable of independent origin? The question has been partially answered by modern electron microscope observations and other methods, although it is far from being solved.

1. Nucleus

One of the cell organelles which certainly cannot originate independently is the cell nucleus. Details of nuclear division are discussed elsewhere (Chapter 6). The molecular phenomenon behind nuclear division is replication of DNA, which is best explained by the Watson and Crick model (see Cavalieri and Rosenberg, 1962). Much less is known about the organization of chromosomes, and therefore the molecular events in their duplication elude us completely. While the nucleus and its chromosomes are self-duplicating, this is less certain for the nucleolus and nuclear membrane. In many fungi both the nucleolus and nuclear membrane persist during nuclear division (Chapter 6), but are lost during meiosis, when "de novo" formation should occur.

Chromosomes possess one or more nucleolar organizers (McClintock, 1934), which seem to function either as direct producers of at least part of the nucleolar material, or as gatherers of material elaborated elsewhere in the nucleus. No genetic information on nucleolar organizers is available in fungi, and nucleolar function has never been studied because of the small size of this organelle.

The nuclear membrane in higher plants and animals is reconstituted from the endoplasmic reticulum with which it remains continuous.

2. Mitochondria

Fungi are one of the classical objects for the study of mitochondria and their origin (Guilliermond, 1941). Guilliermond stated that the chondriosomes are transmitted by division from cell to cell and never arise *de novo*, but Dangeard (1958) maintained that there is *de novo* origin of mitochondria from submicroscopic particles. The *de novo* formation of mitochondria was observed in yeast buds with phase contrast optics (Müller, 1956). It has also been demonstrated with the electron microscope that anaerobically grown *Torulopsis* possesses no mitochondria, but that a membrane system becomes organized, during aeration, into such organelles (Linnane *et al.*, 1962).

On the other hand, Guilliermond's views have been supported recently by a quite different method by Luck (1963). Mitochondria were labeled with radioactive choline during the logarithmic growth phase of *Neurospora*. When the mycelium continued to grow in the absence of the radio-

active precursor, all mitochondria remained labeled equally, indicating that there was no *de novo* formation from smaller particles. This contradiction in the origin of mitochondria may be only apparent. It is possible that mitochondria multiply by division during active growth, but in other cases they may develop from organelles lacking typical mitochondrial structure.

Whatever their origin, mitochondria are built by a variety of molecules which must fit into the structure at proper places. Green and collaborators (Criddle *et al.*, 1962) were able to break mitochondria into smaller constituent parts and, by combining certain of these, reconstitute some of the original function. Similarly, the succinic oxidase system could be reconstituted from its parts (King, 1963). These studies indicate that structural and functional elements of mitochondria have certain specific affinities, so that even after destruction, they combine in proper arrangement. The forces involved in ultrastructural organization are a subject for future studies, and it is to be hoped that researchers will pay more attention to the excellent mitochondrial material of fungi.

3. Endoplasmic Reticulum and Ribosomes

As in the case of other cellular structures, the study of the origin of these components was done mainly with other organisms. The endoplasmic reticulum is continuous with the nuclear membrane, from which it is derived according to Porter (1961). Ribosomal RNA originates in nuclei, as demonstrated in *Neurospora*, and there is no self-replication of ribosomes in the cytoplasm (p. 395). Nothing is known, however, of the origin of the protein moiety of ribosomes, although it is suspected that in cells of higher plants and animals complete ribosomes are elaborated in nuclei and pass into the cytoplasm. A continuity of ribosomes has been found in *Allomyces* (Turian, 1961), where they became packed in the nuclear cap during spore formation, to be released to the cytoplasm during spore germination.

Sedimentation studies show that ribosomes occur in different sizes, and Britten *et al.* (1962) showed in bacteria that particles of smaller size build up larger particles in the cytoplasm. This sequence of ribosome formation may be true for bacteria, but still has to be demonstrated for other cells, including fungi.

4. Vacuoles

This cell organelle probably can be studied better in fungi than in any other organism (see Guilliermond, 1941; Dangeard, 1956). Growing hyphae are usually free of vacuoles near the tips, and vacuoles appear larger in older parts. Hyphae also become vacuolated under certain, usu-

ally adverse, environmental conditions. Thus, the origin of vacuoles can be studied by examining young and old hyphae. Here, again, classical cytology could not provide an answer since nascent vacuoles were at the limit of visibility with optical microscopy. This led to the controversies between Giulliermond and Dangeard, the first claiming *de novo* origin, and the second the continuity of vacuoles. Observations with the electron microscope indicate that in higher plant cells vacuoles are derived from the endoplasmic reticulum (Poux, 1962).

V. CONTROL MECHANISMS IN CELLULAR PROCESSES

A. General Systems of Control

The normal functioning of a cell depends on proper adjustment of all the biochemical reactions of which the cell is capable. This necessitates a system of controls, the interplay of which is the essence of life itself. We are only beginning to understand some of these controls, and, for the present, we must study isolated cases and guess about their significance in the organism as a whole. Bacteria are the favorite subject for the study of control mechanisms, but many important observations have been made on fungi. Valuable discussions of control mechanism can be found in D. M. Bonner (1961) and in the Cold Spring Harbor Symposium for 1961.

1. Reaction Kinetics

Given an unchanging enzyme system, certain controls can be exerted on metabolism simply by the laws of mass action. A self-regulating system based entirely on chemical kinetics was postulated by Hinshelwood (1946). Such a system of controls is undoubtedly of great importance, but living cells also exert controls at levels of higher complexity.

In reversible reactions, an equilibrium is established between the concentration of reactants and that of the product, so that the reaction does not proceed further, unless the product is removed. This would lead to an establishment of a certain concentration of end products of metabolism in the cell, and the existence of metabolite pools may be due to such a simple regulatory mechanism.

Most of the substrates in a living cell are used in branched and cross-linked reaction pathways. In such reactions, enzymes often compete for a common substrate. In yeast respiration (Holzer, 1961) (Fig. 5), pyruvate can be either decarboxylated by pyruvate decarboxylase into acetaldehyde (anaerobically) or oxidized by pyruvate oxidase to acetyl coenzyme A (aerobically). The affinity of the oxidizing enzyme for pyruvate is much

higher so that, at low substrate concentrations, most of the pyruvate is converted to acetyl CoA. At higher substrate concentrations, however, pyruvate oxidase becomes saturated, and decarboxylation continues to increase, thereby increasing the proportion of fermentation to respiration.

A similar mechanism may be the reason for the "Crabtree effect" in tumor cells, in which fermentation suppresses respiration. In the more

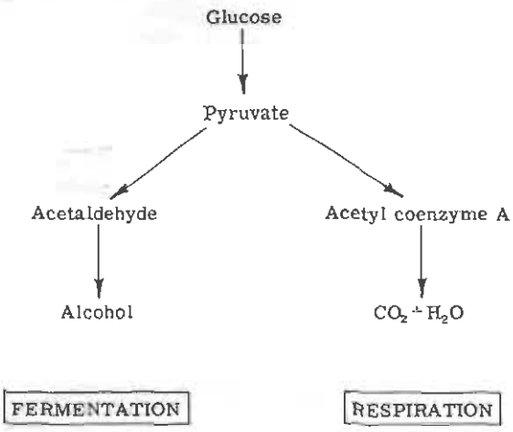


FIG. 5. Branching of glucose breakdown in yeast at the pyruvate level, from Holzer (1961).

familiar "Pasteur effect," the reverse occurs, in that respiration inhibits fermentation. The mechanism of both effects is not yet fully understood, but competition of enzymes for the same substrate must be involved (Van Eys, 1961; Lynen *et al.*, 1959) (cf. Chapter 12).

In a more complex situation, reaction chains are interconnected, so that the product of one chain is used as an intermediate in another chain or is used at the beginning of the same chain in a circular pathway. Such cyclically linked models of enzyme action constitute the basic system of controls in Hinshelwood's discussions.

Interconnected reactions can be found in yeast fermentation (Holzer, 1961) (Fig. 6). At the start of fermentation, α-glycerophosphate accumulates. Phosphoglyceraldehyde can be oxidized to α-phosphoglycerate as long as NAD is available. The NADH produced in this reaction initially is used for the reduction of dihydroxyacetone phosphate to phosphoglyceraldehyde. As the concentration of acetaldehyde builds up, NADH is oxidized in the formation of alcohol at a higher rate and the accumulation of α-glycerophosphate ceases. This case also illustrates control of metabolism by competition for cofactors. If several enzymes use the same

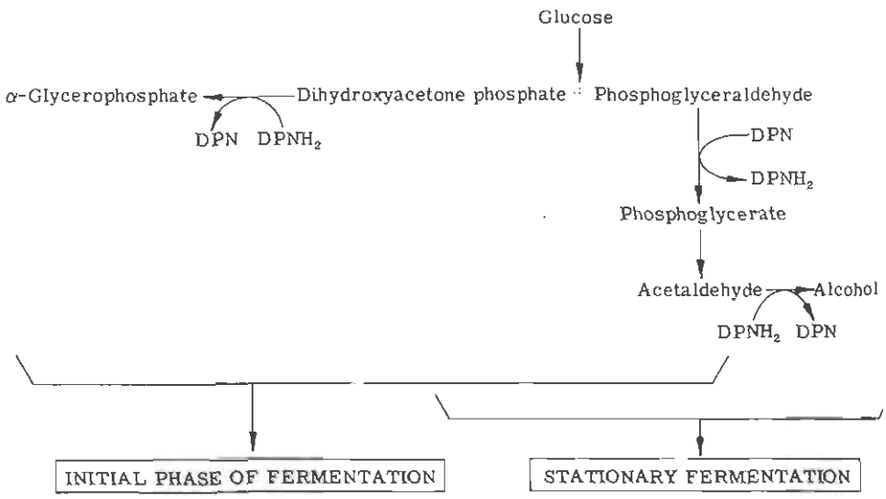


FIG. 6. Scheme of initial phase of fermentation (*Angärung*) and stationary phase of fermentation in yeast, from Holzer (1961).

cofactor, their reaction rates will depend on the availability of the cofactor in its proper form. Inasmuch as cofactors are always in limited supply, they have to be regenerated continuously so that one reaction will proceed only if another reaction runs in an opposite direction.

2. *Controls of Enzyme Function*

The controls by mass action would be ineffective if the reaction rates were not properly adjusted by enzymes. The rate of conversion of a metabolite in the reaction chain is controlled by the slowest link. Thus, it is enough to have one pace-setting enzyme at the beginning of the chain, while the other enzymes work below their full capacity. Most cellular control mechanisms involve regulation of the synthesis or activity of enzymes. In a previous chapter we discussed the control of enzymes by cellular structure. At the biochemical level, enzyme function is controlled by inhibitors or activators.

In many cases, a metabolite can compete with another for the active site of the enzyme. Thus, fructose competes with sucrose for yeast saccharase and can inhibit further hydrolysis of sucrose. Natural or artificial analogs of metabolites can compete for sites on enzymes and prevent their function. This fact has been used extensively in the search for chemicals and drugs that can regulate growth.

In feedback inhibition, the end product of a reaction chain inhibits the

functioning of an enzyme somewhere near the beginning of the chain. The net result is that, as soon as the end product begins to accumulate, its further synthesis is stopped. As it becomes depleted, synthesis begins again upon the freeing of the enzyme from inhibition. Feedback inhibition can be very specific, acting only on one enzyme in the chain and being exerted by only one metabolite. The inhibitor need not to be structurally similar to the substrate, and it appears that the inhibitor does not interfere with the active site of the enzyme. It has been suggested that the inhibitor interacts with another part of the enzyme molecule in such a way that it impairs reversibly the enzyme's function (Monod *et al.*, 1963).

Feedback inhibition was discovered by Novick and Szilard (1954), who noticed that tryptophan inhibited the synthesis of its precursor in *Escherichia coli*. In studies of *E. coli* and yeast, Roberts *et al.* (1955) observed that endogenous production of amino acids was inhibited if the amino acids were added to the medium. Later, feedback inhibition was studied in several biosynthetic chains, mainly in *E. coli* (Umbarger, 1961a.b). No such detailed analysis of feedback exists in fungi, and the best example is tryptophan synthesis in *Neurospora* (G. Lester, 1963; Matchett and DeMoss, 1963), in which the addition of tryptophan blocks the production of anthranilic acid.

3. Induction and Repression

The controls discussed until now act immediately and allow the organism to adjust rapidly to changing substrate levels. More specific, perhaps, but slightly slower in action, is a control that acts on the production of enzymes themselves. When enzyme synthesis is enhanced in the presence of a substrate (inducer), the process is called induction. In the opposite case, which is called repression, enzyme synthesis is repressed by the end product of a reaction chain, or by other specific repressors.

In fungi, various enzyme systems are inducible. In yeast, respiratory enzymes are induced by molecular oxygen (Slonimski, 1956). Fermenting yeast, when aerated, increases its content of cytochrome c oxidase 200-fold, cytochrome c peroxidase 50-fold, catalase 30-fold, and cytochrome c 30-fold. The activity of many other enzymes connected with aerobic metabolism is increased to a lesser extent. If a "petite" strain, not capable of normal respiration, is used for induction experiments, oxygen serves as a gratuitous (see below) inducer.

The inducer normally is a natural substrate for the induced enzyme. Many chemically related substances can also serve as inducers, sometimes more efficiently than the normal metabolite. Thus, methyl-β-glucoside is about forty times more potent an inducer of β-glucosidase in yeast than is cellobiose (Halvorson, 1960).

In some cases, inducers cannot serve as a substrate for enzymes at all, although they are related to natural inducers. Thus, thiogalactosides are strong inducers of β-galactosidase in bacteria, and thioglucosides of β-glucosidase in yeast, without being substrates. Such "gratuitous" inducers are very useful in the study of induction, since they are not metabolized. Conversely, some substrates are not inducers. Some substrate analogs, instead of being inducers, actually act as competitive inhibitors of induction.

Induced enzymes often are contrasted to constitutive enzymes, but the distinction is no longer very clear. Induced enzymes can be produced in negligible quantities even in the absence of induction, and in many cases, an inducer enhances the production of a constitutive enzyme, as in the β-glucosidase of yeast (MacQuillan and Halvorson, 1962a). Some authors believe that some constitutive enzymes actually may be induced by an endogenous inducer which cannot be removed, so that proof is difficult to obtain. In all cases of enzyme induction, the cell possesses the gene necessary to determine the amino acid sequence of the enzyme, and induction only activates the gene. The inducible and the constitutive β-glucosidases of yeast are identical by many enzymologic criteria, indicating that they are determined by the same structural gene.

The repressor normally is the end product of a reaction chain, suppressing the formation of most of the enzymes in the chain. Repression differs from feedback inhibition in that enzyme synthesis, not function, is inhibited. Both phenomena have the same end effect and can be distinguished only after careful study. Often, both repression and feedback mechanisms can coexist in the same enzyme system. Thus, in *Neurospora,* tryptophan acts as feedback inhibitor, but also represses the formation of tryptophan synthetase in certain auxotrophic mutants (Matchett and DeMoss, 1962).

In the special case of catabolite repression (Magasanik, 1961), a metabolite not directly connected with enzyme action can function as a repressor. This is evident in the so-called "glucose effect," where the addition of glucose represses the formation of certain enzymes. Such repression may be useful by preventing the production of metabolites through other pathways, when they can be obtained more readily by the metabolism of glucose. In yeast, glucose inhibits the formation of β-glucosidase, and also of succinic and isocitric dehydrogenases and of several other enzymes of citric acid cycle (MacQuillan and Halvorson, 1962b).

It is believed now that both induction and repression involve a similar mechanism, acting at the genetic locus which controls the formation of the RNA necessary for enzyme synthesis. The study of induction has led to the concept of an unstable "messenger" RNA, produced by genes that interact with ribosomes in enzyme synthesis. After the addition of an in-

ducer, a short lag of a few minutes occurs before enzyme synthesis starts. This is the time necessary to build new messenger RNA. Since, in the absence of inducer enzyme synthesis ceases rapidly, it is assumed that this messenger RNA is very unstable and disappears from the cytoplasm, unless more is formed continuously.

Jacob and Monod (1961), based on their studies of β-galactosidase induction in *Escherichia coli,* proposed the following model to explain both induction and repression. The cell has structural genes for β-galactosidase and β-galactoside permease, or any other enzymes belonging to the same induction sequence. These genes determine the amino acid sequence of the enzymes and are situated in adjacent cistrons. The cistrons are preceded by an "operator" gene which initiates RNA synthesis. Another gene, the "regulator," produces a substance which interacts with the "operator" and keeps it inactive. During induction, the inducer combines with this substance and derepresses the operator, thereby initiating new RNA synthesis. During repression, the substance produced by the regulator does not normally interact with the operator, but the repressor makes the interaction possible, shutting off further RNA synthesis. This rather complicated model explains most of the facts of induction and repression studied in bacteria, but still has to be proved to apply more generally.

B. Controls in Growth and Development

1. Germination

When a fungal spore falls on fertile ground, with enough moisture available, many changes occur in its cytoplasm, setting the biosynthetic machinery in motion. When the spores were formed, many processes slowed down or stopped entirely, and many intermediates of metabolism were depleted. On the other hand, spores have accumulated reserve substances in the form of glycogen, proteins, nucleic acids, and others, to allow resumption of growth without immediate need for external food. The mechanisms involved in these controls will be discussed in Volume II, Section II).

After germination, there is a lag before growth assumes its normal fast rate. During the lag period, all metabolic reactions become equilibrated through various control mechanisms. This includes restoration of the enzyme system and a build-up of the proper concentration of intermediary metabolites which is necessary for enzymatic reactions to proceed at full pace according to the laws of mass action. An example can be found in yeast fermentation, which does not proceed normally until there is a build-up of the acetaldehyde concentration. The proper level of intermediates can be established only slowly in cyclic reaction sequences such as the Krebs

cycle, which cannot get into full swing until all its intermediates reach a steady-state concentration.

2. Exponential Growth (Steady State)

After this initial lag period, in the presence of abundant food supply, growth proceeds at an exponential rate. This means that the living substance duplicates at regular intervals. The growth rate can be very fast in some fungi: *Neurospora crassa* doubles its protein content every 2 hours but some fungi, like those forming lichens, grow extremely slowly. During exponential growth, every component of the living protoplast is exactly doubled in a given time. All metabolic reactions of the cell must be so adjusted that none proceeds at a rate that would result in the exhaustion or accumulation of a metabolite. This dynamic equilibrium is called a steady state, and we can imagine how the different control mechanisms discussed above might function to maintain it.

In reality, the steady state does not exist throughout the reduplication cycle. Many processes are continuous during growth, but others proceed sporadically. In a synchronized culture of yeast and in slime molds, the synthesis of proteins and RNA increases continuously whereas the replication of DNA is completed in a fraction of the generation time. New cell walls are formed only at certain stages in the course of cell division. Exponential growth, therefore, comprises a series of rhythmical events giving the appearance of a steady state.

The rate of growth during the exponential phase is dependent on the source of food. It is easy to see how the deficiency of a metabolite can affect growth, but the fact that all processes are slowed down proportionally needs explanation. Is the reduction of growth due to a general overall slowing of synthetic processes, or do only a few biosynthetic enzymes continue to work at full capacity? When bacteria (*Aerobacter*) are grown on peptone medium, growth is about ten times faster than on glutamic acid (Neidhardt and Fraenkel, 1961; Kjelgaard and Kurland, 1963). In cells grown at a slow rate the ratio of DNA to protein, and DNA to transfer RNA remained the same, whereas the ratio of ribosomes to DNA decreased. This suggests that the slowing of protein formation is due to a reduction in the amount of ribosomes which continue to produce proteins at the same rate as in fast growing cells. Ribosome synthesis must somehow be connected with the level of a nutrient (or its metabolic product). It has been shown that the presence of amino acids is necessary for the synthesis of RNA (Gros and Gros, 1958; Kurland and Maaløe, 1962), and this may be part of the mechanism involved.

The steady state is altered as the growth medium becomes modified by the activities of the growing organism. It is only through the "chemostat"

and unicellular organisms (Novick and Szilard, 1954) that unchanging growth conditions can be maintained. Most fungi, however, produce a mycelium in which the older parts remain in a medium modified or exhausted by growth. Only the peripheral hyphae continuously invade fresh medium and remain in a steady state.

Theoretically, indefinite uniform growth of mycelia can be obtained in growth tubes (Ryan *et al.*, 1943) on solid medium. In liquid medium, the exponential growth phase lasts only as long as hyphae are freely suspended in the medium and not overcrowded (Zalokar, 1959a). Later, hyphae branch in all directions to produce a mycelium growing as a sphere, which increases its radius at a constant rate, so that the cube root of the dry weight increases linearly (S. Emerson, 1950).

3. Aging and Differentiation

Unless hyphae can creep continuously into a fresh medium, their environment changes as a result of their own growth and growth slows down in an aging colony. At this time, many controls cannot cope fully with the changing conditions and profound changes occur in the cells. One visible effect of aging in hyphae is the increase in the number and size of vacuoles and the accumulation of different products of metabolism, such as fat droplets or pigments. In many molds, like *Penicillium,* pigments accumulate whenever growth slows down, and this may lead to concentric pigmented rings in a colony in which growth is periodic owing to fluctuations of light or temperature (cf. Chapter 27). In *Neurospora,* the slowing or stopping of growth induces the formation of tyrosinase (Horowitz *et al.,* 1960) which leads to the accumulation of melanin. It is difficult to decide whether such phenomena are merely side effects of metabolism or whether they are useful to the aging organism.

Reproductive organs often are induced by the cessation of growth. The colored concentric rings of mold colonies are the sites where conidiophores are formed. An increase in the partial pressure of carbon dioxide in the medium induces the formation of sporangia in *Blastocladiella* (Cantino and Horenstein, 1955) (see Volume II, Chapter 3c). A similar effect of this gas has been observed in the animal kingdom, where it induces sexual stages in *Hydra* (Loomis, 1961). In all the cases where an apparently simple external stimulus is associated with drastic changes in the organism, we are tempted to consider it to be the primary cause of the changes. Undoubtedly the stimulus often serves as a trigger, but the response is an inherent quality of the cell. The chain of events leading from the triggering effect to various responses may be long and complicated and difficult to study. The models used to explain induction and repression of enzymes are not sufficient and do not apply in all these cases. Differentiation comprises a complex rear-

rangement of controls, leading to new metabolic patterns, which often result in changed morphology. The intrinsic qualities of the organism, as coded in its genetic system, will determine which condition will initiate such rearrangements and how they will proceed.

C. Malfunctions of Control Mechanisms

The breakdown of control systems during aging is a natural consequence of the life cycle. Often, however, these systems can be thrown out of control by external or internal causes. As a result, certain metabolites will be used at an excessive rate, or metabolic products will accumulate. The organism can readjust to some of these interventions, others may be lethal. An interesting case of natural death in *Neurospora* has been described by Sheng (1951) in which a gene mutation caused colonies to die after a defined period of growth. This effect was ascribed to the accumulation of toxic products of metabolism in the cytoplasm.

One of the early methods in the study of yeast fermentation was to poison certain steps and find which intermediates accumulated as a result. Sodium fluoride inhibits enolase and induces the accumulation of phosphoglyceric acid and glycerophosphate. In the system discussed earlier in this chapter (Fig. 5) acetaldehyde can be trapped with sodium sulfite, thereby preventing the reoxidation of NADH. As a result, the reaction is switched to the accumulation of glycerophosphate, which is hydrolyzed by phosphatases to glycerol. This process has been adapted for the industrial production of glycerol.

Tampering with feedback controls can lead to accumulation of an end product or to other derangements of metabolism. If the feedback mechanism acts upon an enzyme preceding a branch of a biosynthetic chain, both products will be affected. Thus in strain K12 of *Escherichia coli,* the addition of excessive amounts of valine to the medium will inhibit not only valine synthesis, but also isoleucine synthesis, both being dependent on the production of the same intermediate (Umbarger, 1961a). Therefore, the organism will develop a requirement for the other product of the branched pathway if one of them is added.

As for repression, enzyme production is inhibited when the concentration of the end product increases. If this increase is prevented, repression does not occur and excessive enzyme synthesis results. Derepression can be caused by genetically blocking the production of the end product, and adding it in reduced quantities to the medium. In *Neurospora crassa,* tryptophan synthetase production was increased severalfold in a tryptophan-requiring mutant, when the internal concentration of tryptophan fell below 1 μ mole per gram dry weight of mycelium (Matchett and DeMoss, 1962).

In some bacteria, it has been possible to increase enormously the production of certain enzymes by derepression (Vogel, 1961).

One of the very effective ways of inducing accumulation of substances in microorganisms is to block intermediate metabolism genetically. As a result of a mutation, a gene may no longer produce the enzyme necessary for a particular step in a reaction chain, and the substance formed in the preceding step accumulates. Since such interruption of metabolism often deprives organisms of one of their essential metabolites, the mutation is usually lethal. But the lethality may be remedied by adding to the medium the missing end product of the defective chain. This is the basis of the very fruitful analysis of biochemical genetics in *Neurospora* and will be discussed further in other chapters. A comprehensive review of interactions between genes, mutation, and metabolism has been written by Strauss (1955). Many interesting intermediate products can be obtained by this genetic method, some of potential practical value. In *Neurospora,* cystathionine accumulated in a mutant blocked between cystathionine and homocysteine and could be isolated by a simple process (Horowitz, 1947). A practical way to obtain orotic acid could be developed with some *Neurospora* mutants which were blocked in pyrimidine synthesis and accumulated the acid (Mitchell *et al.*, 1948). It is significant that such accumulation of intermediates would not be possible if they served as repressors of prior steps in their biosynthesis. By judicious manipulation of mutants and metabolic controls, it should be possible to direct the production of many interesting compounds by fungi.

REFERENCES

Allfrey, V. (1959). The isolation of subcellular components. *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 1, pp. 193–290. Academic Press, New York.

Anderson, N., and B. M. Pollock. (1952). A comparison between the cytoplasmic components in the myxomycete, *Physarum polycephalum* and in the amoeba, *Chaos chaos. Compt. Rend. Trav. Lab. Carlsberg* **28**:247–264.

Aronson, J. M., and L. Machlis. (1959). The chemical composition of the hyphal walls of the fungus *Allomyces. Am. J. Botany* **46**:292–300.

Bachman, B. J., and D. M. Bonner. (1959). Protoplasts from *Neurospora crassa. J. Bacteriol.* **78**:550–556.

Bartnicki-Garcia, S., and W. J. Nickerson. (1962). Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii. Biochim. Biophys. Acta* **58**:102–119.

Bautz, E. (1955). Mitochondrienfärbung mit Janusgrün bei Hefen. *Naturwissenschaften* **42**:49–50.

Bautz, E. (1956). Zytologische Untersuchungen an "Mitochondrienfraktionen" von Hefen. *Z. Naturforsch.* **11b**:25–31.

Bautz, E., and H. Marquardt. (1953). Das Verhalten oxydierender Fermente in den

grana mit Mitochondrienfunktion der Hefezellen. *Naturwissenschaften* **40**:531–532.

Blondel, B., and G. Turian. (1960). Relation between basophilia and fine structure of cytoplasm in the fungus *Allomyces macrogynus* Em. *J. Biophys. Biochem. Cytol.* **7**:127.

Bolton, A. A., and A. A. Eddy. (1962). The properties of certain particles isolated from yeast protoplasts disrupted by osmotic shock. *Biochem. J.* **82**:16P–17P.

Bonner, B. A., and L. Machlis. (1957). Respiration of the mycelia and mitochondria of the filamentous watermold, *Allomyces macrogynus*. *Plant Physiol.* **32**:291–301.

Bonner, D. M., ed. (1961). "Control Mechanisms in Cellular Processes," p. 248. Ronald Press, New York.

Bonner, J. (1959). Protein synthesis and the control of plant processes. *Am. J. Botany* **46**:58–62.

Brachet, J. (1961). Nucleocytoplasmic interactions in unicellular organisms. *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 771–842. Academic Press, New York.

Britten, R. J., B. J. McCarthy, and R. B. Roberts. (1962). The synthesis of ribosomes in *E. coli*. IV. The synthesis of ribosomal protein and the assembly of ribosomes. *Biophys. J.* **2**:83–93.

Burger, M., E. E. Bacon, and J. S. D. Bacon. (1961). Some observations on the form and location of invertase in the yeast cell. *Biochem. J.* **78**:504–511.

Cantino, E. C., and E. A. Horenstein. (1955). The role of ketoglutarate and polyphenol oxidase in the synthesis of melanin during morphogenesis in *Blastocladiella emersonii*. *Physiol. Plantarum* **8**:189–221.

Cantino, E. C., and E. A. Horenstein. (1956). Gamma and the cytoplasmic control of differentiation in *Blastocladiella*. *Mycologia* **48**:443–446.

Caspersson, T. O. (1950). "Cell Growth and Cell Function," 185 pp. Norton, New York.

Cavalieri, L. F., and B. H. Rosenberg. (1962). Nucleic acids: Molecular biology of DNA, *Ann. Rev. Biochem.* **31**:247–270.

Chantrenne, H. (1961). "The Biosynthesis of Proteins," 220 pp. Macmillan (Pergamon), New York.

Chao, F. C. (1957). Dissociation of macromolecular ribonucleoprotein of yeast. *Arch. Biochem. Biophys.* **70**:426–431.

Chao, F. C., and H. K. Schachman. (1956). The isolation and characterization of macromolecular ribonucleoprotein from yeast. *Arch. Biochem. Biophys.* **61**:220–230.

Corwin, L. M., L. J. Schroeder, and W. G. McCullough. (1957). Studies of lipide synthesis in cell-free yeast extracts. *Arch. Biochem. Biophys.* **72**:112–118.

Cowie, D. B., and E. T. Bolton. (1957). The use of metabolic pools of purine compounds for nucleic acid syntnesis in yeast. *Biochim. Biophys. Acta* **25**:292–298.

Cowie, D. B., and F. T. McClure. (1959). Metabolic pools and the synthesis of macromolecules. *Biochim. Biophys. Acta* **31**:236–245.

Criddle, R. S., R. M. Bock, D. E. Green, and H. Tisdale. (1962). Physical characteristics of proteins of the electron transfer system and interpretation of the structure of the mitochondrion. *Biochemistry* **1**:827–842.

Dangeard, P. (1956). Le vacuome de la cellule végétale. Morphologie. *Protoplasmologia* **III/D**:1–41.

Dangeard, P. (1958). Le chondriome de la cellule végétale. Morphologie du chondriome. *Protoplasmologia* **III/A**:1–35.

Danielli, J. F., ed. (1958). "General Cytochemical Methods," Vol. 1, 471 pp.; (1961) Vol. 2, 297 pp. Academic Press, New York.

de Duve, C., R. Wattiaux, and P. Baudhuin. (1962). Distribution of enzymes between subcellular fractions in animal tissues. *Advan. Enzymol.* **24**:291–358.

DeLamater, E. D. (1950). The nuclear cytology of the vegetative diplophase of *Saccharomyces cerevisiae*. *J. Bacteriol.* **60**:321–332.

de Terra, N., and E. L. Tatum. (1961). Colonial growth of *Neurospora*. *Science* **134**:1066–1068.

Ebel, J. P., J. Colas, and S. Muller. (1958). Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants. *Exptl. Cell Res.* **15**:21–42.

Eberhart, B. M. (1961). Exogenous enzymes of *Neurospora crassa* conidia and mycelia. *J. Cellular Comp. Physiol.* **58**:11–16.

Eddy, A. A. (1958). Presumed nuclear bodies isolated from yeast protoplasts using ultrasonics. *Biochem. J.* **69**:47P–48P.

Eddy, A. A., and D. H. Williamson. (1957). A method of isolating protoplasts from yeast. *Nature* **179**:1252–1253.

Emerson, R., and D. L. Fox. (1940). α-Carotene in the sexual phase of the aquatic fungus *Allomyces*. *Proc. Roy. Soc.* **B128**:275–293.

Emerson, S. (1950). The growth phase in *Neurospora* corresponding to the logarithmic phase in unicellular organisms. *J. Bacteriol.* **60**:221–223.

Emerson, S., and M. R. Emerson. (1958). Production, reproduction and reversion of protoplast-like structures in the osmotic strain of *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **44**:668–671.

Fink, R. M., and K. Fink. (1962). Relative retention of H^3 and C^{14} labels of nucleosides incorporated into nucleic acids of *Neurospora*. *J. Biol. Chem.* **237**:2889–2891.

Freese-Bautz, E. (1957). Mitochondria in living cells of respiratory mutants of *Neurospora*. *J. Cellular Comp. Physiol.* **50**:203–217.

Frey-Wyssling, A. (1959). "Die pflanzliche Zellwand," 367 pp. Springer, Berlin.

Friis, J., and P. Ottolenghi. (1959a). Localization of invertase in a strain of yeast. *Compt. Rend. Trav. Lab. Carlsberg.* **31**:259–271.

Friis, J., and P. Ottolenghi. (1959b). Localization of melibiase in a strain of yeast. *Compt. Rend. Trav. Lab. Carlsberg.* **31**:272–281.

Ganesan, A. T. (1959). The cytology of *Saccharomyces*. *Compt. Rend. Trav. Lab. Carlsberg.* **31**:149–174.

Ganesan, A. T., and C. Roberts. (1959). Observations on the nuclear cytology of *Lipomyces lipofer*. *Compt. Rend. Trav. Lab. Carlsberg.* **31**:175–180.

Gascoigne, J. A., and M. M. Gascoigne. (1960). "Biological Degradation of Cellulose," 264 pp. Butterworths, London.

Glaser, L., and D. H. Brown. (1957). The synthesis of chitin in cell-free extracts of *Neurospora crassa*. *J. Biol. Chem.* **228**:728.

Glick, D. (1961). "Quantitative Chemical Techniques of Histo- and Cyto-Chemistry," Vol. I, 470 pp. Wiley (Interscience), New York.

Green, D. E., and S. Fleischer. (1962). On the molecular organization of biological transducing systems. *In* "Horizons in Biochemistry" (M. Kasha and B. Pullman, eds.), pp. 381–420. Academic Press, New York.

Gros, F. and F. Gros. (1958). Rôle des acides aminés dans la synthèse des acides nucléiques chez *Escherichia coli*. *Exptl. Cell Res.* **14**:104–131.

Gross, S. R. (1962). On the mechanism of complementation at the leu-2 locus of *Neurospora*. *Proc. Natl. Acad. Sci. U.S.* **48**:922–930.

Grunberg-Manago, M. (1962). Enzymatic synthesis of nucleic acids. *Ann. Rev. Biochem.* **31**:301-332.

Grunberg-Manago, M., and J. Wisniewski. (1957). Synthèses de polynucléotides par un système enzymatique isolé à partir de levure. *Compt. Rend.* **245**:750–753.

Guilliermond, A. (1941). "The Cytoplasm of the Plant Cell," 247 pp. Chronica Botanica, Waltham, Massachusetts.

Guilliermond, A., M. Fontaine, and A. Raffy. (1935). Sur l'existence dans *Eremothecium ashbyii* d'un pigment jaune se rapportant au groupe des flavines. *Compt. Rend.* **201**:1077–1080.

Halvorson, H. O. (1960). The induced synthesis of proteins. *Advan. Enzymol.* **22**:99–156.

Hanzon, V., L. H. Hermodsson, and G. Toschi. (1959). Ultrastructural organization of cytoplasmic nucleoproteins in exocrine pancreas cells. *J. Ultrastruct. Res.* **3**:216.

Harold, F. M., and A. Miller. (1961). Intracellular localization of inorganic polyphosphate in *Neurospora crassa*. *Biochim. Biophys. Acta* **50**:261–270.

Hatch, W. R. (1935). Gametogenesis in *Allomyces arbuscula*. *Ann. Botany* (*London*) **49**:623–649.

Hauge, J. G., and H. O. Halvorson. (1962). The role of a large particle fraction from yeast in protein synthesis. *Biochim. Biophys. Acta* **61**:101–107.

Heim, P. (1946a). Sur la localisation des pigments carotinides chez les *Phalloidées*. *Compt. Rend.* **222**:1354–1355.

Heim, P. (1946b). Sur les pigments carotinides des Champignons. *Compt. Rend.* **223**:1170–1172.

Hinshelwood, C. N. (1946). "The Chemical Kinetics of the Bacterial Cell," 284 pp. Oxford Univ. Press (Clarendon), London and New York.

Hogeboom, G. H., and W. C. Schneider. (1952). Cytochemical studies. IV. Physical state of certain respiratory enzymes of mitochondria. *J. Biol. Chem.* **194**:513–519.

Hokin, L. E., and M. R. Hokin. (1960). Studies on the carrier function of phosphatidic acid in sodium transport. *J. Gen. Physiol.* **44**:61–85.

Holzer, H. (1961). Regulation of carbohydrate metabolism by enzyme competition. *Cold Spring Harbor Symp. Quant. Biol.* **26**:227–288.

Holzer, H., and H. W. Goedde. (1957). Zwei Wege von Pyruvat zu Acetyl-Coenzym A. in Hefe. *Biochem. Z.* **329**:175–191.

Horowitz, N. H. (1947). Methionine synthesis in *Neurospora*. The isolation of cystathionine. *J. Biol. Chem.* **171**:255–264.

Horowitz, N. H., M. Fling, H. L. MacLeod, and N. Sueka. (1960). Genetic determination and enzymatic induction of tyrosinase in *Neurospora*. *J. Mol. Biol.* **2**:96–104.

Huebschman, C. (1952). A method of varying the average number of nuclei in the conidia of *Neurospora crassa*. *Mycologia* **44**:599–604.

Hurst, H. M. (1952). An electron diffraction study of the structure and orientation of the lipids in yeast and bacterial cell walls. *J. Exptl. Biol.* **29**:30–53.

Iwasa, K., F. Imamoto, and K. Okunuki. (1959). Adenosine triphosphatase of the intracellular particles of *Aspergillus oryzae*. *J. Biochem.* **46**:113–120.

Jacob, F., and J. Monod. (1961). On the regulation of gene activity. *Cold Spring Harbor Symp. Quant. Biol.* **26**:193–212.

Jensen, W. A. (1962). "Botanical Histochemistry: Principles and Practice," 408 pp. Freeman, San Francisco, California.

Jones, R. C. (1947). The Feulgen reaction as a cytological technique with *Allomyces arbuscula*. *Mycologia* **39**:109–112.

Jordanov, J., I. Grigorov, and A. Bojadžieva-Michajlova. (1962). Über die Zytochemie der Polyphosphate bei *Aspergillus oryzae*. *Acta Histochem.* **13**:165–178.

Kikuchi, G., and E. S. G. Barron. (1959). Electron transport system in *Fusarium lini*. *Arch. Biochem. Biophys.* **84**:96–105.

King, T. E. (1963). Is the reconstitution of respiratory succinate oxidase really a process of reactivation? *Nature* **198**:366–368.

Kitazume, Y., M. Yčas, and W. S. Vincent. (1962). Metabolic properties of a ribonucleic acid fraction in yeast. *Proc. Natl. Acad. Sci. U.S.* **48**:265–282.

Kjelgaard, H. O., and C. G. Kurland. (1963). The distribution of soluble and ribosomal RNA as a function of growth rate. *J. Mol. Biol.* **6**:341–348.

Klein, H. P. (1960). Yeast particle fraction involved in lipid synthesis. *J. Bacteriol.* **80**:665–672.

Kornberg, A. (1960). Biologic synthesis of deoxyribo-nucleic acid. *Science* **131**:1503–1508.

Kurland, C. G., and O. Maaløe. (1962). Regulation of ribosomal and transfer RNA synthesis. *J. Mol. Biol.* **4**:193–210.

Küster, E. (1956). "Die Pflanzenzelle." 986 pp. Fischer, Jena.

Lampen, J. O., P. M. Arnow, Z. Borowska, and A. I. Laskin. (1962). Location and role of sterol and nystatin binding sites. *J. Bacteriol.* **84**:1152–1160.

Landman, O. E., and D. M. Bonner. (1952). *Neurospora lactase*. I. Properties of lactase preparations from a lactose utilizing and lactose non-utilizing strain. *Arch. Biochem. Biophys.* **41**:253–265.

Lederberg, S., and J. M. Mitchison. (1962). Interaction of the ribosomes of *Schizosaccharomyces pombae* and *Escherichia coli*. *Biochim. Biophys. Acta* **55**:104–109.

Lehninger, A. L., and C. L. Wadkins. (1962). Oxidative phosphorylation. *Ann. Rev. Biochem.* **31**:47–78.

Lester, G. (1963). Regulation of early reactions in the biosynthesis of tryptophan in *Neurospora crassa*. *J. Bacteriol.* **85**:468–475.

Lester, G., and O. Hechter. (1959). The relationship of sodium, potassium, and deoxycorticosterone in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **45**:1792–1801.

Lester, R. L., and Y. Hatefi. (1958). Studies on the mechanism of oxidative phosphorylation. IV. Pyridine nucleotide binding and its relation to activity in heart mitochondria. *Biochim. Biophys. Acta* **29**:103–112.

Lindegren, C. C., and M. M. Rafalko. (1950). The structure of the nucleus of *Saccharomyces bayanus*. *Exptl. Cell Res.* **1**:169–187.

Linnane, A. W., and J. L. Still. (1955). The isolation of respiring mitochondria from baker's yeast. *Arch. Biochem. Biophys.* **59**:383–392.

Linnane, A. W., E. Vitols, and P. G. Nowland. (1962). Studies on the origin of yeast mitochondria. *J. Cell. Biol.* **13**:345–350.

Lison, L. (1960). "Histochimie et cytochimie animales, principes et méthodes." 842 pp. Gauthier-Villars, Paris.

Loomis, W. F. (1961). Feedback factors affecting sexual differentiation in *Hydra littoralis*. *In* "The Biology of Hydra" (H. M. Lenhoff and W. F. Loomis, eds.). Univ. of Miami Press, Coral Gables, Florida.

Luck, D. J. L. (1963). Genesis of mitochondria in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **49**:233–239.

Lynen, F. G., G. Hartmann, K. F. Netter, and A. Schnegraf. (1959). Phosphate turnover and Pasteur effect. *Ciba Found. Symp., Regulation Cell Metab., 1959* pp. 256–273. Little, Brown, Boston, Massachusetts.

McClintock, B. (1934). The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z. Zellforsch. Mikroskop. Anat.* **21**:294–328.

McLaren, A. D., and K. L. Babcock. (1959). Some characteristics of enzyme reactions at surfaces. *In* "Subcellular Particles" (T. Hayashi, ed.), pp. 23–36. Ronald Press, New York.

MacQuillan, A. M., and H. O. Halvorson. (1962a). Metabolic control of β-glucosidase synthesis in yeast. *J. Bacteriol.* **84**:23–30.

MacQuillan, A. M., and H. O. Halvorson. (1962b). Physiological changes occuring in yeast undergoing glucose repression. *J. Bacteriol.* **84**:31–36.

Maeda, A. (1960). Preparation and some properties of ribonucleic acid of yeast 80s particles. *J. Biochem.* **48**:363–374.

Magasanik, B. (1961). Catabolite repression. *Cold Spring Harbor Symp. Quant. Biol.* **26**:249–256.

Mandels, G. R. (1953). Localization of carbohydrases at the surface of fungus spores by acid treatment. *Exptl. Cell Res.* **5**:48–55.

Marquardt, H., and E. Bautz. (1954). Die Wirkung einiger Atmungsgifte auf das Verhalten von Hefe-Mitochondrien gegenuber der NADI-Reaktion. *Naturwissenschaften* **41**:361–362.

Maruyama, Y., and M. Alexander. (1962). Localization of enzymes in the mycelium and microconidia of *Fusarium oxysporum*. *J. Bacteriol.* **84**:307–312.

Matchett, W. H., and J. A. DeMoss. (1962). Factors affecting increased production of tryptophan synthetase by a td mutant of *Neurospora crassa*. *J. Bacteriol.* **83**:1294–1300.

Matchett, W. H., & J. A. DeMoss. (1963). Direct evidence for a tryptophan-anthranilic acid cycle in *Neurospora*. *Biochim. Biophys. Acta* **71**:632–642.

Mirsky, A. E., and S. Osawa. (1961). The interphase nucleus. *In* "The Cell" (J. Brachet and A. E. Mirsky, eds), Vol. 2, pp. 677–763. Academic Press, New York.

Mitchell, H. K., M. B. Houlahan, and J. F. Nye. (1948). The accumulation of orotic acid by a pyrimidine-less mutant of *Neurospora*. *J. Biol. Chem.* **172**:525–529.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exptl. Cell Res.* **13**:244–262.

Mitchison, J. M., and P. M. B. Walker. (1959). RNA synthesis during the cell life cycle of a fission yeast *Schizosaccharomyces pombe*. *Exptl. Cell Res.* **16**:49–58.

Monod, J., J.-P. Changeux, and F. Jacob. (1963). Allosteric proteins and cellular control systems. *J. Mol. Biol.* **6**:306–329.

Mudd, S., A. F. Brodie, L. C. Winterscheid, P. E. Hartman, E. M. Benter, and R. A. McLean. (1951). Further evidence of the existence of mitochondria in bacteria. *J. Bacteriol.* **62**:729–739.

Müller, R. (1956). De-novo Entstehung der Hefegranula ("Mitochondrien"). *Naturwissenschaften* **43**:86–87.

Mundkur, B. (1960). Electron microscopical studies of frozen-dried yeast. I. Localization of polysaccharides. *Exptl. Cell Res.* **20**:28–42.

Nečas, O. (1956). Regeneration of yeast cells from naked protoplasts. *Nature* **177**:898–899.

Nečas, O. (1958). Action of ribonuclease on yeast protoplasts. *Expl. Cell Res.* **14**:216–219.

Neidhardt, F. C., and D. G. Fraenkel. (1961). Metabolic regulation of RNA synthesis in bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **26**:63–74.

Nickerson, W. J. (1954). An enzymatic locus participating in cellular division of a yeast. *J. Gen. Physiol.* **37**:483–494.

Nickerson, W. J., and G. Falcone. (1956a). Enzymatic reduction of disulphide bonds in cell wall protein of baker's yeast. *Science* **124**:318–319.

Nickerson, W. J., and G. Falcone. (1956b). Identification of protein disulfide reductase as a cellular division enzyme in yeasts. *Science* **124**:722–723.

Nossal, P. M. (1954). Distribution of enzymes in cell-free yeast extracts. *Biochem. J.* **57**:62–69.

Novelli, G. D. (1960). Protein synthesis in microorganisms. *Ann. Rev. Microbiol.* **14**:65.

Novick, A., and Szilard, L. (1954). Experiments with the chemostat on the rates of amino acid synthesis in bacteria. *In* "Dynamics of Growth Processes" (E. J. Boell, ed.), p. 21. Princeton Univ. Press, Princeton, New Jersey.

Novikoff, A. B. (1961). Mitochondria (Chondriosomes). *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 299–404. Academic Press, New York.

Nygaard, O. F., S. Güttes, and H. P. Rusch. (1960). Nucleic acid metabolism in a slime mold with synchronous mitosis. *Biochim. Biophys. Acta* **38**:298–306.

Nyman, M. A., and E. Chargaff. (1948). On the lipoprotein particles of yeast cells. *J. Biol. Chem.* **180**:741–746.

Oparin, A. I., T. N. Evreinova, T. I. Larionova, and I. M. Davydova. (1962). Synthesis and degradation of starch in coacervate droplets. *Dokl. Akad. Nauk SSSR* **143**:980–983.

Palade, G. E. (1956). The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* **2**: suppl., 85–98.

Paquin, R., and L. J. Coulombe. (1962). Pectic enzyme synthesis in relation to virulence in *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder and Hansen. *Can. J. Botany* **40**:533–541.

Pearse, A. G. E. (1960). "Histochemistry," 998 pp. Little, Brown, Boston, Massachusetts.

Porter, K. R. (1961). The ground substance, observation from electron microscopy. *In* "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. 2, pp. 621–627. Academic Press, New York.

Post, R. L., and C. D. Albright. (1961). Membrane adenosine triphosphatase system as a part of a system for active sodium and potassium transport. *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 219–227. Academic Press, New York.

Poux, N. (1962). Nouvelles observations sur la nature et l'origine de la membrane vacuolaire des cellules végétales. *J. Microscopie* **1**:55–66.

Reich, E., and S. Tsuda. (1961). Isolation of nuclei of *Neurospora crassa*. *Biochim. Biophys. Acta* **53**:574–575.

Reinhardt, M. O. (1892). Das Wachstum der Pilzhyphen. *Jahrb. Wiss. Botan.* **23**: 479–566.

Ritchie, D. (1947). The formation and structure of the zoospores in *Allomyces*. *J. Elisha Mitchell Sci. Soc.* **63**:168–205.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. (1955). Studies of biosynthesis in *Escherichia coli*. *Carnegie Inst. Wash. Publ.* **607**, 535 pp.

Robertson, J. D. (1959). The ultrastructure of cell membranes and their derivatives. *Biochem. Soc. Symp. (Cambridge, Engl.)* **16**:3–43.

Roren, E. S., and L. Machlis. (1957). The ribonucleoprotein nature of large particles in the meiosporangia of *Allomyces*. *J. Biophys. Biochem. Cytol.* **3**:879–888.

Rothstein, A., D. H. Jennings, C. Demis, and M. Bruce. (1959). The relationship of fermentation to cell structure in yeast. *Biochem. J.* **71**:99–106.

Royan, S., and M. K. Subramaniam. (1960). Differential fluorescence of the chromocenters and nucleolar equivalents of the yeast nucleus in acridine orange. *Proc. Indian Acad. Sci.* **B.51**:205–210.

Rustad, R. C. (1958). Identification of the nucleus of a fission yeast with fluorescent dyes. *Exptl. Cell Res.* **15**:444–446.

Ryan, F. J., G. W. Beadle, and E. L. Tatum. (1943). The tube method for measuring the growth rate of *Neurospora*. *Am. J. Botany* **30**:784–799.

Schmidt, G., G. Bartch, M-C. Laumont, T. Herman, and M. Liss. (1963). Acid phosphatase of baker's yeast. An enzyme of the external cell surface. *Biochemistry* **2**:126–131.

Schröder, H. (1961). Der Einfluss von Substrat, Säurebildung und Temperatur auf die amylolytische Aktivität verschiedener Schimmelpilze. *Z. Allgem. Mikrobiol.* **1**: 201–222.

Schulman, H. M., and D. M. Bonner. (1962). A naturally occurring DNA-RNA complex from *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **48**:53–63.

Setterfield, G., and S. T. Bayley. (1961). Structure and physiology of cell walls. *Ann. Rev. Plant Physiol.* **12**:35–62.

Seyfarth, W. (1952). Über das Studium der Chondriosomen der Soorhefe (*Monilia albicans*) und ihr Nachweis mit Triphenyltetrazoliumchlorid. *Naturwissenschaften* **39**:90.

Shatkin, A. J., and E. L. Tatum. (1961). The relationship of m-inositol to morphology in *Neurospora crassa*. *Am. J. Botany* **48**:760–771.

Sheng, T. C. (1951). A gene that causes natural deaths in *Neurospora crassa*. *Genetics* **36**:199–212.

Simpson, M. V. (1962). Protein biosynthesis. *Am. Rev. Biochem.* **31**:333–368.

Singleton, J. R. (1953). Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. *Am. J. Botany* **40**:124–144.

Skou, J. C. (1961). The relationship of a $(Mg^{++} + Na^{+})$ -activated, K^{+}-stimulated enzyme or enzyme system to the active, linked transport of Na^{+} and K^{+} across the cell membrane. *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 228–236. Academic Press, New York.

Slonimski, P. P. (1956). Adaptation respiratoire: développement du système hemoprotéique induit par l'oxygène. *Proc. 3rd Intern. Cong. Biochem., Brussels, 1955* pp. 242–252.

Smith, D. C. (1962). The biology of lichen thalli. *Biol. Rev. Cambridge Phil. Soc.* **37**:537–570.

Steiner, M., and H. Heineman. (1954). Grana mit positiver NADI-Reaktion als Ort der primären Fettbildung in Pilzzelen. *Naturwissenschaften* **41**:40–41.

Storck, R. (1963). Characterization of ribosomes from *Neurospora crassa*. *Biophys. J.* **3**:1–10.

Stout, H. A., and H. Koffler. (1951). Biochemistry of filamentous fungi. I. Oxida-

tive metabolism of glucose by *Penicillium chrysogenum*. *J. Bacteriol.* **62**:253–268.
Strauss, B. S. (1955). A mechanism of gene interaction. *Am. Naturalist* **89**:141–150.
Suomalainen, H., and S. Rihtniemi. (1962). Mitochondrial and cytoplasmic thiamine in baker's yeast during the decline of decarboxylase activity. *Biochim. Biophys. Acta* **59**:420–425.
Svihla, G., and F. Schlenk. (1960). *S*-Adenosylmethionine in the vacuole of *Candida utilis*. *J. Bacteriol.* **79**:841–848.
Svihla, G., J. L. Dainko, F. Schlenk. (1963). Ultraviolet microscopy of purine compounds in the yeast vacuole. *J. Bacteriol.* **85**:399–409.
Takagi, S. (1959). Respiratory system of *Candida albicans*. *Osaka Daigaku Igaku Zasshi* **11**:523–530.
Tarwidowa, H. (1938). Über die Entstehung der Lipoidtröpfchen bei *Basidiobolus ranarum*. *La Cellule* **47**:205–216.
Tissières, A., H. K. Mitchell, and F. A. Haskins. (1953). Studies on the poky strain of *Neurospora*. *J. Chem.* **205**:423–433.
Tsuda, S., and E. L. Tatum. (1961). Intracellular crystalline ergosterol in *Neurospora*. *J. Biophys. Biochem. Cytol.* **11**:171–177.
Turian, G. (1956). Le corps paranucléaire des gamètes géants d'*Allomyces javanicus* traité à l'acide borique, *Protoplasma* **47**:135–138.
Turian, G. (1957). Détection cytochimique de l'acide ribonucléique dans les chromosphères pré- et post-meiotiques des sporanges de résistance d'*Allomyces*. *Experientia* **13**:315.
Turian, G. (1958). Recherches sur les bases cytochimiques et cytophysiologiques de la morphogenèse chez le champignon aquatique *Allomyces*. *Rev. Cytol. Biol. Végétales* **19**:241–272.
Turian, G. (1961). Cytoplasmic differentiation and dedifferentiation in the fungus *Allomyces*. *Protoplasma* **54**:232–327.
Turian, G. (1962). Déficience d'activité de la déhydrogénase succinique dans les mitochondries isolées du *Neurospora* en condition d'induction isocitratasique par culture sur acetate. *Compt. Rend.* **255**:755–757.
Umbarger, H. E. (1961a). End-product inhibition of the initial enzyme in a biosynthetic sequence as a mechanism of feedback control. *In* "Control Mechanisms in Cellular Processes" (D. M. Bonner, ed.), pp. 67–86. Ronald Press, New York.
Umbarger, H. E. (1961b). Feedback control by end-product inhibition. *Cold Spring Harbor Symp. Quant. Biol.* **26**:301–312.
Vanderwinkel, E., R. H. DeDeken, and J. M. Wiame. (1958). Isolation of cytoplasmic structures from yeast. *Exptl. Cell. Res.* **15**:418–421.
Van Eys, J. (1961). Regulatory mechanisms in energy metabolism. *In* "Control Mechanisms in Cellular Processes" (D. M. Bonner, ed.), pp. 141–166. Ronald Press, New York.
Vitols, E., and A. W. Linnane. (1961). Studies on the oxidative metabolism of *Saccharomyces cerevisiae*. II. Morphology and oxidative phosphorylation capacity of mitochondria and derived particles from baker's yeast. *J. Biophys. Biochem. Cytol.* **9**:701–710.
Vogel, H. J. (1961). Control by repression. *In* "Control Mechanisms in Cellular Processes" (D. M. Bonner, ed.), pp. 23–66. Ronald Press, New York.
Wainwright, S. D. (1959). On the development of increased tryptophan synthetase enzyme activity in cell-free extracts of *Neurospora crassa*. *Can. J. Biochem. Physiol.* **37**:1417–1430.

Wainwright, S. D. (1960). Mediation of the genetic control of protein synthesis in cell-free extracts of *Neurospora crassa*. *Nature* **185**:314–315.

Wainwright, S. D., and E. S. McFarlane. (1962). Partial purification of the messenger RNA of *Neurospora crassa* controlling formation of tryptophan synthetase enzyme. *Biochem. Biophys. Res. Commun.* **9**:529–533.

Webster, G. C. (1957). Amino acid incorporation by intact and disrupted ribonucleoprotein particles. *J. Biol. Chem.* **229**:535–546.

Weibull, C. (1958). Bacterial protoplasts. *Ann. Rev. Microbiol.* **12**:1–26.

Wiame, J. M. (1947). Etude d'une substance polyphosphorée basophile et métachromatique chez les levures. *Biochim. Biophys. Acta* **1**:234–255.

Wood, R. K. S. (1960). Pectic and cellulolytic enzymes in plant disease. *Ann. Rev. Plant Physiol.* **11**:299–322.

Woodward, D. O., C. W. H. Partridge, and N. H. Giles, Jr. (1958). Complementation of the AD-4 locus in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **44**:1237–1244.

Yanofsky, C., D. R. Helinski, and B. D. Maling. (1961). The effects of mutation on the composition and properties of the A protein of *Escherichia coli* tryptophan synthetase. *Cold Spring Harbor Symp. Quant. Biol.* **26**:11–24.

Yčas, M., and W. S. Vincent. (1960). A ribonucleic fraction from yeast related in composition to desoxyribonucleic acid. *Proc. Natl. Acad. Sci. U.S.* **46**:804–811.

Yotsuyanagi, Y. (1962). Études sur le chondriome de la levure. I. Variation de l'ultrastructure de chondriome au cours du cycle de la croissance aérobie. *J. Ultrastruct. Res.* **7**:121–140.

Zalokar, M. (1959a). Enzyme activity and cell differentiation in *Neurospora*. *Am. J. Botany* **46**:555–559.

Zalokar, M. (1959b). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* **46**:602–610.

Zalokar, M. (1960a). Cytochemistry of centrifuged hyphae of *Neurospora*. *Exptl. Cell. Res.* **19**:114–132.

Zalokar, M. (1960b). Sites of protein and ribonucleic acid synthesis in the cell. *Exptl. Cell. Res.* **19**:559–576.

Zalokar, M. (1961a). Electron microscopy of centrifuged hyphae of *Neurospora*. *J. Biophys. Biochem. Cytol.* **9**:609–617.

Zalokar, M. (1961b). Kinetics of amino acid uptake and protein synthesis in *Neurospora*. *Biochim. Biophys. Acta* **46**:423–432.

Zalokar, M. (1962). Uptake of ribonucleic acid precursors by *Neurospora*. *Plant Physiol.* **37**:Suppl., xxii.

Zalokar, M., and V. W. Cochrane. (1956). Diphosphopyridine nucleotidase in the life cycle of *Neurospora crassa*. *Am. J. Botany* **43**:107–110.

Nutrition and Growth of Cells

CHAPTER 15

Uptake and Translocation

1. Uptake[1]

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1. INTRODUCTION

No substance can leave or enter a cell without being subjected to the traffic rules that are inherent in the nature of the cell membrane. In fact, the primary function of this thin outer membrane is the regulation of traffic. By carefully determining the rules that apply to various substances, the cell physiologists hope not only to describe the balance and flow of substances into and out of the cells, but to determine the underlying mechanisms within the membrane.

Although the specific characteristics of the membrane of each type of cell are unique, common underlying patterns are quite evident and comparisons are often fruitful. Although much of the early work on membranes was done with plant cells, in recent years most of the new concepts have been derived from studies with animal cells. The only fungal cell which has been studied with any intensity is the yeast cell. The general features of membrane transport will therefore be discussed in relation to cells in general, and the detailed studies of the yeast cell will then be placed in the context of the general pattern.

[1] This study is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

A. Functions of the Cell Membrane

The chief function of the cell membrane is the regulation of cellular composition with respect to diffusible substances. Essential metabolites are retained while waste products escape. Substrates are taken up, while many foreign substances are excluded. In the naked cells of animals, the membrane is also responsible for the regulation of size (Tosteson and Hoffman, 1960). These cells are always in osmotic equilibrium; therefore, in controlling electrolyte composition, the membrane is simultaneously the predominant factor in the regulation of cell volume. In contrast, in the walled cells, including fungi, the wall itself determines cellular volume. Although the membrane does not play a direct role in regulating size, it is responsible for maintaining a high internal osmotic pressure by salt accumulation, so that an outward pressure exists against the wall (Falcone and Nickerson, 1959). When growth occurs, the wall is softened by specific enzymes, and the outward pressure forces an expansion of the protoplast which accommodates the newly forming bud or cell (Rothstein, 1964).

Another difference between certain animal cells and plant cells relates to the role of membrane polarization. In all cells, differences in the distribution of electrolytes between the cytoplasm and the external aqueous environment give rise to membrane potentials. In nerve and muscle, transient changes in the permeability of the membrane result in depolarization phenomena associated with transmission of impulses and with stimulation of contraction. Although action potentials can be induced in large algal cells, the phenomena are undoubtedly of little physiological concern in the fungi.

Another function of the cell membrane which is not directly concerned with exit and entry of substances is its role in the degradation and synthesis of extracellular substances. These activities are related to enzyme localized on the membrane and are perhaps of primary importance in the elaboration of cell wall materials (Rothstein, 1954).

B. Structure of the Cell Membrane

The cell membrane is not visible in the light microscope, and its existence, until recently, was inferred from physiological studies. The cell behaved as though it were covered by a membrane ("plasma membrane" or "plasmalemma" in plants) that acted as a diffusion barrier. From many studies of permeability it was postulated that the membrane was composed of a bimolecular leaflet of lipid, the polar ends being associated with layers of protein (Danielli, 1943). In recent years this postulated model has

been confirmed by visualization with the electron microscopic (Robertson, 1959) and with X-ray diffraction techniques (Finean, 1961). All cells have an outer membrane of the same basic pattern, called the "unit membrane" (Fig. 1). Indeed, many of the internal structures of the cell such as mito-

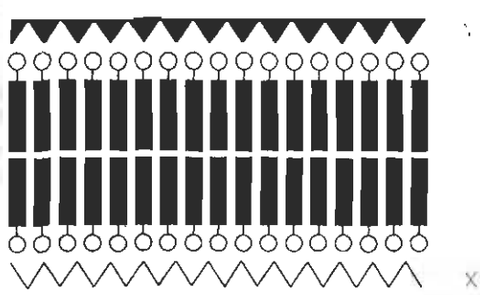

FIG. 1. Highly schematic diagram of the unit membrane structure. The jointed circles and bars represent lipid molecules, and the zigzag lines represent nonlipid molecules designated *X* and *Y*. From Robertson (1961).

chondria, Golgi, nucleus, vacuoles, and endoplasmic reticulum also contain similar membrane structures. The double "track" seen by electron microscopy with heavy metal staining represents a basic structural configuration or "backbone" of lipids and proteins, common to all cells. The detailed structural array associated with specific permeability properties and with specialized transport systems are beyond the present resolution of the electron microscope. Nor can differences in structure which must underlie the physiological differences in the membranes of different types of cells be seen by means of the electron microscope.

C. Mechanisms of Membrane Transfer

A net movement of solute across the cell membrane will take place only if a driving force acts on that particular solute, and if some means exist for the solute molecule to pass through the membrane. In the simplest case, the driving force derives from a difference in the chemical potential of a solute in the two solutions on the two sides of the membrane. For example, with a nonelectrolyte, a difference in concentration (or more precisely, activity) in the two solutions will tend to drive solute in the direction of the lower concentration. In the case of ions, the electrical potential across the membrane as well as the chemical potential can serve as driving forces and the net driving force is usually referred to as the electrochemical potential. If no other forces intervene, the net movement will cease when the electrochemical potential is zero, and the solute will

reach an *equilibrium* distribution. But if solute is continuously removed or added to one side of the membrane (for example, by metabolic production or destruction), then equilibrium is never reached and solute will continue to move. Movements of solutes in response to driving forces outside of the membrane are usually referred to as "downhill" or "passive."

The movements of many physiologically important solutes through the cell membrane cannot be accounted for by the external gradients. Other forces operating within the membrane push the solutes against their electrochemical gradients. Such movements are generally called "uphill" or "active transport," the required energy being derived from metabolic reactions. Usually a solute that can be actively transported in the "uphill" direction can also move in the "downhill" direction as well. In this case a steady state distribution is attained, in which an electrochemical gradient is maintained, and in which the "uphill" movement is exactly balanced by the "downhill" movement. This situation is sometimes referred to as a "pump and leak" system.

The mechanisms by which solutes penetrate the membrane are not completely understood, primarily because the detailed molecular architecture of the membrane is unknown. From studies of the kinetics of transport, three general patterns have emerged which, for convenience, can be labeled the "diffusion pattern," the "pore pattern," and the "carrier pattern," and mechanisms have been postulated for each of them. In addition, entry and exit of macromolecules by pinocytosis or by undefined mechanisms have been observed.

The diffusion pattern was the earliest to be developed, dating back to the nineteenth century, particularly to the work of Overton. The rate of transport follows kinetics that can be described by appropriate modifications of the Fick equation for diffusion. (The rate is proportional to the concentration gradient across the membrane.) In comparing the rate constant (or permeability constant) for various solutes, the predominant factor is the degree of lipid solubility. Presumably the solute penetrates by dissolving in the lipid phase of the membrane, and this pattern is displayed primarily by lipid-soluble nonelectrolytes. The most detailed analysis of the diffusion pattern is given by Danielli (1943), who points out its complexities. The rate of penetration may depend not only on the partition coefficient, but on the viscosity of the membrane lipids, internal bonding, and bonding that may occur between the solute molecule and the lipid molecules.

The concept of pores or aqueous channels through the membrane is an old one which was used to explain the relatively rapid penetration of small, lipid-insoluble (polar) molecules. After many years of neglect, the concept has been revived in a more sophisticated form to explain the move-

nents of water (Solomon, 1961). Thus, the movement of labeled water tritium) in a system which is in osmotic equilibrium, follows a typical liffusion pattern. The tritium-labeled molecules are relatively few in num-)er relative to unlabeled water, and behave like the diffusion of solute in ı solvent. If, however, the water movements are measured in terms of ıet change driven by an osmotic gradient (a difference in the activity of vater on the two sides of the membrane), then it is the solvent itself that noves rather than a solute. The apparent permeability may be three or nore times higher. The water behaves as though it is moving in bulk rather han as individual molecules in random motion. The kinetics follow the low equations (Poiseuille's law) rather than diffusion equations (Fick's aw). From the data an average equivalent pore radius can be calculated. For several kinds of cells it is of the order of 4–5 Å. Two other conse-juences of the pore pattern are the sharp screening effect or cutoff of penetrability for molecules of a size greater than that of the pore, and he phenomenon of "solvent drag." The latter phenomenon involves move-ments of polar solutes across a membrane, driven by the flow of water (Ussing, 1963).

In the "diffusion" and "pore" patterns, the driving forces are considered to be the activity or electrochemical gradients in the bulk solutions on the two sides of the membrane. That is, they are "downhill" movements.

The concept of carriers has developed in the last twenty years primarily to explain the behavior of many physiologically important solutes. The kinetics of transport in this case cannot be explained by a diffusion-limited process, or by bulk flow, but it can be explained by assuming that the solute must form a chemically specific complex with a receptor site in the membrane in order for transfer to occur (Wilbrandt and Rosenberg, 1961). The general properties of carrier systems can be summarized briefly: (1) "saturation" by high concentrations of solute, resulting in a maximal rate of transfer; (2) inhibition by small amounts of specific inhibitors indicating that the carriers occupy only a small fraction of the membrane surface; (3) linkages, often seen between the solute movements in opposite directions. For example, the movement of one sugar into the cell can accelerate the movement of a different sugar in the opposite direction. In the case of ion movements, the linkage may be obligatory; an ion can be transferred in one direction only if another ion of the same charge is simultaneously transferred in the other direction (called "forced exchange"); (4) pairs of chemically similar substances that may compete for the limited numbers of carriers.

Carrier-mediated transfers of solute may result from driving forces arising from the external electrochemical gradient, or from internal gradients generated within the membrane by active transport mechanisms. In the

latter case, metabolic reactions are required to maintain an asymmetry in the distribution of the carriers across the membrane so that solute is transferred faster in one direction than in the other.

D. Compartmentalization within the Cell

In characterizing the uptake of solutes by cells it is important to consider not only the properties of the cell membrane, but also the various subdivisions of the cell and their behavior toward the solute in question. In plants and microorganisms, the cell membrane is covered by the cell wall and in some cases by slime layers. The anatomical interrelationships between the wall and the membrane may be more intimate than merely one layer upon another. In yeast, the membrane is considerably folded and the wall itself consists of three distinct layers (Vitols *et al.*, 1961). The outfoldings of the membrane must represent an intimate molecular attachment of the membrane and wall because if the cell is subjected to media of very high osmotic pressure, no plasmolysis occurs. That is, the protoplast does not shrink away from the wall as in the case of plant cells. If, however, the wall is digested away by appropriate enzymes, the protoplast itself responds osmotically, shrinking in media of high tonicity.

The presence of a cell wall outside of the protoplast imposes some limitations on the study of uptake or release of substance from the cell. Thus, substances associated with water in the cell wall, or with binding groups in the cell wall, have not passed through the cell membrane and are not in the protoplast itself; yet, when the distribution of these substances between the medium and the cells is measured, they are measured as part of the cellular compartment. In yeast cells the cell wall space has been investigated by measuring the volume of distribution of various solutes (Conway and Downey, 1949). Large molecules such as proteins and inulin cannot penetrate into the "cell wall space." In yeast packed by centrifugation these substances distribute into 26% of the volume, presumably in the water between the cells. The value is reasonably close to 22%, the theoretical close packing of spheres. Furthermore, if the yeast is pressed, the distribution space of proteins is redueed to 3 or 4%. Small molecules and ions rapidly distribute into an additional volume equivalent to 12% of the cell volume, and it is this volume which is presumed to be the "cell wall space" outside of the cell membrane.

Within the protoplast are many visible structures, such as vacuoles, nucleus, membranes, and granules, organized in a most complicated fashion. Obviously the cytoplasm is *not* a homogeneous well-mixed solution. Physiologically it is also obvious that the cytoplasm is compartmentalized and that the internal structure is a contributing factor in distribution of solutes.

No systematic study of the intracellular compartmentalization has been made, but some of the factors are recognizable: (1) as much as 30% to 40% of the volume of the protoplast is not in solution (nonsolvent volume); (2) a proportion of the water is involved in hydration of protein and other surfaces and does not behave like bulk water; (3) internal structures such as vacuoles and mitochondria may concentrate or exclude certain solutes; (4) internal barriers may limit rates of diffusion of solutes; (5) solutes may be bound by cellular components and structures; (6) owing to pH differences inside and outside the cell, weak electrolytes may assume anomalous distribution; (7) the nondiffusible components tend to impose a Donnan distribution on all diffusible ions.

II. STRONG ELECTROLYTES

A. Monovalent Cations

The uptake of the alkali metal cations by yeast has been extensively studied, particularly by Conway and Duggan (1958) and by Rothstein (1955, 1959). With any substrate that can be respired or fermented, the K^+ can be rapidly absorbed. The inside:outside ratio of concentrations may be greater than 5000:1, a consequence of a vigorous "uphill" transporting system and very slow "downhill" leakage.

When K^+ is taken up, electrical balance is maintained by a stoichiometric excretion of H^+ so that the transport system behaves as a linked K^+–H^+ exchange system. The H^+ ion is derived directly from metabolic reactions, perhaps in the membrane itself. Conway has proposed a redox-pump theory relating H^+ production and K transport to electron-transport systems (Conway, *1953*).

The kinetics of K transport follow the typical carrier pattern. As the K^+ concentration is raised, the rate of transport increases to a maximum along a curve that can be fitted by the Michaelis-Menten equation for enzyme kinetics (Fig. 2). This implies that K^+ must combine with a receptor present in limited amount, and that the rate of transport is proportional to the amount of K^+–receptor (or K^+–carrier) complex. Other alkali metal cations can also be transported by the K^+–carrier. The relative affinity of the carrier for cations is $K^+ > Rb > Cs > Na > Li$ in the ratio of 1:3:15:20:30. Mg^{++} can also be transported by this same carrier, but its affinity is very low. If ions are present in pairs, they compete with each other for transport in proportion to their affinity constants (Fig. 2). Thus, if K^+ and Na^+ are presented in a concentration ratio of 1:25, they will combine with equivalent amounts of carrier.

Another factor that determines the discrimination between cations is

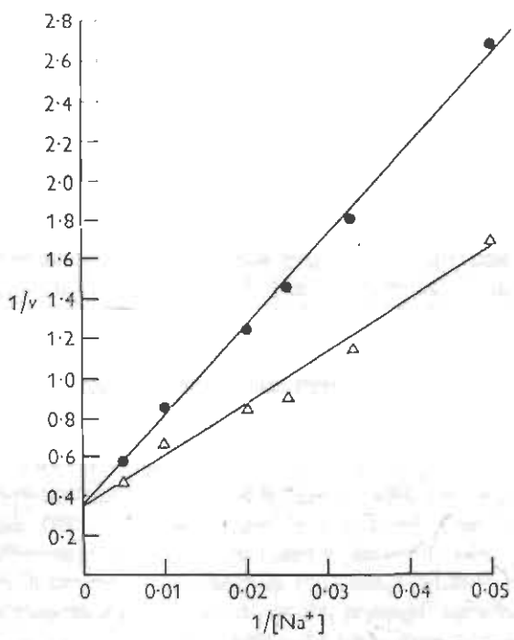

FIG. 2. Demonstration of competitive inhibition of uptake of sodium by potassium during fermentation: △, sodium acetate alone; ●, sodium acetate with 5 m*M* potassium acetate. From Conway and Duggan (1958).

the maximum rate of turnover of the transport system (Armstrong and Rothstein, 1964). At a pH of 6–8, the maximal transport rate for K^+ is higher than that for the other ions by about 50%. At low values of pH, the maximal rates are all reduced, but that of K^+ is reduced to a lesser degree (Fig. 3). Consequently the maximal rate for K^+ is five times as high as for other cations under these circumstances. The factor of discrimination is thereby increased over that predicted from affinity ratios by a factor of 5.

The reduction in maximal transport rates at low pH is due to the interaction of H^+ with a membrane ligand distinct from the transport site itself. For convenience it has been called the modifier site. Its pK is 4.8. This site can also combine with other cations, such as Ca^{++}, with a similar reduction in maximal rates of transport.

The inward-transporting system has about an equal affinity for K^+ and H^+ and a much lower affinity for Na^+, but the outward-directed system discriminates against K^+ in favor of H^+ or Na^+. Thus the influx of K^+ is normally balanced by an efflux of H^+ plus K^+. But the efflux of K^+ is

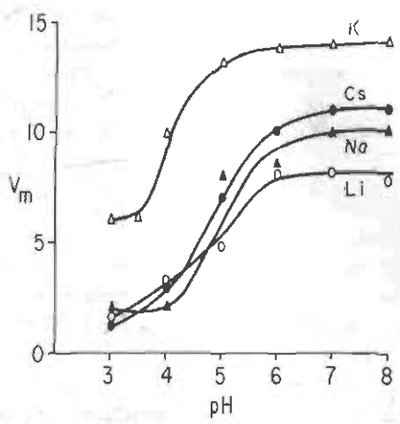

FIG. 3. The effect of pH on the maximal rate (V_m) of transport of the alkali metal cations by yeast. From Rothstein (1964).

very small (20 mmoles/kg/hr) whereas the efflux of H^+ may be as high as 600 mmoles/kg/hr, despite the fact that the K^+ concentration of the cell is about 200 mmoles/kg and that of H^+ only 0.001 mmoles/kg (pH of 6.0). Granted that the local concentration of H^+ at the transport site may be somewhat higher, the ability of the inner face of the membrane to discriminate between H^+ and K^+ must be very large. Similarly if cells are loaded with Na^+, the Na^+ is expelled in preference to K^+. Thus the membrane is asymmetrical, selecting K^+ at the outer face and discriminating against K^+ at the inner face. It is the function of metabolism to maintain this asymmetry, probably by a cyclic conversion of the carrier from K^+-selective to H^+- or Na^+-selective forms.

If the yeast cells are left in K^+-free solution, then the efflux of K^+, though small, is easily measured. In this situation with no other external cation, the K^+ efflux is balanced by H^+ influx. If an appropriately small concentration of K^+ is added to the external medium, no net uptake or loss will take place. The system is in a steady state in which the efflux of K^+ is exactly equal to the influx (Fig. 4). The concentration of K^+ required for such a steady state is dependent on the pH. At pH 4.0 it is of the order of 0.05 m*M* (even though the internal concentration of K^+ is 200 m*M*). At this point the inward-directed "K^+ pump" is balanced by the outward leak. If higher concentrations of K^+ are added, the rate of transport is increased and the cell gains K^+. The uptake of K^+ under these conditions (only glucose and KCl present) continues for only about 10 minutes. In this short time, however, the transport system is so effective and the rate of efflux so slow, that the cellular content of K^+ may double.

The cell is geared for the selective accumulation of K^+ against large gradients, and the accumulated cation is retained for long periods of time because of the relative impermeability of the membrane to ions. If K^+ is absent other cations can be accumulated. For example, cells have been grown in which the primary internal cation is Na^+ or NH_4^+ (Conway

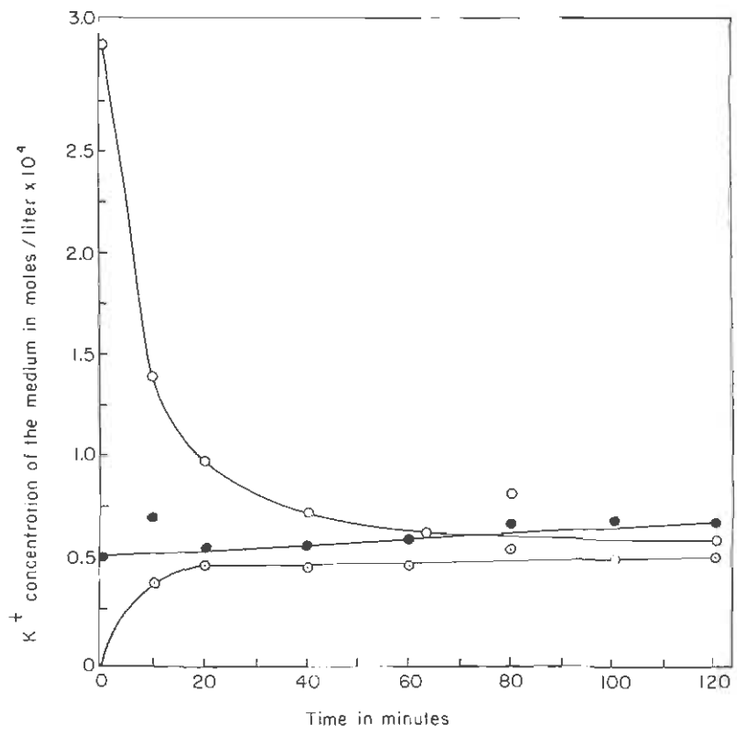

FIG. 4. Changes in the potassium concentration of the medium in the presence of fermenting yeast. The yeast concentration was 10 mg/ml, glucose 0.1 *M*, pH 4.5 with TST buffer. From Rothstein and Bruce (1958).

and Brein, 1945; Conway and Moore, 1954). The limitations on the amount of K^+ that can be taken up derive from effects on the acid-base balance of the cell. The K^+ is exchanged for H^+ derived from metabolism, the balancing anions within the cell being organic anions such as bicarbonate, acetate, and succinate. The accumulation of anion results in an increased pH in the cell (despite the many buffer systems), which in turn shuts off the inward transport of K^+ (Rothstein, 1960).

B. Phosphate Uptake

Phosphate is the most important inorganic anion for the yeast cell, and a special mechanism exists for its transport into the cell. The properties of the mechanism are similar to those of typical carrier-mediated active transport systems, with saturation kinetics, competition (with arsenate), specificity, and dependence on metabolism (fermentation only, in contrast to respiration or fermentation for K^+). In contrast to K^+, the uptake of phosphate proceeds without measurable efflux, and therefore without steady-state relationships (Goodman and Rothstein, 1957). Such one-way movements have been observed also with plant cells, but not in animal cells.

The maximal uptake of phosphate, as with K^+, depends on the acid-base balance of the cell. Only the monovalent form $H_2PO_4^-$ is transported. Consequently the medium becomes more alkaline and the cell more acid, the system behaving as an $H_2PO_4^-$–OH^- exchange system. Potassium has a profound effect on the total amount of phosphate that can be taken up, but not on the initial rate of its uptake. The effect is indirect and can be separated in time. Cells that have previously taken up K^+ can subsequently take up larger amounts of phosphate, presumably because K^+-rich cells have greater buffer capacity against acid. If K^+ and phosphate are presented to the cells together, a much larger quantity of each is taken up even though the transport mechanisms are quite independent. The alkalization of the cell associated with K^+-uptake is counterbalanced by the acidification associated with phosphate uptake (Rothstein, 1961).

Arsenate is taken up by the same transport system as phosphate; if the two ions are present at the same time, they compete with each other for uptake (Rothstein, 1963). In contrast to phosphate, however, arsenate uptake is associated with a second effect, the slow development of an irreversible reduction in the capacity of the transport system. The blocking action is due to the direct interaction of arsenate with the transport system itself, rather than to an indirect effect, such as inhibition of metabolism.

When phosphate is taken up, the level of inorganic phosphate in the cell is not altered appreciably. The absorbed phosphate is converted into inorganic polyphospbates of high molecular weights which are aggregated in granules (volutin or metachromatic granules) (Wiame, 1949). The —P—O—P— bonds in polyphosphates are high-energy bonds. They are formed by metabolic reactions involving ATP at the cost of metabolic energy. It is not clear whether the sequence of events in phosphate uptake involves the direct formation of ATP and polyphosphates at the membrane,

or whether the transport system loads the interior of the cell with inorganic phosphate which is subsequently converted to polyphosphate.

Phosphate-transporting systems have not been as carefully studied as some others. Phosphorylation reactions are probably involved at some stage in the process, but, as is the case in other transporting systems, the specific sequence of reactions is not known.

C. Bivalent Cations

In well-starved cells, the only uptake of the bivalent cations is due to a small amount of binding by negative groups on the outside of the cell membrane (Rothstein, 1955). This binding can be characterized by a simple, reversible, mass-law relationship. Little difference in the strength of binding exists between Ca^{++}, Mn^{++}, Mg^{++}, and Sr^{++}, but the binding of UO_2^{++} is especially strong, whereas that of the alkali metals is very weak. Two kinds of binding sites have been identified, including phosphoryl and carboxyl groups.

If the cells are allowed to absorb phosphate by exposure to that ion plus glucose, another capability appears (Rothstein and Jennings, 1958). In addition to relatively nonspecific surface binding, the cells now display a transport system, which is highly specific for Mg^{++} and Mn^{++} and capable of carrying large amounts of these cations into the cell. Once inside, the cations are no longer exchangeable. The cation-transport system, once induced by phosphate, behaves like all the other transport systems. It requires a substrate, glucose; it displays saturation kinetics, competition between pairs of ions, and a high degree of specificity. It differs from other transport systems not only because it becomes active following phosphate uptake, but also because it disappears again on subsequent starvation. The dependence on phosphate uptake suggests that some phosphorylated intermediate is essential, a conclusion supported by the fact that Mg^{++} transport is inhibited by low concentrations of arsenate (Jennings *et al.*, 1958).

The maximum uptake of magnesium seems to depend on the relative cellular content of K^+ and phosphate. A cell rich in K^+ and low in phosphate cannot absorb much Mg^{++}, but a cell rich in phosphate and low in K^+ can take up a great deal of Mg^{++}.

D. Other Anions

Sulfate can be taken up by a transport system that follows saturation kinetics. As in the case of phosphate, sulfate that is absorbed does not exchange (Kotyk, 1959).

Other inorganic ions have not been extensively studied. The yeast cell behaves as though impermeable to Cl^-.

E. Summary

The yeast cell membrane is a relatively "tight" membrane with a low permeability to ions. Yet it possesses an exceedingly high transport capacity for physiologically important ions, particularly K^+, Mg^{++}, and phosphate. In these respects the yeast cell is much more like roots of plant cells and unlike bacteria and animal cells. The low permeability, high transport capacity is probably related to the adaptation to a very diluted environment. If the cells were permeable, the loss of salts would lead to an intolerable dilution of the cytoplasm. Another feature of yeast cells which is common to all walled cells, in contrast to unwalled cells, concerns osmoregulation. In unwalled cells, the size of the cell is determined by the osmotic content (largely electrolytes). The transport and leakage of ions is therefore a predominant factor in the regulation of cell size (Tosteson and Hoffman, 1960). In walled cells, the size is determined hy the structurally rigid wall. Such cells do not regulate their osmotic content, but in order to grow, the osmotic pressure within the cell must be higher than that outside of the cell. When the wall is softened by enzyme reactions (disulfide splitting in the case of yeast), the difference in osmotic pressure causes an outward swelling and production of a bud (Rothstein, 1964).

With no necessity to regulate electrolyte content, the yeast cell is geared to accumulation and, given the opportunity, can store tremendous quantities of electrolytes. The limiting factor in uptake seems to be the acid-base and anion-cation balances. If given appropriate ratios of K^+, Mg^{++}, and phosphate, the cellular contents can be doubled or tripled even though the cells have no source of nitrogen. The stored ions can at a later time, when nitrogen becomes available, serve as a reservoir for growth and cell division.

III. NONELECTROLYTES AND WEAK ELECTROLYTES

A. Sugars

The uptake of sugars has been extensively studied in many types of cells. In all cases the same general pattern is evident. Cell membranes, in general, are relatively impermeable to the free diffusion of sugars, as would be predicted from molecular size and lipid insolubility. On the other hand, the majority of cells possess a highly specialized transport system that allows rapid entry and exit of certain sugars.

The problem in studying the uptake of sugars is to distinguish between their transport across the membrane and their subsequent metabolism. In yeast the two processes were first distinguished by experiments with uranyl ion (UO_2^{++}). This cation does not penetrate the cell membrane, but it does bind to phosphoryl and carboxyl groups on the outside of the membrane (Rothstein, 1955). Associated with the binding to phosphoryl groups, the uptake of sugars is inhibited whereas the metabolism of stored carbohydrates and of other substrates is not. Thus UO_2^{++} inhibits a membrane step that precedes glycolysis. The first enzymatic step in sugar metabolism is the hexokinase reaction. The membrane step can be distinguished from the hexokinase reaction in two ways: (1) Hexokinase requires Mg^{++} and is inhibited by Ca^{++} whereas the membrane step is not inhibited by Ca^{++}, and indeed its inhibition by UO_2^{++} can be competitively reversed by Ca^{++}. (2) The specificity pattern of uptake is broader than that of the hexokinase reaction and includes some sugars that cannot be phosphorylated by hexokinase (Burger *et al.,* 1959; Cirillo, 1961; de la Fuente and Sols, 1962).

The properties of the sugar transport system can be studied by the use of sugars that can be transported but not metabolized, or by inhibiting metabolism (with iodoacetic acid). All the features attributed to carrier systems have been described: (1) saturation kinetics, (2) specificity, (3) competition of pairs of sugars, (4) inhibition by small concentrations of inhibitors, (5) high temperature coefficient. It is not, however, an "active" or uphill transport system. Metabolic energy is not required and net movement ceases when an equilibrium distribution (zero chemical potential) is attained. With a fermentable sugar, inward movement continues to completion because sugar is removed from inside the cell by metabolic reaction, as rapidly as it is transported.

A kinetic model of a sugar transporting system has been evolved from studies of the red blood cell (Fig. 5), in which the sugar-carrier complex is assumed to move across the membrane (Wilbrandt and Rosenberg, 1961). This model predicts certain unusual behavior: (1) If one sugar is at equilibrium distribution and a second sugar is added to one side of the membrane, the movement across the membrane of the second sugar will cause a transient "uphill" movement of the first sugar (called counter transport). (2) If two sugars have widely different affinities for the transport system, the sugar with the lower affinity will be transported more slowly at low concentrations, but faster at high concentrations. (3) If a competing sugar is added together with the measured sugar, the expected inhibition of uptake occurs at one concentration, but at another, stimulation occurs (competitive acceleration). It is not the purpose here to enter into a detailed discussion of the kinetics of transport, but only to point

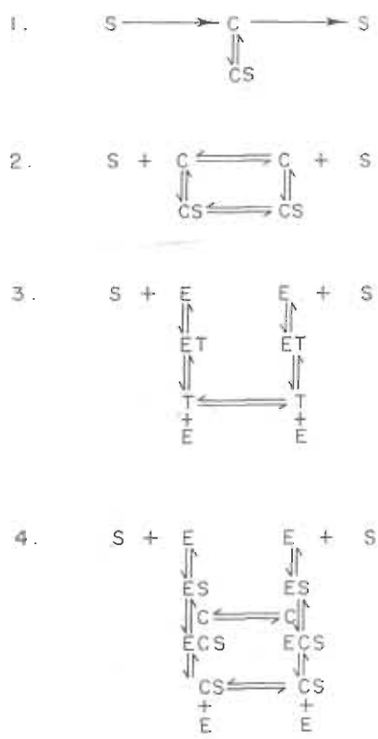

FIG. 5. Schemes of carrier models. From Cirillo (1961).

ıut that the above results can be predicted from a model in which the :arrier moves across the membrane with the sugar. The phenomena listed ıbove have been observed in sugar transport in the yeast cell (Cirillo, 961) as well as in animal cells.

The specificity of sugar transport is much broader than that of sugar-·nzyme reactions. The enzymes are group specific whereas the transport ;eems to depend predominantly on the shape of the sugar molecule (Le-'evre, 1961).

The disaccharides can be utilized by cells in two ways (de la Fuente ınd Sols, 1962): (1) They can be split by saccharases located on the ›uter surface of the cell to monosaccharides. The products are then trans-›orted into the cell by the carrier systems for monosaccharides. (2) Some lisaccharides are moved into the cell by specific membrane transport sys-ems. For example, in *Saccharomyces cerevisiae* sucrose is split to glucose ınd fructose by invertase on the outer surface of the cell (Rothstein,

1954). The products, glucose and fructose, enter the cell by means of a common transport system. The fermentation of sucrose is thus indirect. Maltose, on the other hand, can be transported into the cell as an intact molecule and is then cleaved inside the cell by enzymatic reactions.

The transport system for glucose and related sugars is constitutive in most yeasts, but other sugar transport systems are adaptive, appearing only after an induction period in the presence of the sugar concerned or of some of its analogs (Harris and Thompson, 1961). The adaptation process in yeast is similar to that described for the permease systems of *Escherichia coli* (Cohen and Monod, 1957). Two distinct capacities must be induced if the particular sugar is to be metabolized, the first a membrane transport system (called permease in the case of *E. coli*) and the second, an enzyme responsible for phosphorylation of the sugar inside the cell. The specificity of the two individual systems is different, and each is controlled by independent genes. Because of differences in specificity, the transport system can be measured with sugar analogs that cannot be metabolized. The individual permease systems that have been studied most extensively in yeast are those for galactose and for the disaccharide maltose.

The transport of glucose and its analogs in yeast is not an "uphill" or active transport. The driving force is the concentration difference that is maintained by the metabolism of the sugar within the cell. With a non-metabolizable sugar such as sorbose, an equilibrium distribution is finally attained. In the case of the inducible permeases, on the other hand, the sugars can be accumulated by an "uphill" transport requiring metabolism of a substrate.

B. Amino Acids

In most cells, yeast included, the amino acids can be transported by special mechanisms possessing all the properties of carrier systems. The exact number of carrier systems is, however, not known exactly because the transport of all amino acids has not been studied in detail. However, the number is small—perhaps three or four. For example, in yeast, many α-amino acids compete for the same mechanism even though the amino acids are not structurally related (Halvorson and Cowie, 1961). They include L-isoleucine, L-proline, L-valine, DL-methionine, L-phenylalanine, D-phenylalanine, and DL-*p*-fluorophenylalanine. With substitution of the amino or carboxyl groups the transport ability disappears. Thus the transport system has a distinct specificity, but it is much broader than that of enzyme systems for amino acids. Both D- and L-forms can be transported in the case of phenylalanine, and so can the fluorine analog, which cannot be metabolized.

The relationship between the transport and the metabolism of amino acids is complicated. Yeast can synthesize amino acids; it can degrade them; it can interconvert them; it can synthesize proteins; it can transport exogenous amino acids; and it can lose amino acids by leakage into the medium. The overall balance is shown in Fig. 6. The endogenous amino

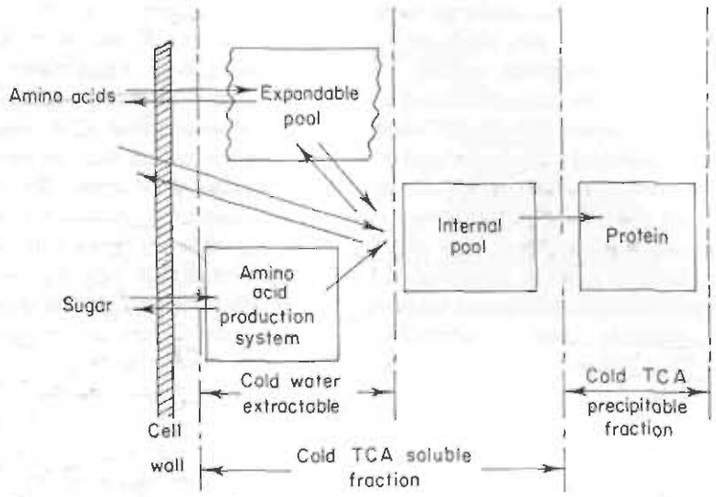

FIG. 6. Carbon flow in yeast. From Halvorson and Cowie (1961).

acids produced by the cell enter an internal pool which serves as a source for protein synthesis. Exogenous amino acids are transported into another pool, which can vary considerably in size (expandable pool), from which they can flow into the internal pool. The amount of amino acids in the expandable pool depends on a balance between leakage into the medium, the transport into the cell, and the rate of protein synthesis. For many amino acids the inward transport is an active (uphill) transport supported by metabolic energy, and the leakage is small. Consequently these amino acids are accumulated in concentration ratios approaching 1000:1.

C. Organic Acids

Organic acids can enter the cell by two mechanisms. The simplest mechanism, and the most rapid, is by diffusion as an undissociated molecule. The dissociation constant of the acid and the pH of the medium are predominant factors because the organic anions penetrate only very slowly, whereas the undissociated form penetrates rapidly (Foulkes, 1955; Suo-

malainen and Oura, 1958). If the pH is more than one unit above the pK so that virtually all the acid is dissociated, the acids can enter the cell slowly, if at all, and the metabolism is minimal. If, however, the pH is below the pK, the undissociated form enters rapidly and metabolism is rapid. If the rate of penetration of various undissociated acids is compared, the rate is proportional to the lipid solubility (Oura *et al.*, 1959).

With high concentrations of organic acids (for example, acetic acid), rapid entry, at low pH, leads to inhibitory effects on the rate of fermentation and of phosphate uptake (Samson *et al.*, 1955). Presumably the acetic acid enters the cell more rapidly than it can be metabolized, and acidification of the cytoplasm results. The same concentration of acetate at higher pH (where all the acid is in ionic form) is nontoxic. A similar pattern holds for those metabolic inhibitors that are weak acids. Thus the inhibitory effect of DNP (Simon, 1953), of iodoacetate (Aldous, 1948), and fluoracetate (Aldous and Rozee, 1956) at different pH's follows a curve identical to their dissociation curves, indicating that only the undissociated molecule can penetrate and exert toxicity (Fig. 7). The rate of

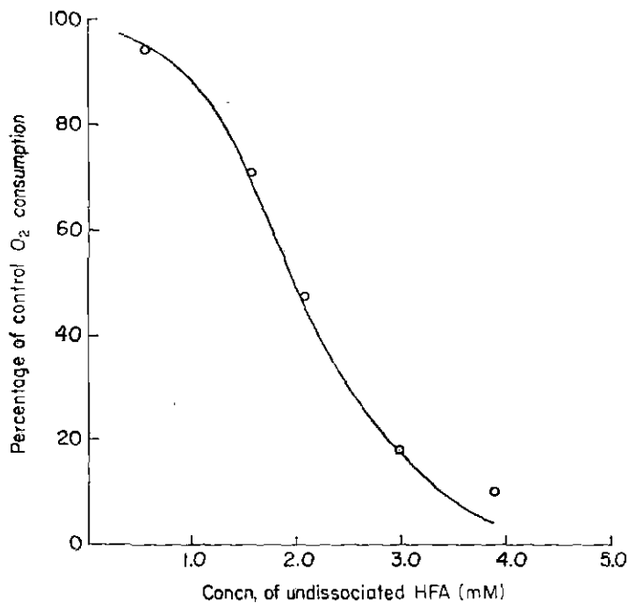

FIG. 7. Inhibition of oxygen consumption of yeast cells by HFA, plotted as a function of the concentration of undissociated HFA in the suspension medium. Concentration of HFA $5 \times 10^{-3}M$; pH varied from 2.15 to 3.50. Duration of experiment 90 minutes, temperature 25°. From Aldous and Rozee (1956).

penetration of the membrane is the determining factor in the inhibitory effects of these substances.

A second mechanism of entry of organic anions is by way of specific carrier systems. Such a system has been demonstrated in the case of pyruvate (Foulkes, 1955) and in the case of acids of the tricarboxylic acid cycle (Barnett and Kornberg, 1960).

D. Nucleotides

Recent studies with a series of purines indicate that some, such as uric acid, can be actively accumulated by inducible transport systems, whereas others enter or leave the cell by a diffusion process (Harris and Thompson, 1962; Roush and Shieh, 1962).

E. Other Substances

For most physiologically important substrates the cell possesses special transport systems in the membrane which allow rapid entry or which, coupled to metabolism, allow uphill transport. The number of such mechanisms is not large compared, for example, to the number of enzymes because the transport systems have very broad specificity. The total number of known transport systems in yeast may be of the order of a dozen, three or four for inorganic ions, two or three for sugars, and a few for amino acids, perhaps two or three for organic acids, and small numbers for nucleotides and growth factors. Many of these are not normally present, but are induced only in the presence of a specific substrate. For those systems that have been studied in detail, the models which seem best to account for the kinetic behavior, involve mobile carriers that move across the membrane with the substrate and release it at the other face of the membrane. The chemical nature of the carriers and the physical nature of their movements are unknown. In the case of the cation-transporting systems, specific ATPases in the membrane have been implicated (Skou, 1963).

IV. ROLE OF METABOLISM

A. Source and Sump for Metabolites

The distribution of some substances may be independent of metabolism. Such substances, regardless of their mechanisms of penetration through the membrane, will tend to reach an equilibrium distribution of the permeating form (electrochemical potential is zero). At equilibrium the flux (or rate) of movement into the cell will be equal to the rate of movement out of the

cell. For nonelectrolytes equilibrium occurs when the concentrations are equal, but for ions the presence of nondiffusible charged molecules results in a Donnan equilibrium in which the distribution ratio is not 1 (Fig. 8,

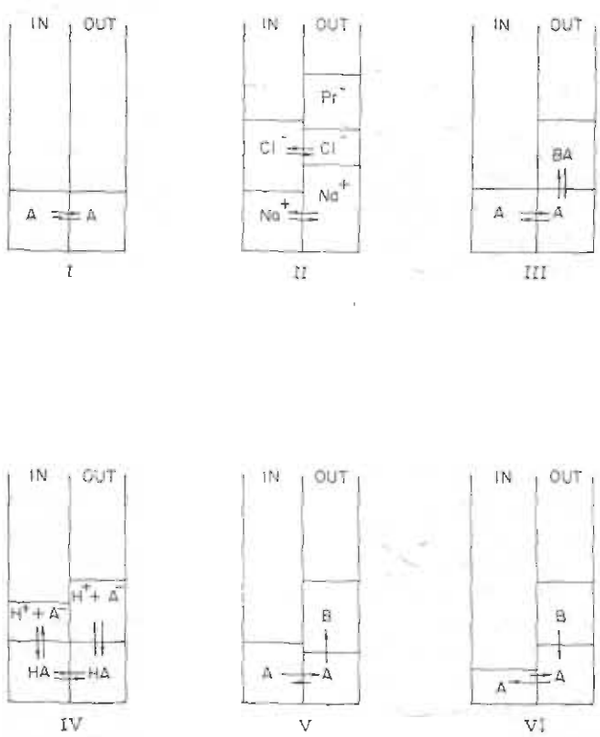

FIG. 8. Distribution of solutes between cell and medium.

II). Only the diffusing form of a solute reaches equilibrium. Thus, an apparent accumulation can occur if the chemical form of the solute is altered by binding to cellular constituents or, in the case of weak electrolytes, if the pH within the cell results in dissociation to a higher degree than outside of the cell (Fig. 8, III and IV).

If, on the other hand, an entering substance is continuously converted into products within the cell, then equilibrium is not attained. The amount of substance within the cell will approach a steady-state level, lower than that in the medium, in which the rate of entry and rate of utilization are

equal (Fig. 8, IV). With glucose the level in the cell is usually low because the rate of its metabolism is potentially faster than its entry. If the metabolic conversion is inhibited by iodoacetic acid, however, equilibrium is attained as in Fig. 8 (I). The reverse situation obtains for substances that are continuously produced. The level in the cell will be higher than that in the medium.

In the case of the cation-transporting system, the uptake of K^+ is balanced electrically by a stoichiometric secretion of H^+, derived from the metabolism of a substrate such as glucose. The net result is the accumulation of K^+, balanced within the cell by an equivalent amount of an organic acid anion. The accumulation of K^+ may continue until the ratio of concentrations is 1000:1. Finally a steady state is reached in which the influx and outflux are equal, the inward active transport being exactly balanced by the outward leakage.

B. Metabolism as an Energy Source for Active Transport

In the preceding section, the effects of metabolism related to events occurring within the interior of the cell that influenced the electrochemical gradients across the membrane. Metabolism also plays a more direct role within the membrane itself, as a source of energy for "uphill" or active transport. The exact nature of the energy coupling is not understood, and even the kind of metabolism may be different for different transport systems. For example, K^+ transport can be supported equally well by respiration or fermentation of glucose, or by respiration of 2- and 3-carbon substrates. Phosphate uptake, on the other hand, is much more rapid with fermentation of glucose than with respiration of 2- or 3-carbon substances.

Although the respiratory or fermentative pathways may be the ultimate supply of energy for the active transport systems, a direct connection or coupling of the carrier to an enzyme system and substrate is required. The exact chemical nature of the carriers and of the associated metabolic steps are not known, but a number of general models have been proposed. For example, a scheme for the forced exchange of K^+ and H^+ would involve an enzymatic conversion of the carrier from a H^+-specific form (Y) at the outer face of the membrane to a K^+-specific form (X) at the inner face (Fig. 9). The conversion of X to Y at one face and of Y to X at the other, by a metabolic reaction, prevents the back-transport of K^+ and H^+ and results in both ions moving uphill. The nature of the conversion step is not specifically known, but two possibilities have been suggested: that the interactions involve an oxidation-reduction [Conway's (1953) redox pump] and, more recently, an accumulating body of evidence linking ATP and a

specific transport-ATPase, with cation transport. The evidence in the latter case is derived primarily from studies with animal cells (Skou, 1963).

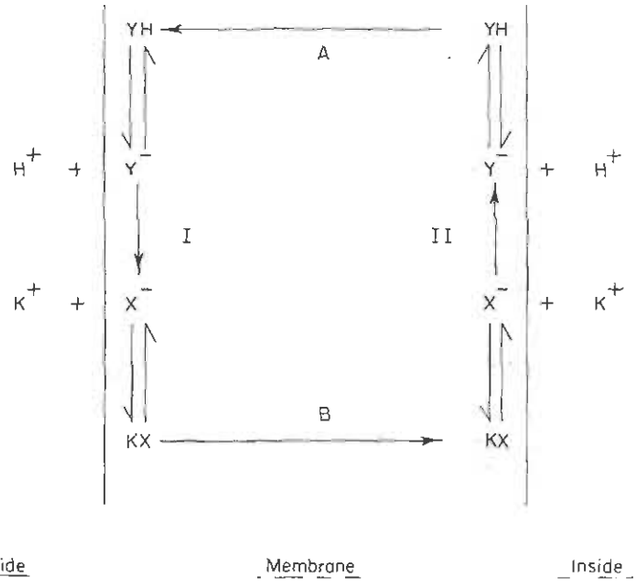

FIG. 9. Diagram of carrier system for K–H forced exchange. X is a K^+-specific form. Reactions I and II are coupled to metabolism, for example ATPase or redox. Reactions A and B represent the translocation through the membrane; the mechanism is unknown at the present time.

C. Location of Enzymes in the Cell Surface

A number of enzymes have been localized on the outer surface of the cell membrane of various kinds of cells (Rothstein, 1954). In yeast cells, they include saccharases and phosphatases. Such enzymes have not, however, been directly implicated in transport. They are hydrolyzing enzymes that convert nonutilizable substances, or nonpenetrating substances, into products that the cell can use. Some of these enzymes may be located in the cell wall rather than in the protoplast membrane. For example, on digestion of the cell wall, much of the yeast invertase is liberated as a soluble enzyme (Islam and Lampen, 1962).

In animal cells, a specific membrane ATPase has been directly implicated in an active transport system. Other transport enzymes will undoubtedly be isolated in the future.

V. TOXIC AGENTS

The cell membrane is the first point of contact of any chemical agent with the cell. As such it can protect the sensitive enzymes within the cell by denying access to the interior of the cell. At the same time, however, it is the first potential target. In fact, membrane systems are often the first, and most sensitive, systems in the cell to respond to toxic agents (Rothstein, 1962).

The simplest case is that of uranyl ion (UO_2^{++}) which cannot penetrate into the cell. It does, however, bind in a reversible manner to two kinds of ligands on the cell surface. The most stable complex is formed with phosphoryl groups and the less stable with carboxyl groups. Associated with the binding to phosphoryl groups, the transport system for glucose is specifically inhibited. The cell can use endogenous carbohydrate, but exogenous sugars can no longer enter the cell. Associated with the binding to carboxyl groups, the enzyme invertase, located in the outer surface of the cell, is inhibited (Fig. 10). The inhibitory effects of UO_2^{++} are completely reversed by the addition of chelating agents or other cations that displace it from the binding sites.

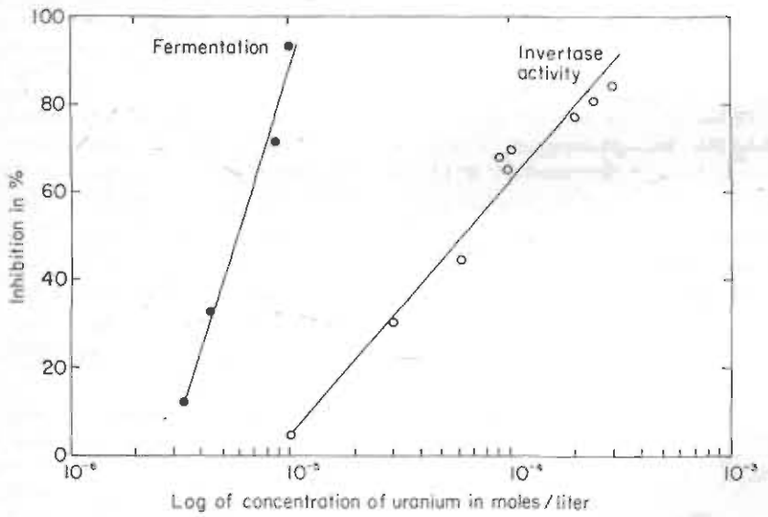

FIG. 10. A comparison of the inhibitory effect of uranium on fermentation of glucose and on invertase activity of living cells. Yeast concentration was 20 mg/ml. The glucose concentration was 0.1 *M* and the sucrose concentration 0.16 *M*. From Demis *et al.* (1954).

Another more complicated case is the action of arsenate. Arsenate is transported into the cell by the same carrier system that transports phosphate, but in the process the carrier system is gradually inactivated. At the same time arsenate that has reached the inside of the cell causes partial inhibition of metabolism (Rothstein, 1963).

A number of agents including mercury, copper, cationic redox dyes, cationic detergents, X-irradiation, and UV irradiation produce a drastic change in the membrane that allows K^+, phosphate, amino acids, and other small molecules to leak out of the cell. The response is all-or-none for individual cells. That is, the membrane of a given cell is relatively normal, or it is very leaky. With increasing dosage of the agents the threshold is exceeded in an increasing number of cells, so the response of the total population is a normal curve against log of dose. In the case of cationic detergents and cationic dyes, binding to phosphoryl groups of the membrane is a prerequisite to the toxic effect. For this reason the cells can be protected by other cations such as Ca^{++}, Mg^{++}, and UO_2^{++}. These cations do not, however, protect against the action of mercury or UV.

Many metabolic agents can inhibit transport systems directly. For example, K transport is inhibited by azide, dinitrophenol, and iodoacetate. Other metabolic inhibitors may have an indirect effect by inhibiting metabolism, thereby shutting off the supply of energy for the transport systems.

VI. CONTROL OF MEMBRANE SYSTEMS

It has already been pointed out that many of the transport systems are inducible by appropriate substrates. Furthermore, the inducible systems are under genetic control and many mutants have been reported. The nutritional state of the cell may also influence the membrane with respect to electrolytes. In *E. coli,* for example, ion transport is markedly different in log-phase and stationary-phase cells (Schultz and Solomon 1961). In yeast, preliminary experiments indicate that smaller changes occur in the transition from log to stationary phase, but that after a period of several hours in the stationary phase, a marked increase in discrimination between Na^+ and K^+ occurs in favor of K^+.

Little work has been done on the genetic control of electrolyte metabolism, but mutants have been selected that are defective in K transport in bacteria (Lubin, 1964).

REFERENCES

Aldous, J. G. (1948). The effect of pH upon the toxicity of iodoacetic acid to yeast cells. *J. Biol. Chem.* 176:83–88.

Aldous, J. G., and K. R. Rozee. (1956). The effect of pH on the toxicity of fluoroacetic acid. *Biochem. J.* **62**:605–610.

Armstrong, W., and A. Rothstein. (1964). The effect of pH on cation transport in yeast. *J. Gen. Physiol.* **48**:61–71.

Barnett, J. A., and H. L. Kornberg. (1960). The utilization by yeasts of acids of the tricarboxylic acid cycle. *J. Gen. Microbiol.* **23**:65–82.

Burger, M., L. Hejmová, and A. Kleinzeller. (1959). Transport of some mono- and di-saccharides into yeast cells. *Biochem. J.* **71**:233–242.

Cirillo, V. P. (1961). Sugar transport in microorganisms. *Ann. Rev. Microbiol.* **15**:197–216.

Cohen, G. N., and J. Monod. (1957). Bacterial permeases. *Bacteriol. Rev.* **21**:169–194.

Conway, E. J. (1953). "The Biochemistry of Gastric Acid Secretion." American Lecture Series. Thomas, Springfield, Illinois.

Conway, E. J., and J. Brein. (1945). An ammonia yeast and some of its properties. *Biochem. J.* **39**:368.

Conway, E. J., and M. Downey. (1949). An outer metabolic region of the yeast cell. *Biochem. J.* **47**:347–355.

Conway, E. J., and F. Duggan. (1958). A cation carrier in the yeast cell wall. *Biochem. J.* **69**:265–274.

Conway, E. J., and P. T. Moore. (1954). A sodium-yeast and some of its properties. *Biochem. J.* **57**:523–528.

Danielli, J. F. (1943). Theory of penetration of a thin membrane. Appendix of "Permeability of Natural Membranes" (H. Davson and J. F. Danielli, eds.), pp. 341–352. Cambridge Univ. Press, London and New York.

de la Fuente, G., and A. Sols. (1962). Transport of sugars in yeasts. II. Mechanisms of utilization of disaccharides and related glycosides. *Biochim. Biophys. Acta* **56**:49–62.

Demis, D. J., A. Rothstein, and R. Meier. (1954). The relationship of the cell surface to metabolism. X. The location and function of invertase in the yeast cell. *Arch. Biochem. Biophys.* **48**:55–62.

Falcone, G., and W. J. Nickerson. (1959). Enzymatic reactions involved in cellular divisions of microorganisms. *Proc. 4th Intern. Congr. Biochem., Vienna, 1958* Vol. 6, pp. 65–70. Pergamon Press, New York.

Finean, J. B. (1961). "Chemical Ultrastructure in Living Tissues." Thomas, Springfield, Illinois.

Foulkes, E. C. (1955). Studies in cell Permeability. The uptake of pyruvate by yeast. *J. Gen. Physiol.* **38**:425–430.

Goodman, J., and A. Rothstein. (1957). The active transport of phosphate into the yeast cell. *J. Gen. Physiol.* **40**:915–923.

Halvorson, H. O., and D. B. Cowie. (1961). Metabolic pools of amino acids and protein synthesis in yeast. *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 479–487. Academic Press, New York.

Harris, G., and C. C. Thompson. (1961). The uptake of nutrients by yeasts. III. The maltose permease of a brewing yeast. *Biochim. Biophys. Acta* **52**:176–183.

Harris, G., and C. C. Thompson. (1962). The uptake of adrenine by brewer's yeast and a respiratory-deficient mutant. *Biochim. Biophys. Acta* **56**:293–302.

Islam, M. F., and J. O. Lampen. (1962). Invertase secretion and sucrose fermentation by *Saccharomyces cerevisiae* protoplasts. *Biochim. Biophys. Acta* **58**:294–302.

Jennings, D., D. Hooper, and A. Rothstein. (1958). The participation of phosphate

in the formation of a "carrier" for the transport of Mg^{++} and Mn^{++} ions into yeast cells. *J. Gen. Physiol.* **41**:1019–1026.

Kotyk, A. (1959). Uptake of inorganic sulphate by the yeast cell. *Folia Microbiol. (Prague)* **4**:363–373.

LeFevre, P. (1961). Sugar transport in the red blood cell: Structure-activity relationships in substrates and antagonists. *Pharmacol. Rev.* **13**:39–68.

Lubin, M. (1964). Cell potassium and the regulation of protein synthesis. *In* "The Cellular Functions of Membrane Transport," Eighteenth Annual Meeting of the Society of General Physiologists. Woods Hole, Massachusetts (J. Hoffman, ed.), Prentice-Hall, Englewood Cliffs, New Jersey. In press.

Oura, E., H. Suomalainen, and R. Collander. (1959). Die permeabilität der Hefezellen für Säuren. *Physiol. Plantarum* **12**:534–544.

Robertson, J. D. (1959). The ultrastructure of cell membranes and their derivatives. *Biochem. Soc. Symp. (Cambridge, Engl.)* **16**:3–43.

Robertson, J. D. (1961). New unit membrane organelle of Schwann Cells. *In* "Biophysics of Physiological and Pharmacological Actions" (A. M. Shanes, ed.), pp. 63–96. Am. Assoc. Advance. Sci., Washington, D.C.

Rothstein, A. (1954). Enzymology of the cell surface. "Protoplasmatologia Handbuch der Protoplasmaforschung," pp. 1–86. Springer, Vienna.

Rothstein, A. (1960. Factors determining the inorganic ion content of yeast cells. *In* "Regulation of the Inorganic Ion of Cells." *Ciba Found. Study Group* **5**:53–68. Churchill, London.

Rothstein, A. (1962). Functional implications of interactions of extracellular ions with ligands of the cell membrane. *Circulation* **26**:1189–1200.

Rothstein, A. (1963). Interactions of arsenate with the phosphate-transporting system of yeast. *J. Gen. Physiol.* **46**:1075–1085.

Rothstein, A. (1964). Membrane function and physiological activity of microorganisms. *In* "The Cellular Functions of Membrane Transport," Eighteenth Annual Meeting of the Society of General Physiologists, Woods Hole, Massachusetts (J. Hoffman, ed.), pp. 23–39. Prentice-Hall, Englewood Cliffs, New Jersey.

Rothstein, A., and M. Bruce. (1958). The potassium efflux and influx in yeast at different potassium concentrations. *J. Cellular Comp. Physiol.* **51**:145–160.

Rothstein, A., and D. Jennings. (1958). The active transport of Mg^{++} and Mn^{++} into the yeast cell. *J. Gen. Physiol.* **41**:585–94.

Rothstein, A. (1955). Relationship of the cell surface to electrolyte metabolism in yeast. *In* "Electrolytes in Biological Systems" (A. M. Shanes, ed.), pp. 65–100. Am. Physiol. Soc., Washington, D.C.

Rothstein, A. (1959). Role of the cell membrane in the metabolism of inorganic electrolytes by microorganisms. *Bacteriol. Rev.* **23**:175–201.

Rothstein, A. (1961). Interrelationships between the ion transporting systems of the yeast cell. *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 270–284. Academic Press, New York.

Roush, A. H., and T. R. Shieh. (1962). Diffusion, active transport and metabolism of purines in the yeast *Torulopsis candida*. *Biochim. Biophys. Acta* **61**:255–264.

Samson, F. E., A. M. Katz, and D. L. Harris. (1955). Effects of acetate and other short-chain fatty acids on yeast metabolism. *Arch. Biochem. Biophys.* **54**:406–423.

Schultz, S. G., and A. K. Solomon. (1961). Cation transport in *Escherichia coli*. I. Intracellular Na and K concentrations and net cation movement. *J. Gen. Physiol.* **45**:355–369.

Simon, E. W. (1953). Mechanisms of dinitrophenol toxicity. *Biol. Rev.* **28**:453–479.

Skou, J. C. (1963). Enzymatic aspects of active linked transport of Na^+ and K^+ across the cell membrane. *In* "Mode of Action of Drugs," First International Pharmacological Meeting (C. Adrian, M. Hogben, and P. Lindgren, eds.). Vol. 4, pp. 41–69. Macmillan, New York.

Solomon, A. K. (1961). Measurement of the equivalent pore radius in cell membranes. *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.). pp. 94–99. Academic Press, New York.

Suomalainen, H., and E. Oura. (1958). Cell permeability and decarboxylation of α-keto acids by intact yeast. *Biochim. Biophys. Acta* **28**:120–127.

Tosteson, D. C., and J. F. Hoffman. (1960). Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* 44:169–194.

Ussing, H. H. (1963). Passage of materials across biological membranes. *In* "Mode of Action of Drugs," First International Pharmacological Meeting (C. Adrian, M. Hogben, and P. Lindgren, eds.), Vol. 4, pp. 15–21. Macmillan, New York.

Vitols, E., R. J. North, and A. W. Linnane. (1961). Studies on oxidative metabolism of *Saccharomyces cerevisiae*. 1. Observations on the fine structure of the yeast cell. *Biophys. Biochem. Cytol.* 9:689–699.

Wiame, J. M. (1949). Occurrence and physiological behavior of two metaphosphate fractions in yeast. *J. Biol. Chem.* **178**:919–929.

Wilbrandt, W., and T. Rosenberg. (1961). The concept of carrier transport and its corollaries in pharmacology. *Pharmacol. Rev.* **13**:109–183.

CHAPTER 16

Uptake and Translocation

2. Translocation

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I. INTRODUCTION

Intracellular transport of substances implies the movement within a hypha from their point of entry to any given point bounded by the cell wall of that hypha. A hypha is considered here as a protoplasmic system bounded or limited from the external environment by a continuous cell wall. This includes the septa within the confines of the vegetative hyphae and the partitions that separate the conidia or other sporulating structures from the vegetative phase. It is assumed that the protoplasm is continuous throughout a given fungal system.

Although the term protoplasmic streaming is most frequently used to indicate the movement of protoplasm within a hypha, other terms such as translocation, transport, intracellular transport, and streaming appear in the literature. These terms will be used more or less interchangeably in this chapter.

Protoplasmic streaming is considered to be the main method of transport of substances from one part of a hypha to another. The direction of streaming is usually from older to younger hyphae, that is, unidirectional. The synthesis of protoplasm is believed to take place in old hyphae and transport is toward the growing front by protoplasmic streaming. Possible causes of streaming are (1) increase in vacuole size effected by osmotic pressure changes and (2) synthesis of protoplasm. In the first instance the pressure of the enlarging vacuole upon the protoplasm forces it to move in the direction of least resistance resulting in the movement. The increase in vacuole size may be due to osmotic changes. Increase in the amount of protoplasm, coupled with the increase in the size of the vacuole, may force

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the protoplasm to move. Septations in hyphae apparently do not stop the flow but do reduce its rate. All the cytoplasm is not in the moving phase; a small portion may be fixed to the cell wall and remain stationary. The movement of materials into spore-bearing bodies is achieved by protoplasmic streaming (Buller, 1933). Buller (1933) has described in great detail the phenomenon of protoplasmic streaming in fungi. Even though his report was based entirely upon microscopic observations there has been relatively little work to support, extend or refute his findings. Quantitative data on the phenomenon of translocation in the fungi per se are very sparse.

II. INTRACELLULAR TRANSPORT OF NONLABELED SUBSTANCES

A. Methods

Schütte (1956) employed a technique of qualitatively determining translocation by using media deficient in the substance whose movement was tested. A petri dish was completely filled with a nutrient agar that contained all the necessary ingredients for growth. This was placed inside a larger container that was filled with nutrient agar, minus the substance to be translocated, so that the level of agar in the larger container reached the top of the petri dish. The system was sterilized and the test organism was inoculated onto the complete medium. Unidirectional translocation was judged according to whether growth occurred, or did not, over the deficient medium. Using this technique, translocation of glucose, sucrose, nitrate, phosphorus, and fluorescein was studied. The test organisms commonly used were: *Aspergillus niger, A. flavus, A. oryzae, Penicillium notatum, Rhizopus oryzae,* and *R. stolonifer.* On the basis of such information all of these fungi except *A. niger* were found to be able to translocate.

Variations of this technique have been employed by other workers (Lucas, 1960; Grossbard and Stranks, 1959) by partitioning and/or cutting out part of the agar. On one side of the partition or cut was placed the substance to be translocated while the other side lacked the substance.

Bidirectional translocation is apparently more difficult to demonstrate than unidirectional. Attempts to show bidirectional flow of substances using media deficient in two components required by the organism has not been too successful. Impaired growth resulting from such experiments probably does not give an indication of bidirectional translocation unless a positive result is obtained. However, since growth is the measure of translocation in each direction, a negative result would not be very revealing because death of the mycelium under deficient conditions would preclude transport.

Fluorescein has been used with some success as a means of circumventing the problem involving deficient media. Simultaneous bidirectional flow using fluorescein as a marker, along with deficient medium, was demonstrated in *R. oryzae* but not in *A. niger* (Schütte, 1956).

B. Hormones

Growth-promoting substances are synthesized in one area of the fruiting body of *Agaricus bisporus* and translocated to another area. Removal of a portion of the lamellae after the fruiting body has matured results in curvature of the stipe. These substances are extractable in aqueous and non-aqueous solvents from the lamellae and have the capacity to diffuse through agar. Agar blocks soaked in the extracted substance and placed unilaterally on the stipe cause the stipe to curve. The substances are apparently produced in the lamellae and translocated through the trama into the stipe (Hagimoto and Konishi, 1960).

It is still not clear whether the hormone moves across the lamellae directly into the stipe, or into the trama before reaching the stipe, or whether both pathways are used. On the other hand, the possibility is not excluded that some of the growth factor may be produced at the apical portion of the stipe itself and has no bearing on the translocation of the substance from the lamellae (Gruen, 1963). It is clear from these and other data (Urayama, 1956; Hagimoto, 1963) that growth-promoting substances are produced in the sporophores of hymenomycetes, notably *A. bisporus,* and that most, if not all, are produced in the lamellae and translocated to the stipe. There was no evidence to suggest that this hormone was translocated intracellularly rather than intercellulary, although Hagimoto and Konishi (1960) demonstrated that the substance could diffuse through a cellophane membrane, a result suggesting a low molecular weight.

C. Transpiration

The movement of water through a transpiration pathway has been studied by Plunkett (1958) and Schütte (1956) in basidiomycetes. Both employed various dyes as a marker of transpirational flow. Schütte concluded that fluorescein and Rose Ediol moved up the interior portion of the stipe and laterally to the edge of the pileus. "Mycelial pressure" may be a controlling factor in upward transport into the fruiting bodies of substances absorbed by mycelium submerged in the substrate. Mycelial pressure refers to the protoplasmic streaming resulting from the vacuolar pressure that is observed in most fungi. This pressure builds up in older cells forcing

substances to move toward paths of least resistance. In attempts to visualize dye ascent in *Polyporus brumalis* there was little evidence indicating that it was localized inside the cell, as ascertained by the light microscope. These findings suggest that the ascent of materials through the stipe can take place intercellularly, as well as intracellularly. That the upward flow of materials is not due only to physical forces such as transpiration was ascertained in growth experiments with *P. brumalis*. Dye was translocated in some specimens even though stipe elongation and pileus development was impeded or stopped for unknown reasons, suggesting that factors affecting translocation of materials, other than transpiration, are in force in these fungi (Plunkett, 1958). The "vital phenomenon" suggested by Plunkett to explain these facts may merely be metabolic energy which is required for many cellular processes (Plunkett, 1958). Transpiration, together with mycelial pressure and vacuolar enlargement, may account for the movement of substances into the spore-bearing structures of most fungi (Buller, 1933; Plunkett, 1958). That substances other than water move into the fruiting body of a fungus from underground mycelium is suggested by the data of Bonner *et al.* (1956).

III. INTRACELLULAR TRANSPORT OF RADIOACTIVELY LABELED SUBSTANCES

Labeled compounds may be incorporated into the medium upon which the fungus is grown, and the mycelium grown over a support of cellophane or nylon. After various time intervals the mycelium is transferred to "cold" medium and autoradiographed or extracted. In this manner a colony that has been incubated on labeled and nonlabeled media can be roughly separated (Zalokar, 1959; Yanagita and Kogané, 1963b; Lucas, 1960; Grossbard and Stranks, 1959). The latter workers incubated *Phytophthora cactorum, Corticium solani, Phycomyces blakesleeanus,* and *Rhizopus stolonifer* in Cs^{137} and Co^{60} in liquid medium and transferred the organisms to soil cultures in an attempt to measure the translocation of labeled compounds into newly formed mycelium. Lucas (1960) used a modification of Schütte's deficient medium technique by removing agar plugs and filling the excavation with $KH_2P^{32}O_4$; the organism was inoculated either before or after addition of the labeled compound. In this instance a trough of agar was removed so that the organism had to bridge this gap in order to overgrow the container. One of the major disadvantages of these techniques is the excessive dilution of the label, a problem that has not been completely overcome.

C^{14}-Proline was not translocated in any significant amounts farther than

2 mm from the tip of the hypha while the quantity of C^{14}-uridine was greatest at this point in *Neurospora.* However, these data do not distinguish between translocation and uptake. On the other hand, it has been suggested that translocation might be a factor in finding C^{14}-uridine concentrated 2 mm away from the tip of the hypha than at points closer to the tip. If indeed this is the case, then a bidirectional translocation system exists in *Neurospora* (Zalokar, 1959). Thrower and Thrower (1961) used Shütte's dish technique to demonstrate that of the 20 species representing all classes of fungi, 10 were translocating fungi as defined by Shütte, 6 were nontranslocating fungi, and 4 were indeterminate. All translocating fungi were capable of moving compounds labeled with C^{14} when the mycelium grew onto either deficient or complete medium. However, the nontranslocating species were also able to transport labeled compounds when the mycelium grew onto complete medium. These findings are at variance with Shütte's hypothesis that growth on a deficient medium was evidence for translocation in fungi.

Autoradiographs from *Phycomyces blakesleeanus* showed that Cs^{137} and Co^{60} were concentrated in the sporangia after the mycelia had been incubated with labeled substances (Grossbard and Stranks, 1959). Autoradiography indicated that all the labeled Co^{60} was expelled from a hypha after rupture and no labeled Co^{60} was shown to be attached to, or incorporated into, the cell wall (Grossbard, 1958).

On the basis of autoradiography labeled P^{32} is apparently translocated upward into the fruiting bodies of *A. niger,* while a lesser amount is translocated laterally. Incorporation of labeled P^{32} is greatest in the rapidly growing areas of the colony. Translocation of S^{35}-sulfate in *A. niger* could not be demonstrated (Yanagita and Kogané, 1963a,b). The data of Lucas (1960) indicate that mycelium of *P. nitens, Absidia glauca,* and *Chaetomium* spp. are able to translocate P^{32} under certain limited conditions. He states: "Distinction needs to be made between translocation through established mycelium independently of growth as in the case of transpiration and dye movement in agarics and translocation by the uptake at one site and its transference in the mycelium to another site due to hyphal extension. . . ." Herein lies an important distinction in interpreting translocation in fungi.

IV. CONCLUSIONS

Although the literature on intracellular transport is sparse it is clear that substances are translocated from one part of the mycelium to another. Translocation is toward the growing front and/or into spore-producing

structures. Translocation is usually unidirectional—from old hyphae to growing or sporulating areas—but bidirectional flow has been reported. In the case of translocation in basidiomycetes it appears that several systems may be involved in transporting water and other materials from the underground mycelium upward into the fruiting body. Growth-promoting substances are apparently synthesized in the fruiting body (lamellae) and translocated to the stipe, causing a curvature of the latter.

A neglected area of investigation is in the mechanism of upward movement not only of water but of organic and inorganic substances into the macroscopic fruiting bodies of the higher fungi. Since these structures lack a vascular system such as is present in the higher plants, the mechanisms of upward transport are much less obvious. Furthermore, it is not at all certain whether upward transport is inter- or intracellular. In any event, knowledge of this process may provide insights into the mechanism of translocation in higher plants as well.

REFERENCES

Bonner, J. T., K. K. Kane, and R. H. Levey. (1956). Studies on the mechanics of growth in the common mushroom, *Agaricus campestris*. *Mycologia* **48**:13–19.

Buller, A. H. R. (1933). "Researches on Fungi," Vol. 5, 416 pp. Longmans, Green, New York.

Grossbard, E. (1958). Autoradiography of fungi through a layer of soil and in agar culture. *Nature* **182**:854–856.

Grossbard, E., and D. R. Stranks. (1959). Translocation of cobalt-60 and caesium-137 by fungi in agar and soil cultures. *Nature* **184**:310–314.

Gruen, H. E. (1963). Endogenous growth regulation in carphophores of *Agaricus bisporus*. *Plant Physiology* **38**:652–666.

Hagimoto, H. (1963). The mushroom growth hormone and the geotropic response of fruit body in *Agaricus bisporus*. *Botan. Mag. (Tokyo)* **76**:363–365.

Hagimoto, H., and M. Konishi. (1960). Studies on the growth of fruit body of fungi. II. Activity and stability of the growth hormone in the fruit body of *Agaricus bisporus* (Lange) Sing. *Botan. Mag. (Tokyo)* **73**:283–287.

Lucas, R. L. (1960). Transport of phosphorus by fungal mycelium. *Nature* **188**:763–764.

Plunkett, B. E. (1958). Translocation and pileus formation in *Polyporus brumalis*. *Ann. Botany (London)* [N.S.] **22**:237–250.

Schütte, K. H. (1956). Translocation in the fungi. *New Phytologist* **55**:164–182.

Thrower, S. L., and L. B. Thrower. (1961). Transport of carbon in fungal mycelium. *Nature* **190**:823–824.

Urayama, T. (1956). Das Wuchshormon des Fruchtkörpers von *Agaricus bisporus* L. (Vorläufige Mitteilung). *Botan. Mag. (Tokyo)* **69**:298–299.

Yanagita, T., and F. Kogané. (1963a). Site of biosyntheses of sulfur compounds in growing colonies of *Aspergillus niger*. *J. Gen. Appl. Microbiol. (Tokyo)* **9**:331–336.

Yanagita, T., and F. Kogané. (1963b). Cellular differentiation of growing and mold

colonies with special reference to phosphorus metabolism. *J. Gen. Appl. Microbiol. (Tokyo)* 9:313–330.

Zalokar, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* 46:602–610.

CHAPTER 17

The Chemical Environment for Fungal Growth

1. Media, Macro- and Micronutrients

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I. INTRODUCTION

Fungi are dependent upon the medium, or substrate, for all the elements and compounds they require or utilize except molecular oxygen and, possibly, a little carbon dioxide, which are obtained from the atmosphere. From these elements and compounds they synthesize their cellular constituents and obtain the energy necessary for their life processes. Some compounds, such as vitamins and some amino acids, enter the cells without modification. High molecular weight compounds, including cellulose, starch, and proteins, are hydrolyzed before their constituents can be utilized.

The minimal composition of a medium, or substrate, must include all the elements that are essential. In addition, the essential elements must be present in compounds that can be utilized. Since the nutritional requirements of fungi differ, it follows that no one medium, or substrate, will be suitable for all fungi. On a given medium, one fungus may thrive and another starve. No universal medium has yet been devised.

The genetic constitution of a fungus determines what it can do, but the expression of its potentialities is dependent upon the composition of the medium on which it grows and the environment to which it is exposed. The choice of the media and the environmental conditions depend on the purpose of the investigator, and on the fungus. Generalizations should not be based on one medium and one set of environmental conditions.

The common practice of designating media by name, e.g., Czapek's medium, is unfortunate and should be avoided. Lilly and Barnett (1951),

among others, recommend that media be designated by naming the carbon and nitrogen sources used. This has the virtue of focusing attention on two salient features of every synthetic medium. Thus, sucrose-nitrate medium (Steinberg, 1941), glucose-nitrate medium (Nicholas, 1952), glucose-ammonium sulfate-potassium acetate medium (Lilly *et al.*, 1960) are suitable ways of citing media. The practice of using personal names for media without a citation to the literature is inexcusable. It is as essential to know the composition of the medium used as it is to know the species of fungus employed.

II. CLASSIFICATION OF MEDIA

Media may be classified as natural, semisynthetic, or synthetic. In terms of composition, these correspond roughly to unknown, partially known, and known. In addition, media may be classified as liquid or semisolid (agar, gelatin, silica gel).

A. Natural Media

Media of this class are composed of natural products of plant and animal origin. The exact composition of such media is unknown. A wide selection of natural products have been used as substrates for culturing fungi. Potato plugs, carrot slices, twigs, stems, roots, and leaves of various plants are used. Partially processed natural products, such as malt and yeast extracts, peptone, and casein hydrolyzate are useful and widely used. It is not surprising that such media are suitable for the cultivation of many species, since these and similar materials are the substrates in, or on, which fungi grow in nature.

Natural media differ in value, and thus certain of them are especially useful for the study of some species. Certain parasitic fungi are host specific. The identification of the metabolites in such natural media which are required by fastidious parasitic fungi has progressed far enough in some instances to make it probable that specific metabolites are involved. Thus, natural media made from legume seed are especially useful for the production of oospores by some species of *Phytophthora,* and the active principle is found in the nonsaponifiable fraction of the lipids (Leonian and Lilly, 1937).* *Calcarisporium parasiticum* is found in nature growing on a few species of *Physalospora;* it was shown by Barnett and Lilly (1958) that *C. parasiticum* was capable of axenic growth when extracts of its natural hosts (and additional species not parasitized) were added

* Recently, Haskins *et al.* (1964) showed a *Pythium* species to require β-sitosterol, or a related sterol, for sexual reproduction. Similar sterol requirements were demonstrated for sexual reproduction in a number of species of *Phytophthora* (Elliott *et al.*, 1964; Leal *et al.*, 1964).

to a glucose-yeast extract medium. *Pilobolus* species require chelated iron compounds found in dung or other sources (Page, 1962).

Natural media are complex in composition. It follows that fungi find at hand many metabolites that are suitable for use without modification. Roberts *et al.* (1957) in studying amino acid transformation in *Torulopsis* [*Candida*] *utilis* and *Neurospora crassa* found that certain amino acids served as parents of a family of amino acids. It is plausible to assume that, in a natural medium where these parent amino acids, and others, are present, the biosynthetic work involved in the synthesis of proteins would be greatly reduced. These ideas receive support from the observation that a mixture of amino acids is frequently a better nitrogen source than a single compound. Mechanisms involved in the control of synthetic pathways are discussed on pp. 408–417.

Natural media have a number of serious disadvantages: (1) The composition of natural media is unknown, thereby excluding their use in exact nutritional studies. (2) The composition of a natural medium composed of plant parts is fixed for any given sample of material. (3) While the concentration of the constituents of a natural medium composed of extracts may be varied by dilution, for any given extract the ratios among the constituents are fixed unless two or more extracts are used. (4) Within limits, the reproducibility of the composition of a natural medium is poor because of variability in natural materials. Thus, Grant and Pramer (1962) analyzed the mineral composition of five lots of yeast extract produced by the same company. They found that the molybdenum content varied between 2.6 and 9.1 μg/gm, and that of zinc between 1.0 and 6.1 μg/gm.

B. Semisynthetic Media

Media in this category are composed in part of natural products and in part of chemical compounds of known composition. Thus, considerable freedom exists in controlling the constituents and their concentrations. Such media are useful and widely used. Potato-glucose-agar medium (PDA), which is much used by plant pathologists is an example. The investigator may vary the concentration of glucose between zero and the upper limit of solubility. In our experience, malt extract should be fortified with thiamine for the cultivation of thiamine-requiring fungi. Since the composition of semisynthetic media is unknown, they too are unsatisfactory for exact nutritional investigations. Any medium that contains agar is at best a semisynthetic medium. Purdy and Grogan (1954) made a direct comparison between the growth of *Sclerotinia sclerotiorum* in liquid medium and on the same medium solidified with agar and concluded that the agar medium was unsatisfactory for studying the mineral element

nutrition of this species. Agar contains various nutrients; Miller (1956) reported a sample of 2% water agar to contain the following (in ppm): potassium, 0; calcium, 120; magnesium, 24; phosphorus, 8; sodium, 20; manganese, 0; iron, 2; aluminum, 3.8; copper, 0.08; and boron, 2.6. Agar contains organic nutrients, as the growth of fungi on water agar shows.

C. Synthetic Media

The ideal synthetic medium is one in which every constituent and its concentration is known. This ideal situation is never attained in practice, but may be approached by the use of suitable methods and techniques. The uncertainties in composition are most serious when the quantity of essential metabolite required is small, e.g., metallic ions and growth factors. The requirement for a specific metabolite is detected by its absence. Additional factors affecting the composition of media will be considered in the next section.

Synthetic media are essential for studying the nutritional requirements of fungi. Within limits, the investigator has a wide choice of constituents and concentrations. A synthetic medium may satisfy the minimal nutritional requirements of a fungus, or it may have a more elaborate composition. The common use of one salt to furnish two essential elements introduces a fixed ratio between these two elements. By using salts which furnish one essential element, increased flexibility in composition is obtained. The price for this increased flexibility is the introduction of still other elements into the medium.

D. Liquid and Agar Media

Liquid media are essential for most nutritional investigations. Agar media are useful for many purposes, especially when it is desirable to make microscopic examinations of the same culture over a period of time. While the rate of growth of a colony growing on an agar medium is easily determined, frequently the measurements have little value (N. Fries, 1943).

Raulin introduced in 1863 the use of synthetic media for the study of nutrition of fungi. For an evaluation of the significance of Raulin's work, see Schopfer (1949).

III. GENERAL CONSIDERATIONS

Some of the possible changes in composition of media will be noted in this section as well as a number of topics that deserve serious consideration when planning or reporting physiological studies.

A. On the Identity of the Compounds Used

If the identity of the compounds used to prepare a medium is not stated, it is impossible for the reader to ascertain the composition of the medium. Either specific names, or formulas, or both, should be presented. D-, DL-, and L-arabinose are not equivalent in the nutrition of fungi (Lilly and Barnett, 1956).

B. On Units of Concentration

For many purposes, concentration is most simply expressed in terms of weight per unit volume, e.g., grams per liter, mg/25 ml, μg/ml, etc. Per cent is ambiguous unless the basis is given, e.g., w/w, w/v, v/v. Moles must be used when it is necessary to compare the activity of the same number of molecules or ions. In comparing the value of different carbon and nitrogen sources, it is essential that this comparison be made on the basis of carbon or nitrogen content.

C. Aeration

Oxygen, the one essential element not added to media, is obtained from the atmosphere. Since the solubility of oxygen in water is low and the rate of diffusion slow, the area: volume ratio is important in determining rate and amount of growth in still cultures. The deeper the medium, the longer it takes to establish surface growth. Norkrans (1953) developed the useful technique of pregrowing inocula from agar blocks by placing them on plates of fresh media for 1 day. They could then be lifted off and floated on the surface of liquid media. Early growth was more rapid, and uniformity among cultures was improved. Ranzoni (1951) found *Anguillospora longissima* and *A. gigantea* to produce more mycelium in the same volume of medium in 250-ml than in 125-ml Erlenmeyer flasks in still culture. The content of dissolved oxygen may be increased by either agitation (shake-culture) or by blowing sterile air into the medium. Both techniques are in wide use.

D. Hydrogen-Ion Concentration

Most fungi grow within the pH range 4–8. Many fungi will grow over a wider range, and a few have been reported to have a narrower range (Lilly and Barnett, 1951; Hawker, 1950; Tandon, 1961). For a list of optimum pH values for various fungi, see Cochrane (1958).

The internal pH of fungus cells has seldom been measured. Conway and Downey (1950) in a careful study of yeast cells found the internal pH to vary within rather narrow limits. They reported the internal pH of resting yeast cells to be 5.81; during fermentation of glucose, the internal pH rose to between 6.12 and 6.55. The external pH fell during fermentation, during which the excretion of hydrogen ions was balanced by an uptake of cations, principally potassium.

During incubation, the external pH of the medium may traverse a pH range of 4 or more units depending on the composition of the medium and the fungus. In general, the change in pH of the medium results from two types of metabolic activity: (1) the production of organic acids, or the release of ammonia; and (2) the unequal utilization or excretion of cations and anions. Gladtke and Bruckner (1958) cultured *Phycomyces blakesleeanus* in a glucose-glycine medium and found the pH to decrease from an initial value of 5.0 to 3.2 after 10 days' incubation. Trace amounts of organic acids were produced, which accounted for a minor part of the observed decrease in pH. Since more cations (33.2×10^{-4} equiv/50 ml) than anions were utilized, the major part of the decrease in pH was attributed to their disappearance from the medium.

Buffers are commonly used in determining the pH range of fungi and to maintain culture media at a suitable pH range. Excessive concentrations of buffer salts may restrict the growth (L. Fries, 1956a) of some fungi. For those media which become acid, calcium carbonate may be used as a neutralizing agent. Suitable internal indicators, or glass electrode assemblies, coupled with the periodic addition of sterile acid and base are used. In order to avoid great changes in composition and pH values, the technique of using flowing media may be used (L. Fries, 1956a).

Changing the pH of a medium may also change its composition if it contains weakly ionized constituents. The physiological effects of dissociated and undissociated species are quite different. *Chaetomium globosum* and *Fusarium lycopersici* were shown to be unaffected by a wide range of concentrations of acetate ion, but failed to grow if the concentrations of the undissociated molecule exceeded about 6 and 12 mg/25 ml, respectively (Lilly and Barnett, 1961). *Chaetomium globosum*, in a potassium acetate-ammonium sulfate medium, had a pH minimum which ranged from 3.6 to 6.8, depending on the content of potassium acetate in the medium.

Precipitates may form in neutral and alkaline media, especially when they are autoclaved. The conversion of certain constituents into insoluble compounds alters the composition of the medium. L. Fries (1956b) analyzed the precipitates that formed on autoclaving a glucose-asparagine medium at pH values of 6.9, 7.4, and 8.0. Iron, aluminum, calcium, barium,

and magnesium were found in the precipitate formed at pH 6.9, and zinc and manganese were found in the precipitates formed at pH 7.4 and 8.0. More to the point, the supernatant medium was analyzed for iron and zinc. A trace of iron was found in the medium autoclaved at pH 6.9, but none was found in the supernatant medium at pH 7.4 and 8.0. Traces of zinc were found at each pH, and the lowest concentration was present in the medium autoclaved at pH 7.4. *Coprinus ephemeris* grew poorly in the medium autoclaved at pH 7.4.

In order to culture fungi in media having pH values of 7 or higher, chelating agents should be present. L. Fries (1956b) found the hydroxy-organic acids (citric, tartaric, and lactic) to be especially useful. Ammonium tartrate was reported to dissolve calcium and zinc phosphates. Some of the precipitation in neutral or alkaline media may be avoided by autoclaving the phosphate and other salts separately, or media may be sterilized by filtration. Synthetic chelating agents, especially ethylenediaminetetraacetic acid (EDTA), have been used to prevent metal ion precipitation. Reischer (1951) used EDTA (0.5 gm/liter) to study the microelement nutrition of *Achlya klebsiana*. Fries (1956b) investigated the use of EDTA (0.2 gm liter) as a chelating agent in a study of *Coprinus* and obtained no growth at pH values lower than 6.0; in some instances severe inhibition occurred at pH 7. She suggested that undissociated EDTA was toxic. These results suggest that EDTA be used with caution.

E. Methods of Sterilization

Four methods of sterilization, each having certain advantages and disadvantages, may be used: (1) Autoclaving is rapid and efficient, but chemical changes may occur; e.g., certain oligo- and polysaccharides may be partially hydrolyzed under slightly acid conditions. Bretzloff (1954) reported better growth of *Sordaria fimicola* on autoclaved medium containing sucrose than on the same medium sterilized by filtration. Decomposition products of sugars produced by autoclaving stimulate the growth of some fungi and inhibit the growth of others. Machlis (1953) reported that *Allomyces javanicus* var. *macrogynus* failed to grow within 12 days when glucose was autoclaved with the medium. (2) Steaming on 3 successive days. This method is not as destructive as autoclaving. (3) Filtration is a nondestructive method of sterilization. Various filters including Millipore and sintered glass filters may be used. (4) Gaseous sterilizing agents such as ethylene and propylene oxides are used. Judge and Pelczar (1955) reported ethylene oxide to be equivalent to filtration in the sterilization of sugars.

F. Balanced and Unbalanced Media

If the ratios among the constituents of a medium are such that the constituents are exhausted at the same time, such a medium may be considered to be balanced. Steinberg (1939) developed a medium for *Aspergillus niger* so well balanced that a decrease in the concentration of any constituent resulted in diminished growth. Within limits, less exquisitely balanced media, on dilution, should produce proportionally less mycelium. *Chaetomium convolutum* in full-strength, one-fourth- and one-sixteenth-strength glucose-casein hydrolyzate medium produced 194, 68, and 16 mg dry mycelium per 25 ml, respectively (Lilly and Barnett, 1949).

More commonly, a single constituent is varied, as in the assay of vitamins and growth factors and microelements, and in the production of metabolic products of interest. Nicholas (1952) published curves relating the concentrations of iron, zinc, copper, molybdenum, and magnesium to the production of mycelium by *A. niger*. For the effect of vitamin concentrations on growth and sporulation, see Chapter 12c.

The concentration of a metabolite which is optimal for growth may not be optimal for sporulation or for the production or accumulation of a metabolic product. Kalyanasundaram and Saraswathi-Devi (1955) found for zinc 100 μg/25 ml to be optimal for the production of mycelium by *Fusarium vasinfectum*, but 6 μg/25 ml was optimum for the production of an antibiotic, and increasing the zinc concentration to 10 μg/25 ml resulted in a ninefold decrease in antibiotic production. *Phycomyces blakesleeanus* produced about 25 mg dry mycelium per 25 ml on a glucose-ammonium sulfate medium; upon the addition of potassium acetate, optimum growth (105 mg/25 ml) was obtained at 0.02 *M;* however, optimum production of carotene was obtained when the acetate concentration was between 0.0075 and 0.01 *M* (Lilly *et al.*, 1960). Many additional examples could be cited. The optimum concentration means little unless it is related to a physiological or morphological marker.

IV. THE CONSTITUENTS

A medium must contain the essential elements in forms that can be utilized. Riker and Riker (1936) give the formulas for a number of commonly used media. It is beyond the scope of this section to discuss concentrations since these vary widely depending on the fungus and the purpose of the investigator.

A. Water

As a basic requirement, the water used should not contain toxic materials. Some well and tap waters are suitable for preparing natural media. For exacting work, deionized distilled water, or distilled water from glass or quartz stills may be required. Water from metal stills may be very impure (Nicholas, 1952).

B. Oxygen

Truesdale *et al.* (1955) measured the solubility of oxygen from a wet atmosphere in water at 0–35° C. The solubility from 0 to 35 °C. decreased from 14.16 to 7.04 mg/liter in a nonlinear fashion. The values at 20, 25, and 30°C were 8.84, 8.11, and 7.53 mg/liter. The solubility of oxygen in media, which are solutions, would be less. A 25-ml aliquot of medium at 25°C., at saturation, would contain about 0.2 mg of oxygen, which on inoculation would be expected to be rapidly utilized. The rate of diffusion of oxygen into media is slow and is dependent on factors other than the intrinsic rate of diffusion. Rahn and Richardson (1941) measured the rate of diffusion of oxygen into a glucose-peptone medium and found that diffusion into alkaline media is much slower than into acidic media, presumably because glucose and peptone were more rapidly oxidized under alkaline conditions.

C. Carbon Sources

This subject is discussed in detail on Chapter 18. In general, 2–3 gm glucose is required to produce 1 gm dry mycelium. The more dilute the medium, the more efficiently the carbon source is converted into mycelium. The economic coefficient usually decreases as the culture ages. In commercial fermentations where the substrate is converted into a product, a high concentration of carbon source is used and the economic coefficient is very low.

D. Nitrogen Sources

Nicholas reviews nitrogen metabolism in Chapter 13. Robbins (1937) classified organisms upon the basis of types of nitrogen sources utilized, as shown in the accompanying tabulation. Organisms in class 1 utilize all four types of nitrogen sources. It has been questioned (Cochrane, 1958) whether any fungus belongs in class 1.

Class	N_2	NO_3^-	NH_4^+	Organic
1	+	+	+	+
2	−	+	+	+
3	−	−	+	+
4	−	−	−	+

Class 2 fungi that utilize nitrate N commonly utilize ammonium N and organic N. A few fungi have been reported to utilize nitrate N, but not ammonium N. *Cryptococcus nigricans* is reported to be such a fungus (Rich and Stern, 1958).

Class 3 fungi utilize ammonium N and organic N, but not nitrate N. This class includes most of the fungi that have been studied. When inorganic ammonium salts are used as nitrogen sources, the pH of the culture medium usually falls, in many instances to inhibitory levels. This difficulty may be avoided, or minimized, by using ammonium salts of organic acids, e.g., diammonium tartrate, or by adding salts of fumaric acid, or other 4-carbon dicarboxylic acids to a medium containing inorganic ammonium salts (Leonian and Lilly, 1940). The available evidence indicates that ammonium N is used in preference to nitrate N (Converse, 1953; Morton and MacMillan, 1954).

All fungi appear to utilize one or more sources of organic nitrogen. Many of the species thought to require organic N when Robbins proposed his classification in 1937 have since been shown to require either vitamins or specific amino acids. It appears doubtful whether there are many fungi which require organic N *sensu* Robbins. However, Goldstein (1963) reported two marine phycomycetes, *Thraustochytrium motivum* and *T. multirudimentale,* as being unable to utilize nitrate N or ammonium N. These fungi utilized glutamate, L-aspartic acid, and L-asparagine. Mixtures of amino acids, such as casein hydrolyzate, or artificial mixtures of amino acids ordinarily are good sources of nitrogen.

Potassium, sodium, and calcium nitrates have been used as sources of nitrate N. The use of ammonium N was discussed above. Sources of ammonium N include ammonium chloride, ammonium sulfate, and ammonium phosphates (diammonium hydrogen phosphate loses ammonia on exposure to air). Diammonium tartrate is a commonly used source of ammonium N. Of the organic nitrogen sources, peptone and casein hydrolyzate are suitable complex sources. As single sources of organic nitrogen, asparagine is commonly used, as are other amino acids.

Synthetic amino acids (except glycine) are mixtures of D- and L-forms. While both forms are known to occur in nature, the L-forms are the more common, and would be expected to be utilized more readily. Newton

(1956) reported that L-alanine was utilized by *Phytophthora parasitica* whereas D-alanine was not. *Verticillium alboatrum* utilized D-alanine slowly and incompletely whereas L-alanine was a good source of nitrogen.

E. Sulfur

Volkonsky (1933) designated those fungi that are able to utilize sulfate sulfur as *euthiotrophic*, and those that required reduced sulfur as *parathiotrophic*. Most species utilize sulfate sulfur. The parathiotrophic fungi may be divided into two groups including (1) those that require a sulfur-containing amino acid such as methionine or cysteine, and (2) those that utilize inorganic reduced sulfur compounds as well as reduced sulfur-containing organic compounds. Crasemann (1957) reported that *Blastocladia pringsheimii* requires methionine whereas *B. ramosa* utilized methionine, cystine, and cysteine. A trace of growth occurred on thioacetamide, and sulfate sulfur was not utilized. *Aphanomyces euteiches*, studied by Davey and Papavizas (1962) would be classified as a parathiotrophic fungus, class 2. Two isolates were used and neither utilized sulfate sulfur or sulfite sulfur. Thiosulfate (one sulfur atom has a valence of −2), elemental sulfur, sulfide, thioglycolic acid, DL-cystine, and DL-methionine were utilized. The sulfur requirements for oospore formation were more restricted than those for growth.

F. Phosphorus

Any soluble nontoxic phosphate may be used as a source of phosphorus. The most commonly used forms are potassium dihydrogen phosphate or dipotassium hydrogen phosphate, or both. Dox (1911–1912) found that the phosphorus in sodium phosphite and sodium hypophosphite was not used by *Aspergillus niger*. The two potassium phosphates also serve as sources of potassium. The necessity of adjusting the pH of media may be avoided by using mixtures of these two phosphates.

G. Potassium

This element is frequently supplied as a phosphate. Other sources include potassium chloride, potassium sulfate, and potassium nitrate.

H. Magnesium

Various soluble nontoxic magnesium salts are used. The most common probably is magnesium sulfate heptahydrate. Magnesium chloride hexahydrate and nitrate hexahydrate are deliquescent.

I. Microelements

For routine use, it is convenient to prepare a microelement solution of such strength that 1 ml/liter will supply the concentration desired. The following salts have been used with satisfaction in this laboratory: iron, $Fe(NO_3)_3 \cdot 9H_2O$; zinc, $ZnSO_4 \cdot 7H_2O$; manganese, $MnSO_4 \cdot H_2O$; copper, $CuSO_4 \cdot 5H_2O$; molybdenum, $Na_2MoO_4 \cdot 2H_2O$; calcium, $CaCl_2 \cdot 6H_2O$. The stock solution should be acidified to prevent precipitation of iron.

J. Effects of Impurities

The chemicals used in making media contain traces of essential and nonessential elements. Methods of removing metallic impurities are noted in Chapter 8 (Section II, B). For less exacting work, chemically pure or reagent grade chemicals are satisfactory. In general, it is necessary to add microelements to media made with such chemicals. The concentrations needed will depend on the fungus and the purpose of the investigator. For the value of such techniques, see Omvik (1951).

REFERENCES

Barnett, H. L., and V. G. Lilly. (1958). Parasitism of *Calcarisporium parasiticum* on species of *Physalospora* and related fungi. *West Va., Univ. Agr. Expt. Sta., Bull.* **420T:** 37 pp.

Bretzloff, C. W., Jr. (1954). The growth and fruiting of *Sordaria fimicola*. *Am. J. Botany* **41**:58–67.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Converse, R. H. (1953). The influence of nitrogenous compounds on the growth of *Helminthosporium gramineum* in culture. *Mycologia* **45**:335–344.

Conway, E. J., and M. Downey. (1950). pH Values of the yeast cell. *Biochem. J.* **47**:355–360.

Crasemann, J. M. (1957). Comparative nutrition of two species of *Blastocladia*. *Am. J. Botany* **44**:218–224.

Davey, C. B., and G. C. Papavizas. (1962). Growth and sexual reproduction of *Aphanomyces euteiches* as affected by the oxidation state of sulfur. *Am. J. Botany* **49**:400–404.

Dox, A. W. (1911–1912). The phosphorus assimilation of *Aspergillus niger*. *J. Biol. Chem.* **10**:77–80.

Elliott, C. G., M. E. Hendrie, B. A. Knights, and W. Parker. 1964. A steroid growth factor requirement in a fungus. *Nature* **203**:427–428.

Fries, L. (1956a). Studies in the physiology of *Coprinus*. 3. Cultivation experiments with running media. *Botan. Notiser* **109**:12–20.

Fries, L. (1956b). Studies in the physiology of *Coprinus*. 2. Influence of pH, metal factors, and temperature. *Svensk Botan. Tidskr.* **50**:48–96.

Fries, N. (1943). Die Einwirkung von Adermin, Aneurin, und Biotin auf das Wachstum einiger Ascomycetes. *Symbolae Botan Upsalienses* **7**(2):1–73.

Gladtke, E., and W. Bruckner. (1958). Über die Ansäuerung der Nährflüssigkeit durch *Phycomyces blakesleeanus*. *Arch. Mikrobiol.* **29**:53–64.

Goldstein, S. (1963). Development and nutrition of new species of *Thraustochytrium*. *Am. J. Botany* **50**:271–279.

Grant, C. L., and D. Pramer. (1962). Minor element composition of yeast extract. *J. Bacteriol.* **84**:869–870.

Haskins, R. H., A. P. Tulloch, and R. G. Micetich. (1964). Steroids and stimulation of sexual reproduction of a species of *Pythium*. *Can. J. Microbiol.* **10**:187–195.

Hawker, L. E. (1950). "Physiology of Fungi," 360 pp. Univ. of London Press, London.

Judge, L. F., Jr., and M. J. Pelczar, Jr. (1955). The sterilization of carbohydrates with liquid ethylene oxide for microbiological fermentation tests. *Appl. Microbiol.* **3**:292–295.

Kalyanasundaram, R., and L. Saraswathi-Devi. (1955). Zinc in the metabolism of *Fusarium vasinfectum* Atk. *Nature* **175**:945.

Leal, J. A., J. Friend, and P. Holliday. (1964). A factor controlling sexual reproduction in *Phytophthora*. *Nature* **203**:545–546.

Leonian, L. H., and V. G. Lilly. (1937). Partial purification of a vitamin-like substance which stimulates sexual reproduction in certain fungi. *Am. J. Botany* **24**:700–702.

Leonian, L. H., and V. G. Lilly. (1940). Studies on the nutrition of fungi. 4. Factors influencing the growth of some thiamin-requiring fungi. *Am. J. Botany* **27**:18–26.

Lilly, V. G., and H. L. Barnett. (1949). The influence of concentration of nutrients, thiamin, and biotin upon growth, and the formation of perithecia and ascospores by *Chaetomium convolutum*. *Mycologia* **41**:186–196.

Lilly, V. G., and H. L. Barnett. (1951). "Physiology of the Fungi," 464 pp. McGraw-Hill, New York.

Lilly, V. G., and H. L. Barnett. (1956). The utilization of D- and L-arabinose by fungi. *Am. J. Botany* **43**:709–714.

Lilly, V. G., and H. L. Barnett. (1961). Acetate as a carbon source for fungi. *Proc. West Va. Acad. Sci.* **33**:5–10.

Lilly, V. G., H. L. Barnett, and R. F. Krause. (1960). The production of carotene by *Phycomyces blakesleeanus*. *West Va., Univ. Agr. Expt. Sta., Bull.* **441T**: 80 pp.

Machlis, L. (1953). Growth and nutrition of water molds in the subgenus *Euallomyces*. 1. Growth factor requirements. *Am. J. Botany* **40**:189–195.

Miller, P. M. (1956). Spectrographic analysis of water agar. Potato-dextrose agar, and V-8 juice agar. *Phytopathology* **46**:526.

Morton, A. G., and A. MacMillan. (1954). The assimilation of nitrogen from ammonium salts and nitrates by fungi. *J. Exptl. Botany* **5**:232–252.

Newton, W. (1956). The non-utilization of D-alanine and growth stimulation by L-alanine in the nutrition of *Phytophthora parasitica*. *Can. J. Botany* **34**:759–761.

Nicholas, D. J. D. (1952). The use of fungi for determining trace metals in biological materials. *Analyst* **77**:629–642.

Norkrans, B. (1953). The effect of glutamic acid, aspartic acid, and related compounds on the growth of certain *Tricholoma* species. *Physiol. Plantarum* **6**:584–593.

Omvik, A. (1951). Experiments on manganese, zinc, and calcium as constituents of nutrient solutions for fungi. *Uni. Bergen Arbok, Naturvitenskap. Rekke* **8**: 85 pp.

Page, R. M. (1962). Light and the asexual reproduction of *Pilobolus*. *Science* 138: 1238–1245.

Purdy, L. H., Jr., and R. C. Grogan. (1954). Physiological studies of *Sclerotinia sclerotiorum* in liquid and agar culture. *Phytopathology* 44:36–38.

Rahn, O., and G. L. Richardson. (1941). Oxygen demand and oxygen supply. *J. Bacteriol.* 41:225–249.

Ranzoni, F. V. (1951). Nutrient requirements for two species of aquatic Hyphomycetes. *Mycologia* 43:130–141.

Reischer, H. S. (1951). Growth of Saprolegniaceae in synthetic media. 1. Inorganic nutrition. *Mycologia* 43:142–155.

Rich, M. A., and A. M. Stern. (1958). Studies on *Cryptococcus nigricans*. 2. The effects of physical, chemical, and nutritional factors. *Mycopathol. Mycol. Appl.* 10:83–90.

Riker, A. J., and R. S. Riker. (1936). "Introduction to Research on Plant Diseases," 117 pp. John S. Swift, St. Louis, Missouri.

Robbins, W. J. (1937). The assimilation by plants of various forms of nitrogen. *Am. J. Botany.* 24:243–250.

Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten. (1957). Studies of biosynthesis in *Escherichia coli*. *Carnegie Inst. Wash. Publ.* 607: 521 pp.

Schopfer, W. H. (1949). Les repercussions hors de France de oeuvre de Jules Raulin (1836–1896) relative au zinc, oligoelement. *Ann. Univ. Lyon.*, Fasc. spéc. 39 pp.

Steinberg, R. A. (1939). Relation of carbon nutrition to trace-element and accessory requirements of *Aspergillus niger*. *J. Agr. Res.* 59:749–763.

Steinberg, R. A. (1941). Sulfur and trace element nutrition of *Aspergillus niger*. *J. Agr. Res.* 63:109–127.

Tandon, R. N. (1961). "Physiological Studies on Some Pathogenic Fungi," 80 pp. Scientific Research Committee, Allahabad, India.

Truesdale, G. A., A. L. Downing, and G. F. Lowden. (1955). The solubility of oxygen in pure water and sea-water. *J. Appl. Chem.* 5:53–63.

Volkonsky, M. (1933). Sur l'assimilation des sulfates par les champignons: euthiotrophie et parathiotrophie. *Compt. Rend.* 197:712–714.

CHAPTER 18

The Chemical Environment for Fungal Growth

2. Carbon Sources

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I. INTRODUCTION

Fungi are among the most ubiquitous microorganisms, growing in a large variety of media and under many conditions. Some utilize chemically complex carbon-containing compounds for growth, whereas others are much more selective in their requirements. Selection of the conditions for growth will depend in part on the purpose of the study. If cells of a definite composition (enzymatic or chemical) are desired, the carbon source used may well be different from that used for production of a certain metabolite. The examples cited in this review are only illustrative of many observations reported in the literature. Stress will be placed on methodology, not on detailed information.

II. TYPES OF CARBON COMPOUNDS UTILIZED FOR CELL PRODUCTION

A list of some of the carbon-containing compounds reported to be utilized by fungi for growth in synthetic media is given in Table I. This list, compiled from the literature, includes compounds which often were reported to have been tested as the *sole* carbon-containing compound in the medium. Included are a wide variety of amino acids, organic acids, sugars, sugar derivatives, alcohols, and more complex organic compounds. In most instances the authors measured only cell formation and did not report the

TABLE I

SOME COMPOUNDS UTILIZED BY FUNGI AS THE SOLE SOURCE OF CARBON

Carbohydrates and related compounds

Adonitol	Galactosamine	Kojic acid	Phytic acid
Amylose	Galactose	Lactose	Polygalacturonate
Arabinose	Gentiobiose	Lyxose	Raffinose
Arabitol	Gluconic acid	Maltose	Rhamnose
Cellobiose	Glucosamine	Mannitol	Ribose
Chitin	Glucose	Mannose	Salicin
Dextran	Glucose pentaacetate	Melibiose	Sorbitol
Dextrin	Glucuronic acid	Melizitose	Sorbose
Dulcitol	Glycerol	α-Methylgalactoside	Starch
Erythritol	Glycogen	α-Methylglucoside	Sucrose
Erythrose	Inositol	α-Methylmannoside	Trehalose
Fucose	Inulin	α-Methylxyloside	Turanose
Fructose	2-Ketogluconic acid	Panose	Xylan
Galactitol	5-Ketogluconic acid	Pectin	Xylose

Amino acids and related compounds

Alanine	Betaine	Glutamic acid	Norleucine	Taurine
α-Aminoadipic acid	Citrulline	Glutamine	Norvaline	Threonine
	Creatine	Glycine	Ornithine	Tryptamine
α-Aminobutyric acid	Cysteine	Hippuric acid	Phenaceturic acid	Tryptophan
	Cystine	Histamine		Tyramine
Amygdalin	Diaminopimelic acid	Histidine	Phenylalanine	Tyrosine
Anthranilic acid		Isoleucine	Phenylglycine	Valine
Arginine	Djenkolic acid	Leucine	Proline	
Asparagine	Ephedrine	Lysine	Sarcosine	
Aspartic acid	Ethionine	Methionine	Serine	

Organic acids and related compounds

Acetic acid	Formic acid	Mandelic acid	Pyruvic acid
Adipic acid	Fumaric acid	Methylricinoleate	Stearic acid
Arachidonic acid	Gentisic acid	Myristic acid	Stipitatic acid
Butyric acid	Glutaric acid	Oleic acid	Succinic acid
Capric acid	Glycerylricinoleate	Oxalic acid	Tartaric acid
Caproic acid	Homogentisic acid	Palmitic acid	Triacetin
Citric acid	α-Ketoglutaric acid	Phenylacetic acid	Tributyrin
Di-*n*-butyrl sebacate	Lactic acid	Phenylpyruvic acid	Triolein
	Lauric acid	Pimelic acid	Tristearin
Ethyl acetate	Linoleic acid	Propionic acid	Valeric acid
Ethanol			

Polycyclic compounds and alkaloids

Androsterone	Digitonin	Ergosterol	Stigmasterol
Cholesterol	Digitoxigenin	Methylreserpate	Testosterone
Cholic acid	Diosgenin	Nicotine	Tetracycline
Cortisone	Ergotamine	Progesterone	Yohimbine

rate or extent of disappearance of the given carbon-containing compound from the medium. However, where these compounds were the sole carbon source, it is obvious that the carbon-containing compounds in the cells were derived from this source. The importance of this tabulation lies in the listing of the variety of compounds metabolized rather than a consideration of the mechanisms of dissimilation of these compounds by the fungi. Most of the information supplied in the literature is inadequate to permit formulation of theories on microbial metabolic patterns.

In most experimental programs studying growth, the production of desired metabolites, or the effects of metabolic poisons or stimulants on fungal growth, media containing a mixture of carbon-containing compounds are used. Very often the components of the mixture are metabolized at different rates. For example, when *Penicillium chrysogenum* was grown in a medium containing glucose, lactose, acetate, and lactate (together with mineral salts), the acetate was metabolized first, the lactate second together with some of the glucose, and lactose was used last (Jarvis and Johnson, 1947). These observations, and those in related studies, emphasize the usefulness of knowledge of the utilization patterns of carbon sources by microorganisms of interest in fermentation processes.

Perusal of the information in Table I leads to the conclusion that fungi may be found that will metabolize any carbon-containing compound and produce cell substance from this material (perhaps the only exceptions are certain plastic, fluorine-containing compounds and "nonbiodegradable" detergents).

This diversified metabolic activity is of practical importance in a beneficial manner in certain industrial situations where carbon-containing waste products accumulate and have to be destroyed by microbial oxidation prior to introduction of these wastes into sewage systems. The activity is detrimental in other industrial processes when the fungal mycelium destroys the usefulness of the product, e.g., food materials, pharmaceutical formulations, motor fuels, industrial chemicals, and measures have to be taken to eliminate these cultures and prevent mycelium formation.

Strain specificity in utilization of carbon sources is not uncommon. For example, mutants of *Aspergillus niger* and *Penicillium chrysogenum,* and of streptomycetes often have different carbohydrate utilization patterns than those of the parent strains. On the other hand, some mutants will attack carbon sources not used by the parent strain, or will metabolize these more efficiently. This has been exploited in the selection of fungi effecting useful transformations of steroids.

Glucose is utilized by more fungi than is any other sugar and is nearly a universal carbon source. In attempting to culture fungi of unknown nutritional requirements on synthetic or semisynthetic media, glucose should

be the first carbon source used. However, there are a few fungi that are unable to utilize glucose: *Leptomitus lacteus* is unable to utilize glucose, fructose, galactose, or sucrose (Schade, 1940). Cheo (1949) found certain isolates of *Ustilago striiformis* to be unable to grow on glucose when freshly transferred from sucrose media. These cultures grew after incubation for 2–4 weeks in these media, and Cheo concluded that adaptive enzyme formation was involved.

III. MEASUREMENT OF CONVERSION OF CARBON SOURCES TO MYCELIUM

There are a number of methods used to determine the efficiency of conversion of carbon sources to mycelium; these include the determination of (1) total weight of mycelium formed and correlation of this with the amount of carbon source utilized; (2) the amount of carbon in the medium converted to mycelium carbon; (3) the amount of substance directly incorporated into the mycelium.

A. Conversion of Carbon to Mycelium (Economic Coefficient)

The first method, often termed the "economic coefficient," is the most widely used. It is defined by the formula

$$\frac{\text{mycelium dry weight (gm)}}{\text{amount of carbon compound consumed (gm)}} \times 100$$

Some authors have used the ratio, or its reciprocal, without multiplication by 100. The economic coefficient is likely to be maximal when respiratory CO_2 and soluble metabolic products are minimal in quantity. Since both of these are affected by cultural conditions, no one value or even range of values can be set down as characteristic of fungi as a group. Fungi grown on dilute media for no longer than is necessary to utilize the carbohydrate usually have an economic coefficient in the range 20–40 (a few higher values have been reported). Very low values reflect the probable production of significant amounts of soluble carbon compounds, difficulties in analysis of the unconverted carbon compound, and related problems. The economic coefficient is likely to be influenced by temperature of incubation, the metallic ion content of the medium, the nitrogen sources in the medium, the age of the culture, and the method of aerating the culture. All these conditions affect the production of soluble metabolites by the mycelium, and thus indirectly the economic coefficient.

A few examples of the effects of some of these variables on mycelium formation, sucrose utilization rate, and economic coefficient in *Aspergillus*

TABLE II

FERMENTATION OF SUCROSE BY STRAINS OF *Aspergillus niger* IN SUBMERGED CULTURE[a]

Fermentation period (days)	High-yielding citric-producer			Low-yielding citric-producer		
	Sucrose used (gm/l)	Mycelium formed (gm/l)	Economic coefficient	Sucrose used (gm l)	Mycelium formed (gm l)	Economic coefficient
2	23	5.6	25	20	6	30
4	65	10	15	30	18.4	61
6	95	18	19	50	22.5	45
8	98	19	20	72	23.5	33
10	98	20	20	95	23.5	25

[a] Data from Perlman (1949).

niger fermentations are presented in Table II. Of the various variables studied, the strain of *A. niger* used and the length of the incubation period had the most effect on the economic coefficient in these submerged culture fermentations. In the "high-yielding" fermentation the sucrose was rapidly utilized and converted to citric acid (data not shown) resulting in a low economic coefficient, whereas in the "low-yielding" fermentation the sucrose was used more slowly with less accumulation of citric acid and a higher economic coefficient. The economic coefficient varied markedly with the length of the incubation period in the fermentations by the "low-yielding" culture.

A series of experiments in which a fumaric acid-forming strain of *Rhizopus nigricans* [*R. stolonifer*] was the test organism are summarized in Table III. Glucose utilization was greater in the presence of zinc than in

TABLE III

EFFECT OF ZINC ON ECONOMIC COEFFICIENT IN *Rhizopus stolonifer* GROWN IN SURFACE CULTURE[a]

Glucose conc. (gm/100 ml)	Glucose used (gm/100 ml)		Efficiency of fumaric acid production[b]		Economic coefficient[c]	
	Zn absent	Zn present	Zn absent	Zn present	Zn absent	Zn present
2.5	1.70	2.07	45.9	2.5	14.1	40.0
5.0	2.65	3.89	38.3	23.3	13.9	21.3
10.0	2.54	6.49	31.9	21.9	16.0	12.5

[a] From Foster and Waksman (1939).

[b] Grams of fumaric acid produced per gram of glucose consumed × 100.

[c] Grams of mycelium produced per gram of glucose consumed × 100.

its absence, while less fumaric acid accumulated in zinc-containing media. An economic coefficient of 40 was obtained with media having the lowest glucose concentration, and the value dropped when the glucose concentration was raised fourfold. In these experiments the static culture technique was used, and the rate of diffusion of the glucose to the mycelium no doubt affected the rate of utilization of this energy source and its conversion to fungal cells and fumaric acid.

B. Conversion of Carbon Source to Mycelial Carbon

The second method listed measures the conversion of carbon in the medium to carbon compounds in mycelium. Data collected in a carbon-balance study of a citric acid-producing *Aspergillus niger* fermentation are summarized in Table IV. The economic coefficient calculated from the weight

TABLE IV

STUDY OF FERMENTATION OF GLUCOSE BY *Aspergillus niger* IN SURFACE CULTURE[a]

A. Conversion of glucose to metabolites

Fermentation period (days)	CO_2 formed (gm)	Mycelium formed (gm)	Economic coefficient	Glucose used (gm)	Citric acid formed (gm)
4	0.67	0.375	14	2.64	1.61
7	1.55	0.676	8	8.78	5.82
10	2.17	0.825	7	12.12	8.50

B. Distribution of carbon in the citric acid fermentation

Fermentation period (days)	Carbon in			Carbon in		Carbon accounted for (%)	Efficiency of conversion of glucose carbon to mycelium
	CO_2 (gm)	Mycelium (gm)	Residual glucose (gm)	Citric acid (gm)	Solution (gm)		
4	0.18	0.17	4.78	0.60	5.42	98.5	0.17
7	0.42	0.32	2.32	2.18	5.03	98.5	0.09
10	0.60	0.38	0.98	3.19	4.74	97.6	0.08

[a] Data from Wells *et al.* (1936).

of mycelium formed per 100 gm of glucose used decreased from 14 to 7 as the incubation period was extended from 4 to 10 days. The efficiency of conversion of glucose carbon to mycelium carbon ranged from 17% at

4 days to 8% at 10 days. The close relationship of the economic coefficient to the carbon conversion efficiency value shows that the carbon compounds of the mycelium were in the same oxidation state as those of the substrate (glucose), and it is likely that the mycelium contained a large amount of a hexose polymer. If the mycelium contained a large amount of fat the carbon content would have been higher than the 40% observed and the carbon converted to mycelium might be as high as 30%. Raistrick *et al.* (1931) noted that the carbon content of mycelia of 40- to 70-day-old fermentations of various fungi often ranged between 46 and 55%. The advantage of using a carbon balance in studies of efficiency of conversion of substrate to mycelium is the specificity of the method; the disadvantage is the increased time needed to carry out the analyses.

C. Direct Utilization of Carbon-Source

The assumption is made in both the methods mentioned above that the substrate is used to form mycelium directly. Study of fungal metabolic patterns has shown that most, if not all, species degrade the carbon compound added to the medium and form mycelium from the metabolic products. There appear to be no reports of investigations designed to show that the carbon source added to the medium is directly incorporated into the mycelial carbohydrate, lipid, or protein. The availability of C^{14}-labeled compounds makes possible direct determination of the carbon compound, and perhaps experimental studies to be carried out in the future will show the direct incorporation of carbohydrates, amino acids, peptides, and/or lipids into the mycelium. The efficiency of such an incorporation might be an index of usefulness of the carbon-containing compound to the growing cells.

IV. PRACTICAL PROBLEMS ENCOUNTERED IN STUDYING THE USE OF CERTAIN SOURCES OF CARBON

Among the practical problems that affect the utilization of energy sources by fungi are (1) physical availability of the carbon source to the fungal cells; (2) cultural conditions, including presence of other nutrients in the media, aeration conditions, incubation temperature, etc.; and (3) adaptation of the strain to the substrate.

The economic coefficient is often directly related to the rate at which the carbon source is absorbed by the mycelium. When *Aspergillus niger* was grown in static culture in shallow layers of media, higher economic coefficients were obtained than when the depth of medium was increased from 1 cm to 10–15 cm. The rate of diffusion of the sucrose or glucose substrate

to the mycelium was very low in fermentations where the depth of the medium exceeded 2 cm. One of the *A. niger* strains studied produced a deeply invaginated mycelium, and this pattern of growth resulted in a more rapid turnover of the sucrose than was observed in the experiments with the culture which formed a "thin" mycelium (Perlman, 1943).

These problems of diffusion of unmetabolized substrate through the medium to the mycelium are, of course, minimized when shaken cultures are used. However, other difficulties encountered include fragmentation of the mycelium due to fragility and limitations of culture operations. Johnson (1952) summarized some of his studies of the penicillin-producing fermentations and noted that for highest antibiotic production a period of "semi-starvation" of the mycelium (as far as carbohydrate source was concerned) is desirable. This could be achieved by using lactose, a carbon source only slowly metabolized by his strains, or by "slow-feeding" glucose or sucrose to the culture. Careful control of the feeding rate was needed so that the sugar was not completely exhausted. If too much sugar was added more of it was converted to mycelium than was desirable, resulting in a higher economic coefficient and lowered penicillin production.

A number of the carbon sources listed in Table I as utilizable by fungi are relatively insoluble in aqueous media. When fungi are grown in media containing these insoluble materials, the mycelium tends to form balls around the particles, a phenomenon which often leads to poor growth and low economic coefficients. Pan *et al.* (1959) found that the addition to these media of mineral oil, or other nonmetabolized oils, resulted in solubilization of the fats and lipids under examination and the formation of a two-phase liquid system. The fungi grew very well in these media, and apparently the "coating" of the mycelium with mineral oil did not affect the microbial attack of the carbon source. Economic coefficients as high as 60 have been observed in other experiments. However, since lipids contain more than twice the carbon content of sugars, this high economic coefficient is not surprising. When the coefficient was calculated on the basis of the carbon content, the results were of the same order as obtained when hexoses were the source of carbon.

Other cultural conditions affecting the economic coefficient include efficiency of aeration, incubation temperature, presence in the media of required vitamins and of trace metals. The importance of adequate aeration has been repeatedly emphasized, and its indirect and direct effects on fungal growth have been examined in many laboratories. Johnson (1952) has reviewed aspects of the problem as concerned with penicillin production, and many of his observations are also related to conversion of carbon sources to mycelium. Often, higher economic coefficients are obtained when "minimal" aeration is used than when "high aeration" is used.

Incubation temperature usually has an effect on the economic coefficient and will sometimes vary from substrate to substrate with a given organism (Lilly and Barnett, 1953). The effect of increasing the length of the incubation period on the economic coefficient is shown in Tables II and IV. In both cases lower values were obtained when the period was extended.

Other factors that influence the economic coefficient relate to the composition of the medium: The presence or absence of B vitamins sometimes affects growth of fungi which do not have an absolute requirement for these vitamins. For example, Fergus (1952) found that the addition of a vitamin supplement to *Penicillium digitatum* cultures resulted in better growth even though the cultures grew well without this supplement. On the other hand, Lilly and Barnett (1953) point out that the literature prior to 1940 may not be very valuable since the investigators did not realize that the fungi under study had absolute requirements for certain water-soluble vitamins which were not included during the experiments. The importance of trace elements in fungal nutrition was discussed in Chapter 17 of this volume and will not be reviewed here. The effect of a trace amount of zinc on the economic coefficient of *Rhizopus* cultures is shown in Table III and is typical of the many reports in the literature. The effect of trace elements on the economic coefficient varies with the carbon source, the culture under study, and, of course, the trace element.

Adaptation of culture to substrate has been mentioned earlier in this section and deserves further discussion. Some strains apparently "adapt" quickly whereas others do not "adapt" even under the most favorable circumstances. Barnett and Lilly (1951) and Lilly and Barnett (1953) reported that the growth of many fungi was markedly inhibited when sorbose was present in the medium. Some of their data, summarized in part in Table V, showed that certain fungi grew well in media containing mixtures of sorbose and other sugars, and presumably the presence of the second sugar encouraged growth of mycelium which eventually utilized the sorbose. Other strains did not adapt to the presence of sorbose, and growth of these fungi in media containing glucose and sorbose was markedly lower than when only glucose was present. Matsushima and Klug (1958) analyzed this problem genetically, using *Ustilago maydis* and found an interaction between genes governing sorbose utilization. The sexually compatible monosporidial lines and their solopathogenic diploid derivative differed in the ability to metabolize sorbose in the presence of various kinds of nitrogen sources. Similar investigations by Roberts (1963) showed that, in a strain of *Aspergillus nidulans*, D-galactose was oxidized by an inducible enzyme system; D-fucose was a poor inducer of this system.

TABLE V

EFFECT OF SORBOSE ON GROWTH OF SELECTED FUNGI[a]

Culture	Incubation period (days)	Mycelium weight (mg 100 ml of Medium Containing Indicated Sugar)				
				Glucose		Maltose
		Glucose	Sorbose	+ sorbose	Maltose	+ sorbose
Aspergillus niger	6	936	344	1136	828	1304
	14	880	704	1168	768	1096
Fusarium culmorum	4	316	104	412	436	400
	7	480	336	588	540	764
Penicillium chrysogenum	3	636	164	664	276	356
	9	664	552	1500	684	1236
Aspergillus elegans	5	640	128	496	428	968
	8	724	156	904	580	868
Choanephora cucurbitarum	5	300	Trace	416	176	180
	9	260	16	488	380	404
Mucor ramannianus	6	392	60	600	336	488
	11	346	40	744	504	624
Phycomyces blakesleeanus	5	616	Trace	584	172	272
	8	668	Trace	876	720	932
Alternaria tomato	4	120	24	68	204	56
	9	868	172	472	884	348
Aspergillus clavatus	6	444	Trace	420	364	96
	9	732	Trace	664	692	176
Rhizoctonia solani	9	1364	Trace	840	1348	28
	13	1376	Trace	2076	1196	80
Sclerotinia sclerotiorum	9	376	Trace	80	272	Trace
	13	748	Trace	256	676	Trace

[a] Data from Lilly and Barnett (1953).

REFERENCES

Barnett, H. L., and V. G. Lilly. (1951). The inhibitory effects of sorbose on fungi. *Science* 114:439–440.

Cheo, P. C. (1949). Stripe smut of blue grass (*Ustilago striiformis* forma *poae-pratensis*): Spore germination, artificial inoculation, pathological history and growth in artificial media. Ph.D. Thesis. West Virginia University.

Fergus, C. L. (1952). The nutrition of *Penicillium digitatum* Sacc. *Mycologia* 44:183–199.

Foster, J. W., and S. A. Waksman. (1939). The specific effect of zinc and other heavy

metals on growth and fumaric acid production by *Rhizopus*. *J. Bacteriol.* **37**:599–617.

Jarvis, F. G., and M. J. Johnson. (1947). The role of the constituents of synthetic media for penicillin production. *J. Am. Chem. Soc.* **69**:3010–3017.

Johnson, M. J. (1952). Recent advances in penicillin fermentation. *World Health Organ., Monograph Ser.* **10**:99–121.

Lilly, V. G., and H. L. Barnett. (1953). The utilization of sugars by fungi. *West Va., Univ. Agr. Expt. Sta., Bull.* **362T**:58 pp.

Matsushima, T., and R. J. Klug. (1958). Utilization of L-sorbose by monosporidial lines and mutants of *Ustilago maydis*. *Am. J. Botany* **45**:165–168.

Pan, S. C., S. Bonanno, and G. H. Wagman. (1959). Efficient utilization of fatty oils as energy source in penicillin fermentation. *Appl. Microbiol.* **7**:176–180.

Perlman, D. (1943). Factors affecting Production of citric acid by *Aspergillus niger*. M.Sc. Thesis, University of Wisconsin.

Perlman, D. (1949). Mycological production of citric acid—the submerged culture method. *Econ. Botan.* **3**:360–374.

Raistrick, H., J. H. Birkinshaw, J. H. V. Charles, P. W. Clutterbuck, F. P. Coyne, A. C. Hetherington, C. H. Lilly, M. L. Rintoul, W. Rintoul, R. Robinson, J. A. R. Stoyle, C. Thom, and W. Young. (1931). Studies in the biochemistry of micro-organisms. *Phil. Trans. Royal Soc. London* **B220**:1–367.

Roberts, C. F. (1963). The adaptive metabolism of D-galactose in *Aspergillus nidulans*. *J. Gen. Microbiol.* **31**:285–295.

Schade, A. L. (1940). The nutrition of Leptomitus. *Am. J. Botany* **27**:376–384.

Wells, P. A., A. J. Moyer, and O. E. May. (1936). The chemistry of the citric acid fermentation. I. The carbon balance. *J. Am. Chem. Soc.* **58**:555–558.

CHAPTER 19

The Chemical Environment for Fungal Growth

3. Vitamins and Other Organic Growth Factors

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I. INTRODUCTION

The term, *growth factor,* means an organic substance which in minute amounts is necessary or stimulatory for growth and does not serve simply as an energy source. Most substances first discovered as growth factors for fungi (or bacteria) have later proved to function as vitamins for animals, and vice versa. The term vitamin is therefore often used also for the growth factors of the fungi, especially for those factors which function as part of coenzymes.

Certain organic substances, e.g., amino acids, nucleotide constituents, and fatty acids, which serve as building blocks of intracellular structures, often are not included among the growth factors of the fungi, since they are active in higher concentrations than the coenzyme vitamins, i.e., 10–1000 mμg/ml instead of 0.01–1 mμg/ml (Table I). However, it is difficult to make a clear separation between these two categories of essential metabolites, and therefore the concept, growth factor, will be taken in its widest sense in the following presentation.

The limited space available in this survey renders it impossible to account for all known cases of growth factor requirements in fungi. A more complete documentation will be found in the comprehensive papers by Schopfer (1943), Knight (1945), Cochrane (1958), and Fries (1961a).

The growth factors are to be considered indispensable for all fungi—just as vitamins are for animals—and the difference between those species

TABLE I

A COMPARISON OF THE ACTIVITY OF FOUR COMMON GROWTH FACTORS

Growth factor	Amount of growth factor necessary for the production of 1 mg mycelium (dry weight) (μg)	Lowest concentration of growth factor giving maximum growth rate (μg, ml)
Biotin	0.00025	ca. 0.0001
Thiamine	0.0004–0.0025	ca. 0.001
Pyridoxine	0.0003–0.0007	ca. 0.001
Inositol	1.1 –1.7	ca. 0.5

or individuals which need such a substance in their substrate, and those which can do without it, lies in the fact that the former cannot synthezise the growth factor themselves, whereas the latter can. The former type of fungus is called *auxoheterotrophic,* the latter *auxoautotrophic* (Schopfer, 1943).

The correctness of this interpretation is proved by the fact that, in a fungus autotrophic for a certain growth factor, production of this factor can always be demonstrated. Sometimes it may even be a considerable surplus production, which can be utilized commercially, e.g., the synthesis of riboflavine by *Eremothecium ashbyii.* The growth factors produced often diffuse out from the mycelium, thus making it possible for two complementary auxoheterotrophic fungi to grow together in a medium without any added growth factors (syntrophism).

The term growth factor itself indicates that growth is the mode of response ordinarily observed. This response can be estimated by weighing the amount of mycelium produced, by measuring the optical density of a culture (in the case of a yeastlike mode of growth), or in some more indirect way, e.g., by determining the pH change of the culture medium. The hormones are also organic compounds active in small amounts, but in contrast to the growth factors, the hormones influence not the rate of growth, but rather the mode of growth (morphogenesis).

Frequently the growth factor, which as a rule enters as part of a coenzyme, can be exchanged for a precursor in its biosynthesis or—which may mean the same—for a simpler compound constituting only a part of the growth factor molecule. This may be exemplified by thiamine, which sometimes can be substituted for by either the pyrimidine or the thiazole part of its molecule, by pantothenic acid, in certain cases exchangeable for pantoic acid or β-alanine, and by biotin and its precursor dethiobiotin. Although the existence of such interchanges causes certain difficulties in terminology,

it contributes to our understanding of the biosynthesis of the growth factors.

In this connection it should also be mentioned that artificial requirements for growth factors can be induced by the application of appropriate inhibitors or analogs. Thus, *Aspergillus niger* requires *p*-aminobenzoic acid only in the presence of its synthetic, structural analog, sulfanilamide (Hartelius, 1946).

A fungus can be completely incapable of synthesizing a certain growth factor, or the biosynthetic capacity may be more or less reduced (Figs. 1 and 2). To determine the power of biosynthesis in an individual strain of

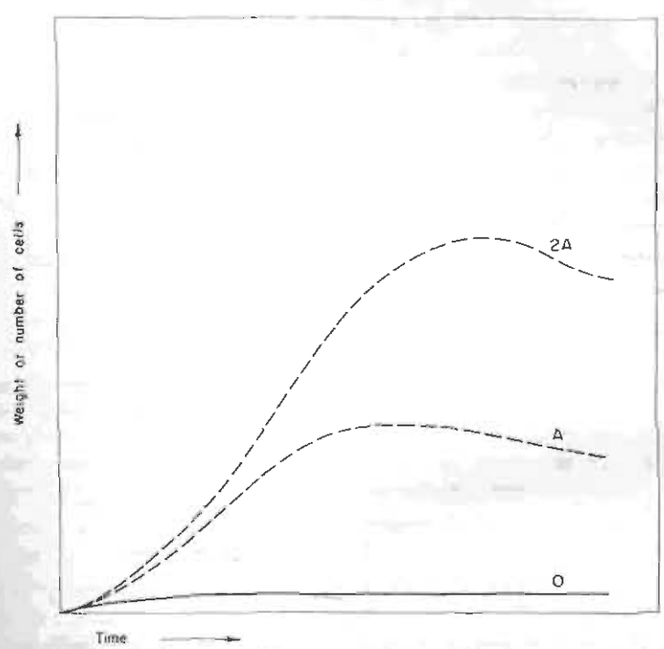


FIG. 1. Action of an *indispensable* growth factor on the growth of a fungus. The curves show the growth without the factor (O) and with suboptimal amounts added (*A* and 2*A*). Growth stops when growth factor is consumed.

a fungus, the growth of a pure culture must be studied under controlled conditions during a certain time of incubation with and without the growth factor included in the medium. In evaluating the result of such experiments, internal (genetic and metabolic) mechanisms as well as environmental conditions have to be considered.

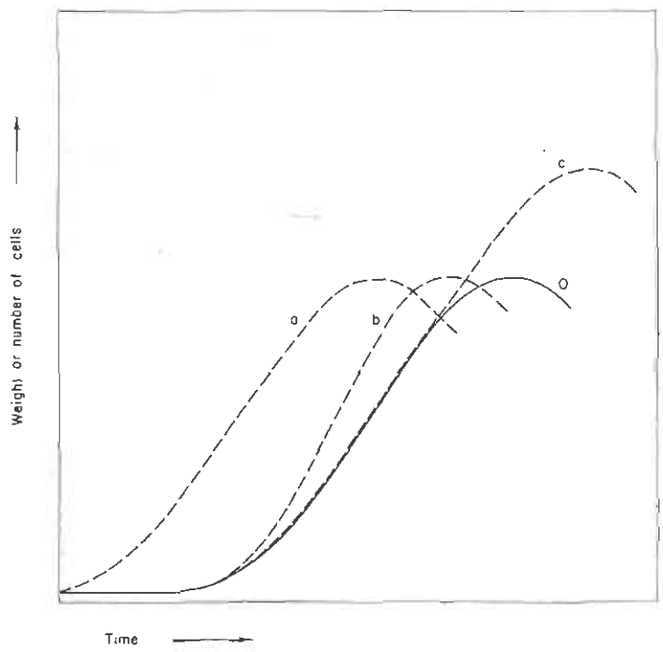


FIG. 2. Action of a dispensable but *promoting* growth factor on the growth of a fungus. The solid line (O) shows the growth without and the dashed lines (*a*, *b* and *c*) the growth with the factor added. Three different types of response are illustrated: (*a*) abbreviated lag phase; (*b*) increased growth rate; (*c*) increased production. Growth stops when one of the nutrients, e.g., the carbon source, of the medium is consumed.

II. VARIATIONS IN BIOSYNTHETIC CAPACITY

The capacity to synthesize a certain growth factor may change with time in one strain as a result of changes in the genetic or metabolic mechanisms of the cells. The biosynthesis of each growth factor is controlled by numerous genes, and mutations in these genes may therefore impede or block the production of the factor in question. The possibility of inducing such mutations by artificial means forms the basis of the modern, experimental branch of biology called biochemical genetics. Undoubtedly, mutations of this sort also arise spontaneously and can be found now and then in wild-type populations, e.g., niacin-heterotrophic strains of *Ophiostoma multiannulatum* (Fries, 1948) and *Polyporus abietinus* (Fries and Aschan, 1952).

This probably explains the sometimes conflicting reports from different authors regarding the auxoheterotrophy of one and the same species, e.g., *Blastocladia pringsheimii* (Cantino, 1948; Crasemann, 1957). The different levels of partial auxoheterotrophy found among strains of the same species are probably also genetically controlled, although according to a rather complicated pattern (Lilly and Barnett, 1948a).

Experimentally induced nutritional mutations are beyond the scope of this survey, which deals only with the naturally occurring growth factor requirements of wild-type (prototroph) strains. However, it may be mentioned that all these natural requirements have their counterparts in induced mutations, as appears from genetic studies in *Neurospora, Ophiostoma, Aspergillus,* and several other fungi (for references see Fincham and Day, 1963; see also Catcheside, Chapter 29 of this volume).

Reversion from auxoheterotrophy to auxoautotrophy seems to be a very rare phenomenon in wild-type strains, where the deficiency constitutes a species character, in contrast to laboratory strains with induced deficiencies. This difference is usually explained by the assumption that in the wild-type strains several genes are involved, in the laboratory strains only one.

Sometimes it is hard to decide whether a change to growth factor independence is the result of mutation in an auxoheterotrophic strain or of a slow adaptation in an intrinsically auxoautotrophic strain. Good examples of these two possibilities can be found in the genus *Exobasidium* (Sundström, 1964).

Most authors seem to agree that the auxoheterotrophic fungi have developed from auxoautotrophic ancestors, spontaneous mutations explaining the evolutionary mechanism. This interpretation finds support in the results of biochemical genetics (Lwoff, 1943; Schopfer, 1944b).

It should also be mentioned that variations in biosynthetic ability may occur during the life cycle of a fungus. In particular the very first stage of development, the germination and outgrowth of the spore, seems to differ in this respect from the fully developed mycelium. Thus the germinated spores of *Myrothecium verrucaria* continue their growth very slowly unless the medium contains biotin, whereas the fully developed mycelium exhibits no requirement for biotin (Mandels, 1955). Even in such typically auxoautotrophic species as *Penicillium digitatum* (Fergus, 1952) and *Aspergillus nidulans* (Strigini and Morpurgo, 1961) initial growth may be strongly promoted by added vitamins. In the dimorphic *Histoplasma capsulatum* it is only the yeastlike phase which requires thiamine and biotin (Pine, 1957).

The number of growth factors required varies from none to at least six, the most exacting species being found among the yeasts (Table II).

TABLE II

EXAMPLES OF FUNGI REQUIRING DIFFERENT GROWTH FACTORS OR COMBINATIONS OF GROWTH FACTORS

Species	Thiamine	Ribo-flavine	Nicotinic acid	Panto-thenic acid	Pyridox-ine	Biotin	PABA	Inositol	Other growth factors	Refer-ences[a]
Phycomyces blakesleeanus	+									1
Trichophyton equinum			+							2
Brettanomyces bruxellansis					+					3
Melanospora destruens						+				4
Rhodotorula aurantiaca	+						+			5
Lophodermium pinastri	+					+				6
Ophiostoma multiannulatum	+				+					7
Torula sphaerica			+			+				8
Saccharomyces logos				+		+				3
Nematospora gossypii						+		+		6
Poria vaillantii	+	+				+			Adenine	9
Candida pseudotropicalis			+	+		+				3
Candida parakrusei	+		+		+	+				12
Ascoidea rubescens	+				+	+				7
Zygosaccharomyces priorianus				+		+		+		3
Z. japonicus	+			+		+		+		3
Kloeckera brevis	+		+	+	+	+		+		3
Eremothecium ashbyii	+					+		+	Leucine, arginine	10
Schizosaccharomyces octosporus			+	+		+		+	Histidine, methionine, adenine	11
Pilobolus kleinii									Hemin	13

[a] Key to references: (1) Schopfer (1934); (2) Georg (1949); (3) Burkholder *et al.* (1944); (4) Hawker (1939); (5) Robbins and Ma (1944); (6) Kögl and Fries (1937); (7) N. Fries (1943); (8) Burkholder and Moyer (1943); (9) Jennison *et al.* (1955); (10) Schopfer and Guilloud (1945); Krneta-Jordi (1962); (11) Northam and Norris (1951); (12) Miyashita *et al.* (1958); (13) Page (1952).

III. THE INFLUENCE OF ENVIRONMENTAL CONDITIONS

A complete requirement for a certain growth factor is usually not influenced by cultural conditions. A partial requirement, on the other hand, may often be more or less pronounced, depending on the external conditions. A few examples of such conditioned requirements may be mentioned.

Close to the upper temperature limit of growth, microorganisms sometimes become more exacting than at lower, more normal, temperatures. This seems to be a common phenomenon in bacteria, but a few cases have also been observed in fungi, e.g., in a strain of *Coprinus fimetarius* stimulated by methionine or an unidentified factor at above 40°C (L. Fries, 1953), and in *Saccharomyces cerevisiae,* which grew well at 30°C in a synthetic medium without pantothenic acid but required this vitamin for growth at 38°C (Lichstein and Begue, 1960). In another study of *S. cerevisiae* it was found that at 40°C no growth occurred unless yeast extract was added to the medium (Sherman, 1959). Oleic acid exerted a sparing effect. Similarly *Aspergillus niger* requires biotin and other supplements for growth above 42.7°C when cultivated on rhamnose as the carbon source (Fries and Källströmer, 1965).

Sometimes the requirement for a certain growth factor depends on the composition of the nutrient medium at any temperature within the biokinetic zone. Thus *Pythium butleri* becomes thiamine-heterotrophic as soon as the mineral salt concentration exceeds a certain level (Robbins and Kavanagh, 1938a). *Pellicularia koleroga* can utilize thiamine or biotin for growth on sucrose but requires thiamine exclusively for growth on glucose (Mathew, 1952). With glycine as the source of nitrogen *Eremothecium ashbyii* requires arginine and leucine for growth; with asparagine there is no such requirement (Krneta-Jordi, 1962).

The presence of other metabolites in the medium may profoundly influence the demand for a growth factor, at least quantitatively. It is obvious that substances functioning as precursors in the biosynthesis of the factor may partly or totally substitute for the factor itself. This is the case in *Trichophyton equinum,* where niacin, otherwise necessary, can be replaced by the amino acid tryptophan (Georg, 1949). On the other hand, a similar effect can be obtained also by adding the metabolite that is the end product of the enzyme system of which the growth factor constitutes an essential component. Such a "by-passing" of the growth factor requirement has been observed in, e.g., *Pityrosporum ovale,* where oxalacetic acid plus α-ketoglutaric acid can be substituted for thiamine (Benham, 1947). In *Torula cremoris* aspartic acid (Koser *et al.,* 1942), and in *Ophiostoma pini* aspartic or oleic acid (Mathiesen, 1950), has a sparing effect upon

the requirement for biotin. The finding that biotin becomes superfluous to *Aspergillus nidulans* and *Neurospora crassa* if fructose or Krebs cycle acids instead of glucose serve as the carbon source may also be due to a "by-passing" mechanism (Strigini and Morpurgo, 1961).

In other cases the mode of interaction cannot yet be satisfactorily explained, e.g., the growth-promoting effect of pyridoxine on *Saccharomyces cerevisiae,* which appears only with an excess of thiamine (Rabinowitz and Snell, 1951; Chiao and Peterson, 1956). More examples of this are mentioned later in Section V on the individual vitamins.

The significance of oxygen can be exemplified by the observation that *Mucor rouxii,* which ordinarily is auxoautotrophic, requires thiamine and niacin when cultured under anaerobic conditions (Bartnicki-Garcia and Nickerson, 1961).

More difficult to explain are the results of Mohan *et al.* (1962), who cultivated *Torulopsis* [*candida*] *utilis* and *S. cerevisiae* in a nutrient medium with D_2O instead of H_2O. Under these conditions both fungi required more growth factors than otherwise.

IV. TAXONOMIC AND ECOLOGICAL ASPECTS

A single strain isolated at random is usually considered representative of the species as far as growth factor requirements are concerned. Rather few extensive investigations have been performed in order to test the validity of this assumption, e.g., the study by Blumer (1940) on *Ustilago violacea,* by Lindeberg (1944) on *Marasmius perforans,* by Lilly and Barnett (1948a) on *Lenzites trabea,* by Halbsguth (1949) on *Tilletia tritici* [*T. caries*], and by Sundström (1964) on *Exobasidium* spp. From these studies it appears that species as a rule are rather homogeneous as regards type of auxoheterotrophy, although occasional, spontaneous mutants with additional requirements occur.

The type of auxoheterotrophy therefore can be looked upon as a physiological character that may be useful for the identification of the species, particularly in cases where morphological characters are few or unreliable, e.g., in *Trichophyton* (Georg and Camp, 1957), *Rhodotorula* (Hasegawa and Banno, 1963), and *Exobasidium* (Sundström, 1964).

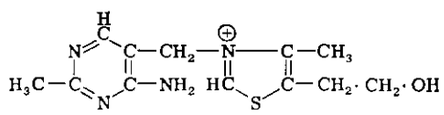
Efforts have also been made to use the type and degree of auxoheterotrophy for the elucidation of phylogenetic relationships among genera and among other taxa of higher orders (Cantino, 1955).

There does not seem to be any obvious correlation between the ecology and the growth factor requirements of a fungus. *Entomophthora apiculata* and *E. coronata,* which are parasites on animals, as well as *Exobasidium cassiopes* and most *Ustilago* species, which are parasites on seed plants, are

auxoautotrophic just as are many ubiquitous saprophytes like the penicillia. On the other hand, fungi living on exactly the same substrate, e.g., pine wood, often exhibit auxoheterotrophy, as well as complete auxoautotrophy. Some obligate parasites, like Erysiphales and Uredinales, still cannot be cultivated under controlled, axenic conditions, but this may not necessarily mean that these fungi are exceptionally exacting for growth factors. There are certain indications, however, that fungi living near the roots in the rhizosphere are more vitamin-dependent than other soil fungi (Cook and Loehhead, 1959).

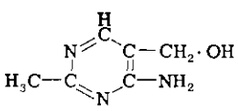
V. THE GROWTH FACTORS

A. Thiamine



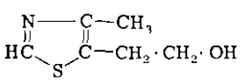
Thiamine

(I)



Pyrimidine moiety

(II)



Thiazole moiety

(III)

In most cases where a fungus does not grow in a simple sugar-mineral-salt-medium the addition of thiamine is sufficient to make the medium suitable for growth. This effect of thiamine was first shown by Burgeff (1934) and Schopfer (1934) for *Phycomyces blakesleeanus*. Since then a great number of investigations, e.g., Schopfer (1935), Robbins (1938), Robbins and Kavanagh (1942), and Fries (1938), have demonstrated that thiamine heterotrophy occurs in the major groups of fungi. This type of auxoheterotrophy seems to be the predominant one, even more common than auxoautotrophy, particularly in the Basidiomycetes. As examples, the following genera may be mentioned in which all or the majority of the investigated species are heterotrophic for thiamine: *Boletus* (Melin and Nyman, 1940, 1941; Melin and Norkrans, 1942), *Clitocybe* (Lindeberg, 1946a), *Coprinus* (L. Fries, 1945, 1955), *Exobasidium* (Sundström,

1960), *Marasmius* (Lindeberg, 1944), *Mycena* (Fries, 1949), *Peniophora* (Fries, 1950), *Polyporus* (Fries, 1938; Noecker, 1938), *Tricholoma* (Norkrans, 1950), and *Lactarius* (Jayko *et al.*, 1962). Among phycomycetes thiamine is the only necessary growth factor for most of the tested species within the genera *Phialophora* (Arêa Leão and Cury, 1950) and *Phytophthora* (Robbins, 1938), whereas the *Mortierella* (Robbins and Kavanagh, 1938b; Schopfer, 1935), *Mucor* (Robbins and Kavanagh 1938b; and others), and *Rhizopus* spp. (Schopfer, 1935) with few exceptions are totally auxoautotrophic. Yeasts and other ascomycetes often require one or more other growth factors, like biotin or pyridoxine, in addition to thiamine (see Table I and Section V, F). The dermatophytes are mostly auxoautotrophic or thiamine-heterotrophic (Arêa Leão and Cury 1950; Drouhet and Mariat, 1953; Georg and Camp, 1957).

The thiamine molecule (I) consists of two units, "moieties," a pyrimidine (II) and a thiazole (III), which can be separated rather easily by heating or by chemical means. Almost all thiamine-heterotrophic fungi grow just as well (sometimes even better: Norkrans, 1950) with the two moieties added together in equimolar concentrations as with the intact thiamine. *Trichophyton discoides* (Robbins *et al.*, 1942), *Kloeckera brevis* (Jones and Finch, 1959) and species of *Phytophthora* are among the very few fungi that require the whole molecule (Robbins, 1938).

On the other hand, many fungi are obviously capable of synthesizing the thiazole half of the molecule and thus require only the pyrimidine, like *Ustilago longissima* (Schopfer and Blumer, 1938), *Polyporus adustus* (Schopfer and Blumer, 1940), *Collybia tuberosa* (Leonian and Lilly 1938), several species of *Coprinus* (L. Fries, 1945), *Marasmius fulvo-bulbillosus* (Lindeberg, 1944), and several species of *Tricholoma* (Norkrans, 1950). Ability to synthesize the pyrimidine, but not the thiazole half, seems to be less common; the following species, however, represent this type: *Mucor ramannianus* (Müller and Schopfer, 1937), *Stereum frustulosum* (Noecker and Reed, 1943), *Endomyces magnusii* (Meissel, 1947) and *Trichophyton concentricum* (Georg and Camp, 1957).

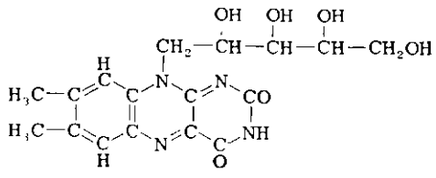
The biosynthesis of thiamine is usually assumed to take place by the condensation of the two above-mentioned precursors, the pyrimidine and the thiazole. However, there is some evidence in favor of another pathway where pyrimidine is first coupled to a thiazole precursor and the compound thus formed is then transformed to thiamine (Harris, 1956).

Certain thiamine-autotrophic species of *Rhizopus* (Schopfer, 1935), *Fusarium* (Elliott, 1949; Esposito *et al.*, 1962), and *Ciborinia* (Lilly and Barnett, 1948b) respond to added thiamine by growth inhibition. This is obviously due to the increased production of ethyl alcohol by the fungus, a change in the metabolism of the mycelium which is caused by the surplus

of thiamine in the medium (Schopfer and Guilloud, 1945). This effect reflects the metabolic function of thiamine as a component of pyruvate decarboxylase, where thiamine pyrophosphate is the prosthetic group. In *Saccharomyces carlsbergensis* the thiamine-induced inhibition can be reversed by pyridoxine (Chiao and Peterson, 1956), and in *Fusarium* by biotin or by vigorous aeration (Esposito *et al.*, 1962).

Very few changes can be made in the constitution of the thiamine molecule without loss of activity (see Robinson, 1951). On the other hand, compounds of apparently rather different constitution are able to serve as sources of the thiazole moiety, e.g., penicillin (Shulman *et al.*, 1957) and bacitracin (Ebringer, 1960). As a growth factor thiamine pyrophosphate (cocarboxylase) seems to be just as effective as free thiamine (Lilly and Leonian, 1940; Sedlmayr *et al.*, 1961). However, this does not necessarily mean that the thiamine pyrophosphate is taken up as such by the cells.

B. Riboflavine

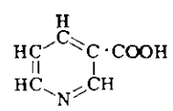


Riboflavine

(IV)

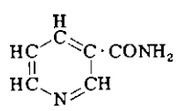
Poria vaillantii is the only fungus so far found which requires an external supply of riboflavine (plus thiamine, biotin, and adenine: Jennison *et al.*, 1955). No further particulars of this case have been published. However, the fact that many bacteria with this requirement are known and that riboflavine-less mutants have been induced and isolated in *Neurospora* and *Aspergillus* makes it quite possible that riboflavine heterotrophy may be found as a natural species character in more fungi than *P. vaillantii*.

C. Nicotinic Acid



Nicotinic acid

(V)



Nicotinamide

(VI)

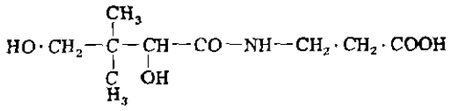
Species requiring nicotinic acid (= niacin; V) or nicotinamide (VI) occur in almost all the main groups of fungi. Among phycomycetes *Phlyctorhiza variabilis* (Rothwell, 1956), *Blastocladia pringsheimii* (Cantino, 1948; Crasemann, 1957), and *B. ramosa* (Crasemann, 1957) require this vitamin together with one or two other growth factors. In various genera of yeasts, e.g., *Saccharomyces*, *Torula*, *Mycotorula*, *Candida*, and *Kloeckera*, niacin heterotrophy seems to be rather common (Burkholder, 1943; Koser and Wright, 1943; Rogosa, 1943; Burkholder *et al.*, 1944; Miyashita *et al.*, 1958). Furthermore, the two dermatophytes *Microsporum audouinii* (Arêa Leão and Cury, 1950) and *Trichophyton equinum* (Georg, 1949) require niacin, this nutritional peculiarity in the latter case probably being the most reliable species character. So far no clear-cut case of niacin heterotrophy has been observed in basidiomycetes. Although *Pholiota aurea* is enabled to grow with niacin as the only growth-factor supplied, growth also occurs, and at a faster rate, with thiamine alone (Bach, 1956).

The biologically active form of niacin is nicotinamide, which constitutes a part of the phosphopyridine nucleotides, NAD and NADP, both of which represent prosthetic groups of enzyme systems for hydrogen transfer. As growth factors for fungi (in contrast to some bacteria), nicotinic acid and its amide seem to be equally active, the transformation to the active amide thus obviously being easily accomplished.

Niacin-less mutants seem to be easily induced in most fungi investigated from this point of view. Mutants of this type obviously also arise spontaneously and can be picked up from cell populations of niacin-independent species: *Ophiostoma multiannulatum* (Fries, 1948), *Glomerella cingulata* (Andes and Keitt, 1950), and *Polyporus abietinus* (a micrurgically isolated neohaplont: Fries and Aschan, 1952).

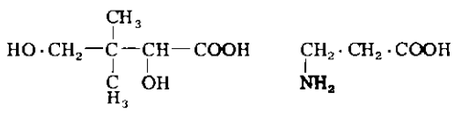
In contrast to many induced niacin-less mutants, the niacin-heterotrophic species usually cannot substitute tryptophan or other indole derivative for niacin. *Trichophyton equinum* constitutes an exception since it is able to utilize tryptophan to some degree (Georg, 1949; Drouhet and Maria, 1953). However, in most cases the effect of niacin precursors and analogs has not been investigated.

D. Pantothenic Acid



Pantothenic acid

(VII)



Pantoic acid (VIII) β-Alanine (IX)

Pantothenic acid (VII) was originally isolated as a growth factor for certain strains of yeasts (Williams, 1940). Later investigations revealed that a great number of species within the Saccharomycetales required pantothenic acid for growth, or at least for good growth, the following genera being represented: *Saccharomyces, Schizosaccharomyces, Zygosaccharomyces, Torula,* and *Candida* (Lochhead and Landerkin, 1942; Leonian and Lilly, 1942; Burkholder, 1943; Burkholder *et al.,* 1944; Schultz and Atkin, 1947; McVeigh and Bracken, 1955; Miyashita *et al.,* 1958; van Uden and Carmo-Sousa, 1959). The requirement can be absolute, as in *Schizosaccharomyces pombe* (McVeigh and Bracken, 1955), or partial, as in most other cases.

Polyporus texanus seems to be the only filamentous fungus hitherto reported as deficient for pantothenic acid (Yusef, 1953). The deficiency, which seemed to be almost complete, is probably a species character since the two isolates tested both responded in the same way. Occasional strains that do not require pantothenic acid have also been found in *Ophiostoma multiannulatum;* probably they originated from spontaneous mutations within this otherwise thiamine-pyridoxine-heterotrophic species (Fries, 1948).

It has furthermore been reported that a shortening of the lag phase in liquid cultures of *Penicillium digitatum* can be induced by pantothenic acid, as well as by some other vitamins (Wooster and Cheldelin, 1945; Fergus, 1952).

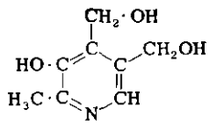
Pantothenic acid, in itself an intermediate in the formation of coenzyme A, is normally synthesized in the cell from the two precursors pantoic acid (VIII) and β-alanine (IX). At least some yeasts, e.g., *Schizosaccharomyces pombe* (McVeigh and Bracken, 1955), can do with β-alanine alone, the synthesis of pantoic acid and the following condensation reaction apparently being performed without difficulty. In *Polyporus texanus* the situation is reversed, since only pantoic acid is required (Yusef, 1953).

An originally vitamin-free medium may after autoclaving contain traces of β-alanine formed through a reaction between glucose and ammonia (Nielsen and Dagys, 1940; Betz, 1960). Another fact which must be considered when interpreting growth experiments with pantothenate-requiring fungi in sterilized solutions is that aspartic and glutamic acid as well as their

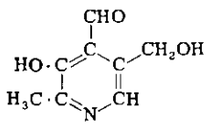
amides act as antagonists to β-alanine, but not to pantothenic acid (Betz, 1960).

Quispel (1944) in his study of lichen symbiosis isolated some sterile mycelia, the growth of which proved to be strongly promoted by β-alanine. They occurred in nature loosely connected with green algae on the bark of trees, and their taxonomic position could not be ascertained.

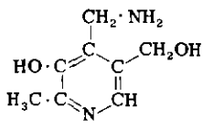
E. Pyridoxine (Vitamin B_6)



Pyridoxine
(X)



Pyridoxal
(XI)



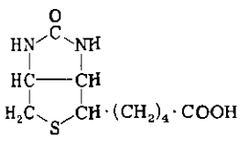
Pyridoxamine
(XII)

As soon as pyridoxine (X) had been isolated as a vitamin for rats, its growth-promoting effect on various microorganisms was demonstrated by several workers independently. As regards fungi the response of *Saccharomyces cerevisiae* to pyridoxine was first observed (Schultz *et al.*, 1938, 1939). Later, several other species of yeast were found to be partially or totally deficient in this vitamin (Burkholder, 1943; Snell and Rannefeld, 1945). The first examples of filamentous fungi requiring pyridoxine were demonstrated in the genus *Ophiostoma* (*Ceratostomella, Graphium*) by various workers at about the same time (Fries, 1942, 1943; Robbins and Ma, 1942b,c) and other cases were then found within various groups of fungi, e.g., *Trichophyton discoides* (Robbins *et al.*, 1942), *Ascoidea rubescens* (Fries, 1943), *Torulopsis dattila* (Burkholder *et al.*, 1944) and *Leptographium* sp. (Leaphart, 1956). So far, pyridoxine heterotrophy has been observed only in the Ascomycetes and the Fungi Imperfecti, this particular deficiency being as a rule combined with a requirement for some other growth factor, usually thiamine.

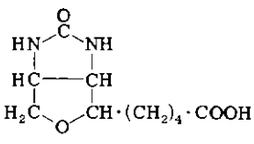
Pyridoxine is only one of the three active, unphosphorylated forms of vitamin B_6 that occur in nature, the other two being pyridoxal (XI) and pyridoxamine (XII). Bacteria are known to respond differently to these three forms and to their phosphorylated derivatives. Very little is known about fungi in this respect. However, *Saccharomyces carlsbergensis* responds to pyridoxine, pyridoxamine and pyridoxal almost equally well (Melnick *et al.*, 1945) and the same can be said about *Ophiostoma multiannulatum* (Wikberg, 1959). *S. cerevisiae*, on the other hand, grows best with pyridoxine (Snell, 1944), and in *O. multiannulatum* mutants occur which specifically require pyridoxamine (Wikberg, 1959).

The effect of pyridoxine on growth often depends on the presence of other metabolites in the medium, notably thiamine (Rabinowitz and Snell, 1951; Harris, 1956; Sakuragi and Kummerow, 1957; Morris *et al.*, 1959). The real nature of these interactions is still obscure.

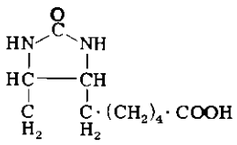
F. Biotin



Biotin
(XIII)



Oxybiotin
(XIV)



Dethiobiotin
(XV)

Like pantothenic acid, biotin (XIII) was first recognized as a growth factor for certain yeasts (Kögl and Tönnis, 1936; Williams *et al.*, 1940). After having become available in pure, crystalline form in 1936, its importance as a growth factor for fungi from many other systematic groups was demonstrated by a long series of investigations by numerous workers. A steadily increasing number of species have been found that require biotin as the only growth factor. The first example recorded was *Melanospora destruens* (Hawker, 1939). Species representing this type of auxoautotrophy have since been found in *Neurospora* (Butler *et al.*, 1941), *Grossmannia* (Robbins and Ma, 1942a), *Mitrula* (Fries, 1943), *Candida* (Burkholder, 1943; Burkholder and Moyer, 1944; Miyashita *et al.*, 1958), *Debaryomyces, Hansenula, Torula,* and *Torulopsis* (Burkholder 1943; Burkholder and Moyer, 1944), *Memnoniella* and *Stachybotrys* (Marsh and Bollenbacher, 1946; Perlman, 1948), *Sordaria* (Lilly and Barnett, 1947; Olive and Fantini, 1961), *Histoplasma* (Salvin, 1949, cf. Pine, 1957), *Allescheria* (Arêa Leão and Cury, 1950), *Blastomyces* (Halliday and McCoy, 1955), *Claviceps* (Taber and Vining, 1957), *Gelasinospora* (Hackbarth and Collins, 1961), *Gloeosporium, Nigrospora, Verticillium* (Esposito *et al.*, 1962) and *Dipodascus* (Batra, 1963).

Very often a biotin-deficient fungus also requires thiamine. Species of this nutritional type have been found in *Lophodermium, Valsa, Hypoxylon,* and *Melanconium* (Fries, 1938), *Marasmius* (Lindeberg, 1939), *Ophiobolus* (White, 1941), *Ophiostoma* (Robbins and Ma, 1942b; Fries, 1943), *Debaryomyces* (Burkholder, 1943), *Saccharomyces* (Burkholder *et al.*, 1944), *Collybia* (Lindeberg, 1946b), *Pyricularia* (Leaver *et al.*, 1947; Sadasivan and Subramanian, 1954), *Chaetomium* (Lilly and Barnett,

1949), *Lachnum* and *Spathularia* (Fries, 1950), *Trichosporon* (Arêa Leão and Cury, 1950), *Endothia* and *Podospora* (Lilly and Barnett, 1951), *Sepedonium* (Painter, 1954), *Isaria* (Taber and Vining, 1959), *Glomerella* (Srinivasan and Vijayalakshimi, 1960), *Gloeocercospora* (Malca and Ullstrup, 1960), *Lactarius* (Jayko *et al.*, 1962), and *Sordaria* (Fields and Maniotis, 1963). In other cases (see Table II) the biotin heterotrophy is combined with some other growth factor deficiency, as in *Ascoidea rubescens* (Fries, 1943), *Kloeckera brevis* (Burkholder *et al.*, 1944), *Poria vaillantii* (Jennison *et al.*, 1955), and several yeasts (Rogosa, 1943; Burkholder and Moyer, 1943; Drouhet and Vieu, 1957; Miyashita *et al.*, 1958). In *Candida* almost all species require biotin, usually together with one or more other growth factors (for references to the comprehensive literature on this genus see Firestone and Koser, 1960). Among the ascomycetes, which normally live together with algae in the lichen symbiosis, biotin- as well as biotin-thiamine-heterotrophic species have been found (Hale, 1958).

In many cases biotin heterotrophy is only partial. Since the growth rate at very low concentrations of biotin usually is rather low, it is often difficult to decide whether the requirement is absolute or not. A very slow adaptation to a biotin-free medium can often be observed, e.g., in *Saccharomyces* (Leonian and Lilly, 1942; Wikén and Richard, 1951).

In oxybiotin (= *O*-heterobiotin) (XIV) the sulfur atom is replaced by oxygen. At least *S. cerevisiae* (Hofmann and Winnick, 1945; Axelrod *et al.*, 1947), *Memnoniella echinata*, and *Stachybotrys atra* (Perlman, 1948), and *Candida albicans* (Firestone and Koser, 1960) are able to substitute this compound for biotin although with some difficulty, the activity of oxybiotin being only 10–25% that of biotin.

On the other hand, in dethiobiotin (XV), the sulfur atom is removed without any replacement. This substance is as active as biotin for some fungi, e.g., *Neurospora crassa* (Lilly and Leonian, 1944), whereas for others, e.g., *Ceratostomella* (*Ophiostoma*) *pini* (Lilly and Leonian, 1944), it acts as an inhibitor. To some degree its mode of action seems to depend on the concentration tested. Dethiobiotin is probably a natural precursor in the biosynthesis of biotin, the biosynthetic chain being blocked either before or after the formation of dethiobiotin in various types of biotin heterotrophy.

An interesting observation is that the biotin-autotrophic *Aspergillus niger* produces not only biotin, but also biotin sulfoxide (Wright *et al.*, 1954). Production of this compound is increased if pimelic or azelaic acid is added to the *Aspergillus* medium. In some fungi the activity of biotin sulfoxide is the same as that of biotin, in others considerably lower.

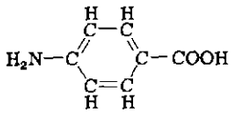
Another biologically active biotin derivative is biocytin, which was isolated from yeast by Wright and collaborators (Wright *et al.*, 1951) and identified as ε-*N*-biotinyl-L-lysine. *Isaria cretacea* responds to biocytin (Taber and Vining, 1959), and *Saccharomyces carlsbergensis* to biocytin as well as to some other derivatives, viz., biotinamide, *N*-biocytin-*p*-aminobenzoic acid and *N*-biotinyl-β-alanine (Wright *et al.*, 1951).

In cellular metabolism biotin is involved in a number of seemingly very different processes, e.g., in the conversion of ornithine to citrulline, in carboxylation reactions, in the hexokinase system, in the synthesis of fatty acids and in the deamination of certain amino acids. This explains why the requirement for biotin sometimes can be at least partially satisfied by the addition of metabolites which constitute products of reactions where biotin functions as a coenzyme. Thus aspartic acid exerts a sparing action in *Torula cremoris* (Koser *et al.*, 1942), in *Memnoniella echinata* and *Stachybotrys atra* (Perlman, 1948), and in *Candida albicans* (Firestone and Koser, 1960), whereas aspartic or oleic acid is able to spare or replace biotin in *Ophiostoma pini* (Mathiesen, 1950). In *C. albicans* biotin can be exchanged for glyceryl monooleate, but not by oleic acid (Nickerson, 1961).

More difficult to understand are the interactions between biotin and niacin (Sundaram *et al.*, 1954), between biotin and inositol (Tirunarayanan and Sarma, 1953) in *Neurospora*, and between biotin and indoleacetic acid in *Nectria* (Quintin-Jerebzoff, 1959).

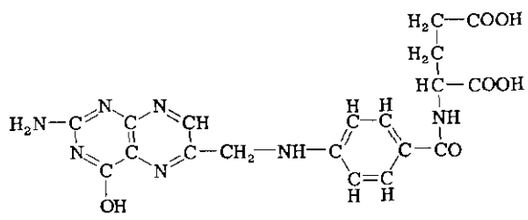
The natural biotin molecule has a side chain containing 4 methyl groups. The two analogs, norbiotin, with 3, and homobiotin, with 5 methyl groups seem to be inactive for most fungi as substitutes for biotin, e.g., in *Claviceps purpurea* (Taber and Vining, 1957), *Isaria cretacea* (Taber and Vining, 1959), and *Candida albicans* (Firestone and Koser, 1960). However, they can replace biotin for *Saccharomyces globosus* and are strong biotin antagonists for *Zygosaccharomyces barkeri* (Belcher and Lichstein, 1949). Most other analogs of biotin have an antagonistic effect.

G. *p*-Aminobenzoic Acid and the Folic Acid Group



p-Aminobenzoic acid

(XVI)



Pteroylglutamic acid

(XVII)

p-Aminobenzoic acid (PABA) (XVI) forms part of the folic acid molecule, derivatives of which serve as coenzymes in reactions where one-carbon compounds are transferred. Folic acid exists in a number of chemically different forms, viz. rhizopterin, pteroylglutamic acid (XVII), pteroyltriglutamic acid, pteroylheptaglutamic acid, leucovorin, and biopterin. Unlike many bacteria, the fungi always seem to be able to synthesize the necessary folic acid coenzyme, at least if PABA is available as a precursor.

Although PABA-less mutants have been artifically induced in several species of fungi, a requirement for PABA in wild-type strains is rare. A few examples of this have been found in the genus *Rhodotorula* (Robbins and Ma, 1944; Hasegawa and Banno, 1959; Nyman and Fries, 1962; Ahearn *et al.*, 1962). Furthermore, single strains of *Saccharomyces cerevisiae* (Rainbow, 1948) and *Blastocladia pringsheimii* (Crasemann, 1957) have been found to require PABA together with other growth factors.

The growth of *S. cerevisiae* is inhibited by concentrations of PABA higher than 25 μg/ml, an inhibition that is accompanied by the accumulation of shikimic acid in the medium (Reed *et al.*, 1959). Normal growth can be restored by the addition of certain aromatic amino acids.

Whether *B. pringsheimii* responds to folic acid (XVII) is not known, but *S. cerevisiae* is unable to utilize it (Woods, 1954). However, at least one *Rhodotorula* strain, when investigated from this point of view, proved capable of growing with folic acid, although 10 to 50 times more of this growth factor (in terms of moles) than of PABA were required for the same amount of growth. There is some evidence indicating that folic acid is not directly incorporated, but that it is first broken down and only the PABA part utilized (Nyman and Fries, 1962).

According to Moser (1960) the growth of several species belonging to the section *Phlegmacium* of *Cortinarius* is strongly stimulated by folic acid, but not by PABA. However, in these cases growth can also occur, although slowly, without folic acid.

In some investigations a mixture of amino acids and purines to an extent could be substituted for PABA (Cutts and Rainbow, 1950; Nyman and

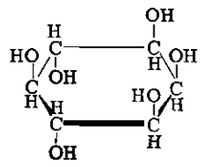
Fries, 1962). This probably means that the biosynthesis of these substances is controlled by folic acid, of which PABA constitutes an essential structural unit.

H. The Vitamin B_{12} Group

It seems clear now that fungi produce substances with vitamin B_{12} activity (Tanner, 1960), and so the conclusion appears justified that one or more of these substances plays a role in the metabolism of fungi. However, mutations involving a deficiency in the biosynthesis of a vitamin B_{12} factor have never been observed. So far only one naturally occurring complete deficiency of this type has been found, viz. in *Thraustochytrium globosum* (Adair and Vishniac, 1958). This marine phycomycete requires cyanocobalamin or vitamin B_{12-III} for growth, four other tested B_{12} factors being inactive. Cyanocobalamin proved to be active at concentrations as low as 5 $\mu\mu$g/ml.

Evidence for a partial deficiency for B_{12} in *Candida albicans* has been presented by Littman and Miwatani (1963), the active concentrations in this case being 1 μg/ml and higher.

I. Inositol



myo-Inositol

(XVIII)

As early as 1928 it was shown (Eastcott, 1928) that the growth of yeast (*Saccharomyces*) is strongly promoted by *myo*-inositol (XVIII) (also called *meso*-inositol). Since then a great number of species within various groups of Ascomycetes has proved to be partially or totally inositol-heterotrophic. As examples may be mentioned members of the genera *Ashbya* (=*Nematospora*) (Buston and Pramanik, 1931), *Lophodermium, Melanconium,* and *Valsa* (Fries, 1938), *Ceratostomella* (*Ophiostoma*) (Robbins and Ma, 1942b), *Kloeckera* (Burkholder, 1943), *Eremothecium* (Schopfer, 1944a,b; Dulancy and Grutter, 1950; Krneta-Jordi, 1962), *Pichia* (Burkholder and Moyer, 1944), *Epichloë* (Lilly and Barnett, 1949), *Sclerotinia* (Lilly and Barnett, 1951), *Torulopsis* (Ribereau-Gayon *et al.,* 1955), and *Diplocarpon* (Shirakawa, 1955). As regards the numerous

publications dealing with inositol requirements of *Saccharomyces, Schizosaccharomyces,* and *Zygosaccharomyces,* and also *Trichophyton,* reference may be made to Fries (1961a).

Inositol heterotrophy is almost always associated with a requirement for thiamine or biotin.

In *Saccharomyces carlsbergensis* the addition of niacin removes the stimulation of inositol, an effect that may be explained as an inhibition of inositol catabolism by niacin (Brackkan and Boge, 1963).

The metabolic function of inositol is still unknown. From experiments with inositol-deficient *S. carlsbergensis* and *Kloeckera apiculata* it has been tentatively concluded that inositol is necessary for maintaining the structure of essential cytoplasmic structures, possibly the mitochondria (Ridgeway and Douglas, 1958).

In all cases where a growth response has been observed the lowest active concentration has been $\sim 1 \mu g/ml$. Therefore inositol should perhaps not be included among the growth factors *sensu stricto*—the vitamins—but belongs rather to the group of other metabolites capable of affecting growth when present in amounts of $\leqq 1$ $\mu g/ml$.

J. Some Other Substances Active in Low Concentration

Hemin is known to form an essential constituent of the nutrient medium for species of *Pilobolus* (Page, 1952). Several hemin derivatives are effective, e.g., coprogen (Hesseltine *et al.,* 1953) and ferrichrome (Neilands, 1953), the chemical constitution not yet having been completely elucidated for all of them. The amount of hemin necessary for optimum growth is somewhat higher than that of the ordinary vitamins, viz. a few milligrams per liter (Page, 1952).

Some vitamins or vitamin-like compounds are known to occur as growth factors only for bacteria, e.g., lipoic acid, mevalonic acid, choline, and vitamin K. Nutritional mutants requiring these factors may occur among fungi, too, and stimulatory effects of, for example, choline have been noted (Andes and Keitt, 1950; Lewis, 1952; Thind and Sharma, 1960). There are also a few observations of a favorable influence of sterols on yeasts (Devloo, 1938; Andreasen and Stier, 1953) and filamentous fungi (Weintraub *et al.,* 1958; Jefferson and Siseo, 1959, 1961; Matkovics, 1960). In yeasts ergosterol seems to be necessary for growth under anaerobic conditions (Andreasen and Stier, 1953). An aliphatic hydrocarbon, 2,3-dimethyl-1-pentene, produced by the mycelium of *Agaricus campestris* was found to stimulate the germination of the spores of this fungus (McTeague *et al.,* 1959). Being a volatile compound, it influenced the spores through the

gas phase. Other metabolites of fungi also are known to affect spore germination (see Volume II).

As regards the effect of the plant hormone, auxin, on fungal growth the numerous reports are conflicting, inhibition as well as stimulation having been observed (cf. Gentile and Klein, 1955).

K. Fatty Acids, Nucleotide Constituents, Amino Acids, and Related Substances

Most of the substances to be dealt with under this heading produce their effects on growth only if present in concentrations of at least a few milligrams per liter ($\sim$0.01 *M*). In this respect they differ from all the growth factors (except inositol) discussed in the preceding paragraphs. They have also other functions than that of forming part of prosthetic groups in enzymes.

The stimulatory activity of organic acids often can be interpreted as a consequence of the buffering or chelating properties of the substance in question. As examples may be mentioned citric acid and glycine. It is sometimes difficult to make sure that the growth-promoting activity of, for example, an amino acid is indeed an expression of a biosynthetic deficiency, especially since these requirements, with few exceptions, are partial rather than total. However, requirements which seem to be truly specific have been found for arginine and leucine in *Eremothecium ashbyii* (Schopfer and Guilloud, 1945), for histidine in *Trichophyton megninii* (Georg, 1952), for methionine in *Candida albicans* (one strain), for cysteine in *C. albicans* (one strain) and *Mycoderma vini* (McVeigh and Bell, 1951), and for histidine and methionine in *Schizosaccharomyces octosporus* (Northam and Norris, 1951). The last-mentioned fungus also requires adenine, like *Fomes officinalis* and *Poria vaillantii* (Jennison *et al.*, 1955).

The requirement for an amino acid may be conditioned by the composition of the medium as in *E. ashbyii*. Arginine and leucine are indispensable for this fungus only if glycine is the source of nitrogen. They become unnecessary when glycine is exchanged for asparagine; in that case, however, methionine or some other suitable source of reduced sulfur becomes an indispensable component of the medium at pH values below 6 (Yaw, 1952; Krneta-Jordi, 1962).

For many aquatic phycomycetes methionine or cystein are seemingly indispensable for growth. In these cases it is not a specific requirement, but only a need for a compound containing reduced sulfur (parathiotrophy) (Volkonsky, 1933; Cantino, 1955).

The demand for methionine, histidine, and adenine in certain strains of

Saccharomyces cerevisiae occurs only in the absence of PABA and is obviously an example of the "by-passing" of a vitamin requirement (Cutts and Rainbow, 1950). Several other analogous cases are known (see page 497).

As regards the numerous reports of the merely stimulatory effects of various metabolites, particularly amino acids and nucleotide constituents, reference may be made to Fries (1961a), where these matters are reviewed.

Pityrosporum ovale is unique insofar as it requires a fatty acid as a growth factor. This was first demonstrated by Benham (1939, 1941, 1947), who cultivated *Pityrosporum* in a synthetic medium supplemented with oleic acid. However, later studies have revealed that pure oleic acid is inactive in itself, whereas myristic or palmitic acid, which occur as contaminants in oleic acid, are able to support growth of the fungus (Shifrine and Marr, 1963). Whether these C_{14} or C_{16} acids are active also in other cases where growth-promoting effects of oleic acid have been observed, e.g., in species of *Polyporus* (Yusef, 1953) and in *Claviceps purpurea* (Taber and Vining, 1957), remains to be tested. Yeasts seem to require oleic acid for anaerobic growth (Andreasen and Stier, 1954; Lennarz and Bloch, 1960); under these conditions 9- and 10-hydroxystearic acid are also active, probably by serving as precursors to oleic acid (Bloch *et al.*, 1961).

In this connection the strong effects of certain aliphatic aldehydes ought to be mentioned. These saturated, straight-chain aldehydes, especially nonanal, but also hexanal, heptanal and octanal, promote the growth of various fungi both when dissolved in the medium and as part of the gas phase (Fries, 1960, 1961b). Nonanal also stimulates the germination of wheat rust spores (French and Weintraub, 1957). The mode of action is so far unknown.

L. Unidentified Growth Factors

Many observations have been published that indicate the existence of growth factors not identical with those already known. Future work will show if the substances postulated in these studies as being respousible for the observed effects can be classified as growth factors in a strict sense. Space does not permit an enumeration of all these cases; a few examples must suffice.

Spore germination and growth of various mycorrhiza-forming fungi is strongly stimulated by a substance, provisionally called "factor M," which is exuded from seed plant roots (Melin, 1954). The mycoparasite *Gonatobotrys simplex* can be grown in pure culture only if an extract from the host or other fungi is added; these extracts contain a growth factor, called "mycotrophein," which has been partially purified but not yet chem-

ically identified (Whaley and Barnett, 1963). Other fungi have been found to respond strongly to extracts from jute (Basu, 1949), malt (Yusef, 1953; Robbins and Hervey, 1955), tomato juice and wood (Robbins and Hervey, 1958, 1959, 1963), agar (von Weihe, 1958, 1961), wood pulp (Brewer, 1961), coconut milk (Johansson, 1962), and yeast extract (Esposito *et al.*, 1962). A volatile compound produced by wood promotes the growth of a number of wood-destroying hymenomycetes (Suolahti, 1951). This compound may be identical with nonanal, although definite proof is still lacking (Fries, 1960, 1961b).

REFERENCES

Adair, E. J., and H. S. Vishniac. (1958). Marine fungus requiring vitamin B_{12}. *Science* **127**:147–148.

Ahearn, D. G., F. J. Roth, and S. P. Meyers. (1962). A comparative study of marine and terrestrial strains of *Rhodotorula*. *Can. J. Microbiol.* **8**:121–132.

Andes, J. O., and G. W. Keitt. (1950). Variability of *Glomerella cingulata* (Stonem.) S. & v.S. from apples. *Phytopathology* **40**:915–925.

Andreasen, A. A., and T. J. B. Stier. (1953). Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cellular Comp. Physiol.* **41**:23–36.

Andreasen, A. A., and T. J. B. Stier. (1954). Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. *J. Cellular Comp. Physiol.* **43**:271–281.

Arêa Leão, A. E. de, and A. Cury. (1950). Deficiencias vitaminicas de cogumelos patogenicos. *Mycopathol. Mycol. Appl.* **5**:65–90.

Axelrod, A. E., B. C. Flinn, and K. Hofmann. (1947). The metabolism of oxybiotin in yeast. *J. Biol. Chem.* **169**:195–202.

Bach, E. (1956). The Agaric *Pholiota aurea*, 220 pp. Thesis, Copenhagen. (Also in *Dansk Botan. Arkiv* **16**:No. 2.)

Bartnicki-Garcia, S., and W. J. Nickerson. (1961). Thiamine and nicotinic acid: anaerobic growth factors for *Mucor rouxii*. *J. Bacteriol.* **82**:142–148.

Basu, S. N. (1949). Effect of water soluble matter in jute on the microbiological deterioration of the fibre. *J. Sci. Ind. Res. (India)* **8B**:73–77.

Batra, L. R. (1963). Habitat and nutrition of *Dipodascus* and *Cephaloascus*. *Mycologia* **55**:508–520.

Belcher, M. R., and H. C. Lichstein. (1949). Growth promotion and antibiotin effect of homobiotin and norbiotin. *J. Bacteriol.* **58**:579–583.

Benham, R. W. (1939). The cultural characteristics of *Pityrosporum ovale*—a lipophylic fungus. *J. Invest. Dermatol.* **2**:187–203.

Benham, R. W. (1941). Cultural characteristics of *Pityrosporum ovale*—a lipophylic fungus. Nutrient and growth requirements. *Proc. Soc. Exptl. Biol. Med.* **46**:176–178.

Benham, R. W. (1947). Biology of *Pityrosporum ovale*. *Ann. Cryptogamici Phytopathologici* **6**:63–70.

Betz, A. (1960). Das Pantothensäurebedürfnis der Hefe. *Arch. Mikrobiol.* **35**:1–33.

Bloch, K., P. Baronowsky, H. Goldfine, W. J. Lennarz, R. Light, A. T. K. Norris,

and G. Scheuerbrandt. (1961). Biosynthesis and metabolism of unsaturated fatty acids. *Federation Proc.* **20**:921–927.

Blumer, S. (1940). Über die Aneurinheterotrophie bei einigen antherenbewohnenden Brandpilzen. *Mitt. Naturforsch. Ges. Bern.* pp. 19–36.

Braekkan, O. R., and G. Boge. (1963). Influence of niacin on the growth-stimulating effect of inositol on *Saccharomyces carlsbergensis*. *Nature* **198**:585–586.

Brewer, D. (1961). Slime accumulations in pulp and paper mills. V. Effect of extracts of spruce and fir on the growth of *Phialophora fastigiata*. *Can. J. Botany* **39**:1579–1583.

Burgeff, H. (1934). Pflanzliche Avitaminose und ihre Behebung durch Vitaminzufuhr. Vorläufige Mitteilung. *Ber. Deut. Botan. Ges.* **52**:384–390.

Burkholder, P. R. (1943). Vitamin deficiencies in yeasts. *Am. J. Botany* **30**:206–211.

Burkholder, P. R., and D. Moyer. (1943). Vitamin deficiencies of fifty yeasts and molds. *Bull. Torrey Botan. Club* **70**:372–377.

Burkholder, P. R., and D. Moyer. (1944). Studies on some growth factors of yeasts. *J. Bacteriol.* **48**:385–391.

Burkholder, P. R., I. McVeigh, and D. Moyer. (1944). Studies on some growth factors of yeasts. *J. Bacteriol.* **48**:385–391.

Buston, H. W., and B. N. Pramanik. (1931). The accessory factor necessary for the growth of *Nematospora gossypii*. I. The chemical nature of the accessory factor. *Biochem. J.* **25**:1656–1670.

Butler, E. T., W. J. Robbins, and B. O. Dodge. (1941). Biotin and the growth of *Neurospora*. *Science* **94**:262–263.

Cantino, E. C. (1948). The vitamin nutrition of an isolate of *Blastocladia pringsheimii*. *Am. J. Botany* **35**:238–242.

Cantino, E. C. (1955). Physiology and phylogeny in the water molds—a re-evaluation. *Quart. Rev. Biol.* **30**:138–149.

Chiao, J. S., and W. H. Peterson. (1956). Some factors affecting the inhibitory action of thiamine on the growth of *Saccharomyces carlsbergensis*. *Arch. Biochem. Biophys.* **64**:115–128.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Cook, F. D., and A. G. Lochhead. (1959). Growth factor relations of soil microorganisms as affected by proximity to the plant root. *Can. J. Microbiol.* **5**: 323–334.

Crasemann, J. M. (1957). Comparative nutrition of two species of *Blastocladia*. *Am. J. Botany* **44**:218–224.

Cutts, N. S., and C. Rainbow. (1950). Studies of a yeast exacting towards *p*-aminobenzoic acid. *J. Gen. Microbiol.* **4**:150–155.

Devloo, R. (1938). Un stérol indispensable à la levure W. *Arch. Intern. Physiol.* **46**:157–188.

Drouhet, E., and F. Mariat. (1953). Nutrition et facteurs de croissance des dermatophytes. *In* "Nutrition and Growth Factors." Symposium, September, 1953. Ist. Superiore di Sanità, Rome.

Drouhet, E., and M. Vieu. (1957). Facteurs vitaminigues de croissance des *Candida*. *Ann. Inst. Pasteur* **92**:825–831.

Dulaney, E. L., and F. H. Grutter. (1950). The nutritional requirements of *Eremothecium ashbyii* Guill. *Mycologia* **42**:717–722

Eastcott, E. V. (1928). Wildiers' bios. The isolation and identification of "Bios I." *J. Phys. Chem.* **32**:1094–1111.

Ebringer, L. (1960). Die Möglichkeit eines Ersatzes von Thiamin durch Bacitracin bei *Phycomyces blakesleeanus*. *Naturwissenschaften* **47**:210.

Elliott, E. S. (1949). Effect of vitamins on growth of some graminicolous species of *Helminthosporium* and *Fusarium*. *Proc. West. Va. Acad. Sci.* **20**:65–68.

Esposito, R. G., H. Greenwood, and A. M. Fletcher. (1962). Growth-factor requirements of six fungi associated with fruit decay. *J. Bacteriol.* **83**:250–255.

Fergus, C. L. (1952). The nutrition of *Penicillium digitatum* Sacc. *Mycologia* **44**: 183–199.

Fields, W. G., and J. Maniotis. (1963). Some cultural and genetic aspects of a new heterothallic *Sordaria*. *Am. J. Botany* **50**:80–85.

Fincham, J. R. S., and P. R. Day. (1963). "Fungal Genetics," 300 pp. Blackwell, Oxford.

Firestone, B. Y., and S. A. Koser. (1960). Growth promoting effect of biotin analogs for *Candida albicans*. *J. Bacteriol.* **79**:674–676.

French, R. C., and R. L. Weintraub. (1957). Pelargonaldehyde as an endogenous germination stimulator of wheat rust spores. *Arch. Biochem. Biophys.* **72**: 235–237.

Fries, L. (1945). Über das Wachstum einiger *Coprinus*-Arten bei verschiedenen Wasserstoffionenkonzentrationen. *Arkiv Botan.* **32A**: No. 10, 1–8.

Fries, L. (1953). Factors promoting growth of *Coprinus fimetarius* (L.) under high temperature conditions. *Physiol. Plantarum* **6**:551–563.

Fries, L. (1955). Studies in the physiology of *Coprinus*. I. Growth substance, nitrogen and carbon requirements. *Svensk Botan. Tidskr.* **49**:475–535.

Fries, N. (1938). Über die Bedeutung von Wuchsstoffen für das Wachstum verschiedener Pilze. *Symbolae Botan. Upsalienses* **3**:Part 2, I–VII, 1–189.

Fries, N. (1942). Adermin (Vitamin B_6) als Wachstumsfaktor für *Ophiostoma ulmi* (Buisman) Nannf. *Naturwissenschaften* **30**:685.

Fries, N. (1943). Die Einwirkung von Adermin, Aneurin und Biotin auf das Wachstum einiger Ascomyceten. *Symbolae Botan. Upsalienses* **7**:Part 2, 1–73.

Fries, N. (1948). Spontaneous physiological mutations in *Ophiostoma*. *Hereditas* **34**:338–350.

Fries, N. (1949). Culture studies in the genus *Mycena*. *Svensk Botan. Tidskr.* **43**:316–342.

Fries, N. (1950). Growth factor requirements of some higher fungi. *Svensk Botan. Tidskr.* **44**:379–386.

Fries, N. (1960). Nonanal as a growth factor for wood-rotting fungi. *Nature* **187**: 166–167.

Fries, N. (1961a). Growth factors: Metabolic factors limiting growth. Bacteria and fungi. *In* Encyclopedia of Plant Physiology (W. Ruhland, ed.), Vol. XIV, pp. 332–400. Springer, Berlin.

Fries, N. (1961b). The growth-promoting activity of some aliphatic aldehydes on fungi. *Svensk Botan. Tidskr.* **55**:1–16.

Fries, N., and K. Aschan. (1952). The physiological heterogeneity of the dikaryotic mycelium of *Polyporus abietinus* investigated with the aid of micrurgical technique. *Svensk Botan. Tidskr.* **46**:429–445.

Fries, N., and L. Källströmer. (1965). A requirement for biotin in *Aspergillus niger* when grown on a rhamnose medium at high temperature. *Physiol. Plantarum.* **18**: 191–200.

Gentile, A. C., and R. M. Klein. (1955). The apparent necessity of indoleacetic acid for the growth of *Diplodia* (Fungi imperfecti). *Physiol. Plantarum* 8:291–299.

Georg, L. K. (1949). Conversion of tryptophane to nicotinic acid by *Trichophyton equinum*. *Proc. Soc. Exptl. Biol. Med.* 72:653–658.

Georg, L. K. (1952). Cultural and nutritional studies of *Trichophyton gallinae* and *Trichophyton megninii*. *Mycologia* 44:470–492.

Georg, L. K., and L. B. Camp. (1957). Routine nutritional tests for the identification of dermatophytes. *J. Bacteriol.* 74:113–121.

Hackbarth, R. D., and R. P. Collins. (1961). A study of the vitamin requirements of three species of the genus *Gelasinospora*. *Am. J. Botany* 48:603–606.

Halbsguth, W. (1949). Über die Bedingungen der Kultur von *Tilletia tritici*-Haplonten auf "definiertem" Substrat und das Verhalten verschiedener Klone gegenüber einzelnen Faktoren im Hinblick auf Wachstum, Konidienbildung und Konidienkeimung. *Planta* 36:551–634.

Hale, M. E. (1958). Vitamin requirements of three lichen fungi. *Bull. Torrey Botan. Club* 85:182–187.

Halliday, W. J., and E. McCoy. (1955). Biotin as a growth requirement for *Blastomyces dermatitidis*. *J. Bacteriol.* 70:464–468.

Harris, D. L. (1956). Interaction of thiamine and pyridoxine in *Neurospora*. II. Competition between pyridoxine and the pyrimidine precursor of thiamine. *Arch. Biochem. Biophys.* 60:35–43.

Hartelius, V. (1946). Antivitamin effect of sulfanilamide on *Aspergillus niger*. *Compt. Rend. Trav. Lab. Carlsberg* 24:178–184.

Hasegawa, T., and J. Banno. (1959). *Rhodotorula*. IV. The vitamin requirement of *Rhodotorula* species. 1 and 2. *Hakko Kogaku Zasshi* 37:171–174, 174–176.

Hasegawa, T., and J. Banno. (1963). Vitamin requirement as a taxonomic key for *Rhodotorula* sp. *J. Gen. Appl. Microbiol.* (*Tokyo*) 9:279–286.

Hawker, L. E. (1939). The nature of the accessory growth factors influencing growth and fruiting of *Melanospora destruens* Shear, and of some other fungi. *Ann. Botany* (*London*) [N. S.] 3:657–676.

Hesseltine, C. W., A. R. Whitehill, C. Pidacks, M. Ten Hagen, N. Bohonos, B. L. Hutchings, and J. H. Williams. (1953). Coprogen, a new growth factor present in dung, required by *Pilobolus* species. *Mycologia* 45:7–19.

Hofmann, K., and T. Winnick. (1945). The determination of oxybiotin in the presence of biotin. *J. Biol. Chem.* 160:449–453.

Jayko, L. G., T. I. Baker, R. D. Stubblefield, and R. F. Anderson. (1962). Nutrition and metabolic products of *Lactarius* species. *Can. J. Microbiol.* 8:361–371.

Jefferson, W. E., and G. Sisco. (1959). The response of *Aspergillus niger* to added steroids. *Federation Proc.* 18:253.

Jefferson, W. E., and G. Sisco. (1961). The influence of exogenous steroids on the growth of *Aspergillus niger* and *Torula utilis*. *J. Gen. Physiol.* 44:1029–1045.

Jennison, M. W., M. D. Newcomb, and R. Henderson. (1955). Physiology of the wood-rotting Basidiomycetes. I. Growth and nutrition in submerged culture in synthetic media. *Mycologia* 47:275–304.

Johansson, M. (1962). Studies in alkaloid production by *Claviceps purpurea*. *Symbolae Botan. Upsalienses* 17:Part 2, 1–47.

Jones, A., and M. Finch. (1959). Plate assay of thiamine. I. Using *Kloeckera brevis*. *Appl. Microbiol.* 7:309–311.

Knight, B. C. J. G. (1945). Growth factors in microbiology. *Vitamins and Hormones* 3:105–228.

Kögl, F., and N. Fries. (1937). Über den Einfluss von Biotin, Aneurin und Meso-Inosit auf das Wachstum verschiedener Pilzarten. *Z. Physiol. Chem.* **249**:93–110.

Kögl, F., and B. Tönnis. (1936). Über das Bios-Problem. Darstellung von krystallisiertem Biotin aus Eigelb. *Z. Physiol. Chem.* **242**:43–73.

Koser, S. A., and M. H. Wright. (1943). Experimental variation of nicotinamide requirement of dysentery bacilli. *J. Bacteriol.* **46**:239–249.

Koser, S. A., M. H. Wright, and A. Dorfman. (1942). Aspartic acid as a partial substitute for the growth-stimulating effect of biotin on *Torula cremoris*. *Proc. Soc. Exptl. Biol. Med.* **51**:204–205.

Krneta-Jordi, M. (1962). Cytologische und physiologische Untersuchungen an *Eremothecium ashbyii* Guill. Wirkstoffbedarf und Synthesevermögen als Funktion der Milieubeschaffenheit. *Arch. Mikrobiol.* **43**:76–108.

Leaphart, C. D. (1956). Physiological studies of some fungi associated with pole blight of western white pine. *Mycologia* **48**:25–40.

Leaver, F. W., J. Leal, and C. R. Brewer. (1947). Nutritional studies on *Piricularia oryzae*. *J. Bacteriol.* **54**:401–408.

Lennarz, W. J., and K. Bloch. (1960). Hydroxystearic acids and the biosynthesis of unsaturated fatty acids. *J. Biol. Chem.* **235**:PC 26.

Leonian, L. H., and V. G. Lilly. (1938). Studies on the nutrition of fungi. I. Thiamin, its constituents, and the source of nitrogen. *Phytopathology* **28**:531–548.

Leonian, L. H., and V. G. Lilly. (1942). The effects of vitamins on ten strains of *Saccharomyces cerevisiae*. *Am. J. Botany* **29**:459–464.

Lewis, R. W. (1952). The vitamin nutrition of *Alternaria solani*. *Phytopathology* **42**:657–659.

Lichstein, H. C., and W. J. Begue. (1960). Increased nutritional requirements of *Saccharomyces cerevisiae* as a result of incubation at 38°C. *Proc. Soc. Exptl. Biol. Med.* **105**:500–504.

Lilly, V. G., and H. L. Barnett. (1947). The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*. *Am. J. Botany* **34**:131–138.

Lilly, V. G., and H. L. Barnett. (1948a). The inheritance of partial thiamine deficiency in *Lenzites trabea*. *J. Agr. Res.* **77**:287–300.

Lilly, V. G., and H. L. Barnett. (1948b). Growth rates, vitamin deficiencies, and sclerotia formation by some Sclerotiniaceae. *Proc. West Va. Acad. Sci.* **20**:69–74.

Lilly, V. G., and H. L. Barnett. (1949). The influence of concentrations of nutrients, thiamin, and biotin upon growth, and formation of perithecia and ascospores by *Chaetomium convolutum*. *Mycologia* **41**:186–196.

Lilly, V. G., and H. L. Barnett. (1951). "Physiology of the Fungi," 464 pp. McGraw-Hill, New York.

Lilly, V. G., and L. H. Leonian. (1940). The growth rate of some fungi in the presence of co-carhoxylase, and the moieties of thiamin. *Proc. West Va. Acad. Sci.* **14**:44–49.

Lilly, V. G., and L. H. Leonian. (1944). The anti-biotin effect of desthiobiotin. *Science* **99**:205–206.

Lindeberg, G. (1939). Über das Wuchsstoffbedürfnis verschiedener Arten der Pilzgattung *Marasmius*. *Svensk Botan. Tidskr.* **33**:85–90.

Lindeberg, G. (1944). Über die Physiologie ligninabbauender Bodenhymenomyzeten. Studien an schwedischen *Marasmius*-Arten. *Symbolae Botan. Upsalienses* **8**: Part 2, 1–183.

Lindeberg, G. (1946a). Thiamin and growth of litter-decomposing hymenomycetes. *Botan. Notiser* pp. 89–93.

Lindeberg, G. (1946b). The effect of biotin and thiamin on the growth of *Collybia dryophila*. *Svensk Botan. Tidskr.* **40**:63–69.

Littman, M. L., and T. Miwatani. (1963). Effect of water soluble vitamins and their analogues on growth of *Candida albicans*. II. Vitamin B_{12}, thiamine, oxythiamine, neopyrithiamine, substituted pyrimidines and thiazoles. *Mycopathol. Mycol. Appl.* **21**:229–314.

Lochhead, A. G., and G. B. Landerkin. (1942). Nutrilite requirements of osmophilic yeasts. *J. Bacteriol.* **44**:343–351.

Lwoff, A. (1943). L'évolution physiologique. Étude des pertes de fonction chez les microorganismes. *Actualités Sci. Ind.* **970**:1–308.

McTeague, D. M., S. A. Hutchinson, and R. J. Reed. (1959). Spore germination in *Agaricus campestris*. L. ex Fr. *Nature* **183**:1736.

McVeigh, I., and E. Bell. (1951). The amino acid and vitamin requirements of *Candida albicans* Y-475 and *Mycoderma vini* Y-939. *Bull. Torrey Botan. Club* **78**:134–144.

McVeigh, I., and E. Bracken. (1955). The nutrition of *Schizosaccharomyces pombe*. *Mycologia* **47**:13–25.

Malca, I., and A. J. Ullstrup. (1960). Vitamin requirements of *Gloeocercospora sorghi*. *Bull. Torrey Botan. Club* **87**:271–275.

Mandels, G. R. (1955). Biotin and interrupted growth of *Myrothecium verrucaria*. *Am. J. Botany* **42**:921–929.

Marsh, P. B., and K. Bollenbacher. (1946). The vitamin requirements of *Memnoniella* and *Stachybotrys*. *Am. J. Botany* **33**:245–249.

Mathew, K. T. (1952). Growth-factor requirements of *Pellicularia koleroga* Cooke in pure culture. *Nature* **170**:889.

Mathiesen, A. (1950). The nitrogen nutrition and vitamin requirement of *Ophiostoma pini*. *Physiol. Plantarum* **3**:93–102.

Matkovics, B. (1960). Über die Einwirkung von Sterinen auf das Wachstum von Mikroorganismen. *Pharmazie* **15**:122–125.

Meissel, M. N. (1947). Hyper- and avitaminosis in micro-organisms. *Nature* **160**: 269–270.

Melin, E. (1954). Growth factor requirements of mycorrhizal fungi of forest trees. *Svensk Botan. Tidskr.* **48**:86–94.

Melin, E., and B. Norkrans. (1942). Über den Einfluss der Pyrimidin- und der Thiazolkomponente des Aneurins auf das Wachstum von Wurzelpilzen. *Svensk Botan. Tidskr.* **36**:271–286.

Melin, E., and B. Nyman. (1940). Weitere Untersuchungen über die Wirkung von Aneurin und Biotin auf das Wachstum von Wurzelpilzen. *Arch. Mikrobiol.* **11**:318–328.

Melin, E., and B. Nyman. (1941). Über das Wuchsstoffbedürfnis von *Boletus granulatus* (L.) Fr. *Arch. Mikrobiol.* **12**:254–259.

Melnick, D., M. Hochberg, H. W. Himes, and B. L. Oser. (1945). The multiple nature of vitamin B. Critique of methods for the determination of the complex and its components. *J. Biol.Chem.* **160**:1–14.

Miyashita, S., T. Miwatani, and T. Fujino. (1958). Studies on the nutrition of *Candida*. I. Vitamin requirements of strains of *Candida*. *Biken's J.* **1**:45–49.

Mohan, V. S., H. L. Crespi, and J. J. Katz. (1962). Nutritional requirements for

the cultivation of fully deuterated yeasts *Torulopsis utilis* and *Saccharomyces cerevisiae*. *Nature* **193**:189–190.

Morris, J. G., D. T. D. Hughes, and C. Mulder. (1959). Observations on the assay of vitamin B_6 with *Saccharomyces carlsbergensis* 4228. *J. Gen. Microbiol.* **20**:566–575.

Moser, M. (1960). "Die Gattung *Phlegmacium*," 440 pp. Julius Klinkhardt, Bad Heilbrunn, Obb.

Müller, W. F., and W. H. Schopfer. (1937). L'action de l'aneurine et de ses constituants sur *Mucor Ramannianus* Möll. *Compt. Rend.* **205**:687–689.

Neilands, J. B. (1953). Biological properties of ferrichrome. *Federation Proc.* **12**:250.

Nickerson, W. J. (1961). Role of biotin in the multiplication of yeasts. *Bacteriol. Proc.* p. 177.

Nielsen, N., and J. Dagys. (1940). Wuchsstoffwirkung der Aminosäuren. VII. Untersuchungen über die Wirkung der Zitronensäure nebst weiteren Untersuchungen über die Wirkungsweise des β-Alanins. *Compt. Rend. Trav. Lab. Carlsberg* **22**:447–480.

Noecker, N. L. (1938). Vitamin B_1 in the nutrition of four species of wood-destroying fungi. *Am. J. Botany* **25**:345–348.

Noecker, N. L., and M. Reed. (1943). Observations on the vitamin requirements of *Stereum frustulosum* (Pers.) Fr. *Am. Midland Naturalist* **30**:171–174.

Norkrans, B. (1950). Studies in growth and cellulolytic enzymes of *Tricholoma*. *Symbolae Botan. Upsalienses* **11**:Part 1, 1–126.

Northam, B. E., and F. W. Norris. (1951). Growth requirements of *Schizosaccharomyces octosporus*, a yeast exacting towards adenine. *J. Gen. Microbiol.* **5**:502–507.

Nyman, B., and N. Fries. (1962). Pteroylglutamic acid as a growth factor for a strain of *Rhodotorula*. *Acta Chem. Scand.* **16**:2306–2308.

Olive, L. S., and A. A. Fantini. (1961). A new, heterothallic species of *Sordaria*. *Am. J. Botany* **48**:124–128.

Page, R. M. (1952). The effect of nutrition on growth and sporulation of *Pilobolus*. *Am. J. Botany* **39**:731–739.

Painter, H. A. (1954). Factors affecting the growth of some fungi associated with sewage purification. *J. Gen. Microbiol.* **10**:177–190.

Perlman, D. (1948). On the nutrition of *Memnoniella echinata and Stachybotrys abra*. *Am. J. Botany* **35**:36–41.

Pine, L. (1957). Studies on the growth of *Histoplasma capsulatum*. III. Effect of thiamin and other vitamins on the growth of the yeast and mycelial phases of *Histoplasma capsulatum*. *J. Bacteriol.* **74**:239–245.

Quintin-Jerebzoff, S. (1959). Blocage de la synthèse de quelques acides organiques chez *Nectria galligena* soumis à des doses inhibitrices d'auxine. Reprise des processus par action de la biotine. *Compt. Rend.* **248**:727–729.

Quispel, A. (1944). The mutual relations between algae and fungi in lichens. *Rec. Trav. Botan. Neerl.* **40**:413–541.

Rabinowitz, J. C., and E. E. Snell. (1951). The nature of the requirement of *Saccharomyces carlsbergensis* for vitamin B_6. *Arch. Biochem. Biophys.* **33**:472–481.

Rainbow, C. (1948). Para-aminobenzoic acid, a growth factor for certain brewer's yeasts. *Nature* **162**:572–573.

Reed, L. J., A. C. Schram, and L. E. Loveless. (1959). Inhibition of *Saccharomyces cerevisiae* by *p*-aminobenzoic acid and its reversal by the aromatic amino acids. *J. Biol. Chem.* **234**:904–908.

Ribereau-Gayon, J., E. Peynaud, and S. Lafourcade. (1955). Dosage microbiologique du mésoinositol à l'aide de *Kloeckera apiculata* et de *Saccharomyces veronae* application aux raisins et aux vins. *Rev. Ferment. Ind. Aliment.* **10**:119–121.

Ridgeway, G. J., and H. C. Douglas. (1958). Unbalanced growth of yeast due to inositol deficiency. *J. Bacteriol.* **76**:163–166.

Robbins, W. J. (1938). Thiamin and growth of species of *Phytophthora*. *Bull. Torrey Botan. Club* **65**:267–276.

Robbins, W. J., and A. Hervey. (1955). Growth substance requirements of *Stereum murraii*. *Mycologia* **47**:155–162.

Robbins, W. J., and A. Hervey. (1958). Wood, tomato, and malt extracts and growth of some *Basidiomycetes*. *Mycologia* **50**:745–752.

Robbins, W. J., and A. Hervey. (1959). Wood extract and growth of *Morchella*. *Mycologia* **51**:356–363.

Robbins, W. J., and A. Hervey. (1963). Unidentified filtrate growth substances for several fungi. *Mycologia* **55**:59–64.

Robbins, W. J., and F. Kavanagh. (1938a). Thiamin and growth of *Pythium butleri*. *Bull. Torrey Botan. Club* **65**:453–461.

Robbins, W. J., and F. Kavanagh. (1938b). Vitamin B_1 or its intermediates and growth of certain fungi. *Am. J. Botany* **25**:229–236.

Robbins, W. J., and V. Kavanagh. (1942). Vitamin deficiencies of the filamentous fungi. *Botan. Rev.* **8**:411–471.

Robbins, W. J., and R. Ma. (1942a). Pimelic acid, biotin and certain fungi. *Science* **96**:406–407.

Robbins, W. J., and R. Ma. (1942b). Vitamin deficiencies of *Ceratostomella* and related fungi. *Am. J. Botany* **29**:835–843.

Robbins, W. J., and R. Ma. (1942c). Specificity of pyridoxine for *Ceratostomella ulmi*. *Bull. Torrey Botan. Club* **69**:342–352.

Robbins, W. J., and R. Ma. (1944). A *Rhodotorula* deficient for paraaminobenzoic acid. *Science* **100**:85–86.

Robbins, W. J., J. E. McKinnon, and R. Ma. (1942). Vitamin deficiencies of *Trichophyton discoides*. *Bull. Torrey Botan. Club* **69**:509–521.

Robinson, F. A. (1951). "The Vitamin B Complex." XI, 688 pp. Wiley, New York.

Rogosa, M. (1943). Nicotinic acid requirements of certain yeasts. *J. Bacteriol.* **46**: 435–440.

Rothwell, F. M. (1956). Nutritional requirements of *Phlyctorhiza variabilis*. *Am. J. Botany* **43**:28–32.

Sadasivan, T. S., and C. V. Subramanian. (1954). Studies in the growth requirements of Indian fungi. *Brit. Mycol. Soc. Trans.* **37**:426–430.

Sakuragi, T., and F. A. Kummerow. (1957). The vitamin B_6 derivatives structurally analogous to thiamine and their biological activity. *Arch. Biochem. Biophys.* **71**: 303–310.

Salvin, S. B. (1949). Cysteine and related compounds in the growth of the yeast-like phase of *Histoplasma capsulatum*. *J. Infect. Diseases* **84**:275–283.

Schopfer, W. H. (1934). Versuche über die Wirkung von reinen kristallisierten Vitaminen B auf *Phycomyces*. *Ber. Deut. Botan. Ges.* **52**:308–312.

Schopfer, W. H. (1935). Etude sur les facteurs de croissance. Action de la vitamine cristallisée B_1 et de l'extrait de germe de blé sur *Rhizopus* et d'autres Mucorinées. *Z. Vitaminforsch.* **4**:187–206.

Schopfer, W. H. (1943). "Plants and Vitamins." Chronica Botanica, Waltham, Massachusetts.

Schopfer, W. H. (1944a). La biotine, l'aneurine et le méso-inositol, facteurs de croissance pour *Eremothecium ashbyii* Guilliermond. La biosynthèse de la riboflavine. *Helv. Chim. Acta* **27**:1017–1032.

Schopfer, W. H. (1944b). Les vitamines, facteurs de croissance pour les microorganismes. *Schweiz. Z. Pathol. Bakteriol.* **7**:314–345.

Schopfer, W. H., and S. Blumer. (1938). Untersuchungen über die Biologie von *Ustilago violacea*. II. Wirkung des Aneurins und anderer Wuchsstoffe vitaminischer Natur. *Arch. Mikrobiol.* **9**:305–378.

Schopfer, W. H., and S. Blumer. (1940). Recherches sur la répartition de l'hétérotrophie par rapport à l'aneurine chez les champignons. *Arch. Microbiol.* **11**:205–214.

Schopfer, W. H., and M. Guilloud. (1945). Recherches expérimentales sur les facteurs de croissance et le pouvoir de synthèse de *Rhizopus cohnii* Berl. et de Toni (*Rhizopus suinus* Neilson). *Z. Vitaminforsch.* **16**:181–296.

Schultz, A. S., and L. Atkin. (1947). The utility of bios response in yeast classification and nomenclature. *Arch. Biochem.* **14**:369–380.

Schultz, A. S., L. Atkin., and C. N. Frey. (1938). Thiamine, pyrimidine and thiazole as bios factors. *J. Am. Chem. Soc.* **60**:490.

Schultz, A. S., L. Atkin, and C. N. Frey. (1939). Vitamin B_6, a growth promoting factor for yeast. *J. Am. Chem. Soc.* **61**:1931.

Sedlmayr, M., E. S. Beneke, and J. A. Stevens. (1961). Physiological studies on *Calvatia* species. I. Vitamin requirements. *Mycologia* **53**:98–108.

Sherman, F. (1959). The effects of elevated temperatures on yeasts. I. Nutrient requirements for growth at elevated temperatures. *J. Cellular Comp. Physiol.* **54**:29–35.

Shifrine, M., and A. G. Marr. (1963). The requirement of fatty acids by *Pityrosporum ovale*. *J. Gen. Microbiol.* **32**:263–270.

Shirakawa, H. S. (1955). The nutrition of *Diplocarpon rosae*. *Am. J. Botany* **42**:379–384.

Shulman, A., M. I. B. Dick. and K. T. H. Farrer. (1957). An investigation of the action of penicillin. *Nature* **180**:657–658.

Snell, E. E. (1944). The vitamin activities of "pyridoxal" and "pyridoxamine." *J. Biol. Chem.* **154**:313–314.

Snell, E. E., and A. N. Rannefeld. (1945). The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. *J. Biol. Chem.* **157**: 475–489.

Srinivasan, K. V., and N. Vijayalakshimi. (1960). Thiamine and biotin requirements of two strains of *Glomerella tucumanensis*. *Current Sci.* (*India*) **29**:103–104.

Strigini, P., and G. Morpurgo. (1961). Biotin requirement and carbon and sulphur sources in *Aspergillus* and *Neurospora*. *Nature* **190**:557.

Sundaram, E. R. B. S., M. O. Tirunarayanan. and P. S. Sarma. (1954). The relation between biotin and tryptophan metabolism studied in *Neurospora crassa*. *Biochem. J.* **58**:469–473.

Sundström, K. R. (1960). Physiological and morphological studies of some species belonging to the genus *Exobasidium*. *Phytopathol. Z.* **40**:213–217.

Sundström, K. R. (1964). Studies of the physiology, morphology and serology of *Exobasidium*. *Symbolae Botan. Upsalienses* **17**:Part 3, 1–100.

Suolahti, O. (1951). Über eine das Wachstum von Fäulnispilzen beschleunigende Fernwirkung von Holz, 95 pp. Dissertation, Helsinki.

Taber, W. A., and L. C. Vining. (1957). A nutritional study of three strains of *Claviceps purpurea* (Fr.) Tul. *Can. J. Microbiol.* 3:1–12.

Taber, W. A., and L. C. Vining. (1959). Studies on *Isaria cretacea*. Nutritional characteristics of two strains and morphogenesis of the synnema. *Can. J. Microbiol.* 5:513–535.

Tanner, F. W. (1960). Process for the production of cobalamins. U.S. Patent No. 2,921,887.

Thind, K. S., and S. R. Sharma. (1960). The influence of different vitamins on the growth of *Cercospora beticola*. *Proc. Natl. Acad. Sci., India* Sect. B 30:109–114.

Tirunarayanan, M. O., and P. S. Sarma. (1953). Studies on biotin. Part V: Influence of biotin on the gammexane—inositol relationship in *Neurospora crassa*. *J. Sci. Ind. Res. (India)* **12B**:251–255.

van Uden, N., and L. D. Carmo-Sousa. (1959). Further studies on the significance of temperature relationships and vitamin deficiency patterns in yeast taxonomy. *Port. Acta Biol. Ser. B* 6:239–256.

Volkonsky, M. (1933). Sur l'assimilation des sulfates par les champignons: euthiotrophie et parathiotrophie. *Compt. Rend.* 197:712.

von Weihe, K. (1958). Effects of exogenous macromolecular growth stimulators. I. Effect of agar and agar hydrolyzates. *Arch. Microbiol.* 30:30–45.

von Weihe, K. (1961). The action of high-molecular growth stimulators. II. The action of agar hydrolyzates and the concentration of the active principle. *Arch. Microbiol.* 39:242–252.

Weintraub, R. L., W. E. Miller, and E. J. Schantz. (1958). Chemical stimulation of germination of spores of *Piricularia oryzae*. *Phytopathology* 48:7–10.

Whaley, J. W., and H. L. Barnett. (1963). Parasitism and nutrition of *Gonatobotrys simplex*. *Mycologia* **55**:199–210.

White, N. H. (1941). Physiological studies of the fungus *Ophiobolus graminis* Sacc. I. Growth factor requirements. *J. Council Sci. Ind. Res.* 14:137–146.

Wikberg, E. (1959). Effects of compounds of the vitamin B_6 group on the growth of *Ophiostoma multiannulatum*. *Physiol. Plantarum* 12:100–117.

Wikén, T., and O. Richard. (1951). Untersuchungen über die Physiologie der Weinhefen. I Mitteilung. Zur Kenntnis der Wachstumsbedingungen einer auxoautotrophen, schweizerischen Kulturweinhefe. *Antonie van Leeuwenhoek, J. Microbiol. Serol.* 17:209–224.

Williams, R. J. (1940). Pantothenic acid. *Enzymologia* 9:387–394.

Williams, R. J., R. F. Eakin, and E. E. Snell. (1940). The relationship of inositol, thiamin, biotin, pantothenic acid and vitamin B_6 to the growth of yeasts. *J. Am. Chem. Soc.* 62:1204.

Woods, D. D. (1954). Metabolic relations between *p*-aminobenzoic acid and folic acid in micro-organisms. *In* "Chemistry and Biology of Pteridines," Ciba Foundation Symposium (G. E. W. Wolstenholme and M. P. Cameron, eds.), pp. 220–236. London.

Wooster, R. C., and V. H. Cheldelin. (1945). Growth requirements of *Penicillium digitatum*. *Arch. Biochem.* 8:311–320.

Wright, L. D., E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant, and K. Folkers. (1951). The elucidation of biocytin. *Science* 114:635–636.

Wright, L. D., E. L. Cresson, J. Valiant, D. E. Wolf, and K. G. Folkers. (1954).

The characterization of biotin l-sulfoxide from a microbiological source. *J. Am. Chem. Soc.* **76**:4163–4166.

Yaw, K. E. (1952). Production of riboflavin by *Eremothecium ashbyi* grown in a synthetic medium. *Mycologia* **44**:307–317.

Yusef, H. M. (1953). The requirements of some hymenomycetes for essential metabolites. *Bull. Torrey Botan. Club* **80**:43–64.

CHAPTER 20

The Chemical Environment for Fungal Growth

4. Chemical Inhibition

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I. GENERAL CONSIDERATIONS

A. Introductory

The study of fungicides is of comparatively recent origin. It is nevertheless beyond the scope of this chapter to provide an exhaustive survey of the developments, and for more detailed treatments reference should be made to the monographs by Horsfall (1956) and Martin (1959).

This review deals with some of the basic mechanisms involved in the chemical inhibition of fungal growth, followed by a brief account of the more important compounds used as fungicides. Most of these materials have been developed empirically, either as a result of chance observation, as in the classical instance of Bordeaux mixture, or else as a result of intensive screening tests in commercial laboratories.

B. Site of Action of Inhibitor

1. Intracellular Action

There is reason to believe that the majority of antifungal compounds act within the cell by the inhibition of vital processes. In order to do this, they must be able to penetrate the cellular membrane and gain access to the subcellular components where these processes occur. This cellular membrane is believed to consist of lipoprotein, as in other organisms (Davson

and Daniellì, 1943), and a degree of lipoid solubility is therefore a necessary property to enable a compound to penetrate into the cell. Examples of antifungal compounds of high lipoid solubility are captan (Horsfall, 1956) and dichlone. In some instances an active group, itself relatively polar in nature, may be rendered more lipoid-soluble by the addition of a fatty alkyl side chain: the toxicity of such a homologous series normally rises to a maximum and then falls away again where diminished aqueous solubility becomes limiting (Albert, 1960). The length of the chain for maximum activity varies from compound to compound and may well be related to the lipoid solubility of the rest of the molecule. Examples of antifungal compounds with such alkyl chain substituents are dodine (*n*-dodecylguanidine), glyodin (2-heptadecyl-2-imidazoline), and dinocap (2,1′-methylheptyl-4,6-dinitrophenyl crotonate).

The uptake of fungicides has been well demonstrated by the use of radioactive tracers, in particular C^{14}. Thus it was shown by Miller *et al.* (1953b) that spores of *Neurospora sitophila* accumulated a 10,000-fold concentration of glyodin from an aqueous solution of 2 μg/ml, and similar, though slightly lower, rates of uptake were recorded for other fungi and other fungicides. Moreover, it was also shown that the uptake is extremely rapid. In the case of captan (Richmond and Somers, 1962) and of a homologous series of *n*-alkyl ethers of 2,5-dimercapto-1,3,4-thiadiazole (Somers, 1958), it seems that the uptake is not a normal diffusion process, but rather an active transport mechanism. Richmond and Somers also gave evidence to suggest that much of the captan taken up may be detoxified by sulfhydryl groups in the cell before reaching vital receptor sites.

It appears likely, from these and other results, that fungicides in current use are characterized not by a high toxicity on a weight/weight basis, but rather by their ability to be accumulated in fungal cells at concentrations

TABLE I

APPROXIMATE LD_{50} VALUES FOR VARIOUS BIOCIDAL AGENTS[a]

Toxicant	Subject	LD_{50}[b] (μg/gm)
Atropine	Man	1.4
Botulinum toxin	Mouse	0.23×10^{-6}
O,O-diethyl *O*-*p*-nitrophenyl phosphorothionate	House fly	0.9
Diethyl *p*-nitrophenyl phosphate	House fly	0.5
2,4-dichlorophenoxyacetic acid	Tomato	10
Penicillin	Staphylococci	2
Various fungicides	Spores	85–10,000

[a] After Miller and McCallan (1956).
[b] Median lethal dose.

greatly in excess of those in the external solution. In this respect they differ markedly from compounds of high toxicity to many other forms of life, as Table I clearly shows.

2. Action at the Cell Surface

A number of compounds of relatively low lipoid solubility are also effective fungicides, however, and evidence is accumulating that these may be acting at the cell surface. It now appears that the toxic effect of metal ions may be related with their electronegativity, and their primary fungistatic action is visualized as being due to nonspecific reactions on or outside the cytoplasmic membrane (Somers, 1961). Surface-active compounds, too, are believed to act at the cell surface: the toxicity of dodine acetate to *Monilinia* [*Sclerotinia*] *fructicola* appears to be due in part to its effects in blocking vital anionic sites at the cell surface (Brown and Sisler, 1960). This compound, and also *s*-triazine fungicides (Burchfield and Storrs, 1957), also gives rise to leakage of essential nutrients from the cell, presumably as a result of disturbed permeability, although there are indications that such leakage is a secondary effect.

3. Extracellular Action

Finholt *et al.* (1952) reported the instance of an aliphatic amine, which was effective against the fungus *Lentinus lepideus* when grown with a cellulose carbon source, but which was inactive when the fungus was grown with glucose as a carbon source. The effect appeared to be due to the inactivation of an extracellular cellulase enzyme upon which the fungus relied for its ability to utilize cellulose. In a similar manner a number of naturally occurring polyphenols, in an oxidized and polymerized form, were shown to inhibit extracellular pectolytic and plant tissue macerating enzymes necessary for infection of apples by a plant pathogenic fungus, *Sclerotinia fructigena* (Byrde *et al.*, 1960): such compounds were much less active in conventional spore germination toxicity tests against the fungus. On the other hand, Reese and Mandels (1957) found that inhibitors of cellulases of three nonpathogenic fungi were active against the growth of the fungus at lower concentrations than against the cellulase, and in general this form of extracellular action is rarely exploited in fungicidal usage.

C. The Inhibition of Vital Processes

1. Inhibition at the Enzyme Level

The action of many fungicides has been attributed to a disruption of cellular metabolism at the enzyme level: such enzyme inhibition effects may

be subdivided into specific inhibition of one enzyme or group of enzymes and those involving a more widespread and nonspecific inactivation of enzymes.

a. Specific Inhibition of Enzymes. There have been at least two reports which have suggested that some fungicides may act as competitive enzyme inhibitors. Thus the fungistatic activity of glyodin to *Sclerotinia fructicola* is reversible by certain purines or purine derivatives (West and Wolf, 1955), whereas the activity of dichlone has been reversed by vitamin K (Woolley, 1945). On the other hand, the cationic surface activity of glyodin suggests that more nonspecific mechanisms than the inhibition of purine synthesis may be involved (Somers, 1962), and dichlone is known to have many nonspecific effects on enzymes (Owens and Novotny, 1958). Although many antimetabolites for fungi have been demonstrated, none of those listed by Horsfall (1956, Table 6) has found extensive use as a fungicide.

b. Nonspecific Inhibition of Enzymes. By far the greater number of instances of fungal enzyme inhibition by chemicals refer to nonspecific effects. For example, Owens (1953a) examined the effect of a range of twenty fungicides on four enzyme systems and showed that, at a fungicide concentration of $10^{-3}M$ about half the possible enzyme-fungicide combinations showed marked reduction in enzyme activity. Other studies by Owens (1953b), Owens and Novotny (1958, 1959), and Owens and Blaak (1960) have also shown inhibition of a wide range of enzymes and coenzymes by captan and dichlone. Heavy metals also are known to affect many enzymes, and in particular those enzymes dependent on sulfhydryl groups.

2. Inhibition at Other Levels

It is not yet clear whether the effect of some fungicides is at the enzyme level and, if it is, what are the enzymes affected. Instead, the effects observed have been restricted to alterations in morphology or in physiology. A number of fungicides exerting morphological effects on fungi have been tabulated by Horsfall (1956, Table 4). Outstanding among these is the antibiotic griseofulvin (Grove *et al.*, 1952), which at extremely low doses produces a characteristic hyphal distortion. This effect seems to be a reflection of some interference with cell wall biosynthesis, and it is probably significant that only fungi with chitinous cell walls are susceptible to this compound (Brian, 1960). The specificity of its action, coupled with the fact that cell wall synthesis appears to be affected, are analagous to the antibacterial action of penicillin.

Reference has already been made to effects of fungicides on the membrane of the fungal cell. Horsfall (1956, Table 3) has tabulated reports of

the effects of fungicidal compounds on nuclei of fungi and other organisms. He suggested that many compounds acting as mitotic poisons—such as ketones, phenols, or amino compounds—may be reacting with vital chromosome constituents.

The effects of some compounds are reflected on sporulation rather than on vegetative growth. Therefore, although this aspect is strictly outside the scope of this paper, it is clear that the prevention of the ability of a single colony to sporulate will limit the spread of a fungal attack, and so indirectly restrict growth. Reavill (1954) has shown the antisporulant effect of chlorinated nitrobenzenes, and Horsfall (1956) has summarized other reports on inhibition of fungal sporulation by applied chemicals. Horsfall and Rich (1959, 1960) have reported on the powerful antisporulant activity of several chlorinated aliphatic compounds, and it seems possible that this type of compound may find application in the future for restricting inoculum levels and thereby rendering easier the task of conventional fungicides.

D. Environmental Factors Affecting Chemical Inhibition

1. Temperature and Light

Comparatively little study appears to have been made of the effects of temperature on antifungal activity. McIntosh (1961), however, examined the toxicity of a number of mercury-containing and other compounds at a range of temperatures, and demonstrated that, in general, an increase in temperature increased toxicity to an extent that varied from compound to compound. Richmond and Somers (1962) examined the effect of temperature on the uptake of captan by *Neurospora crassa* spores and showed a linear increase of uptake with temperature. A few fungicides in use, for example dinocap, have a relatively high vapor pressure and might therefore be expected to show reduced persistence at high temperatures. Sulfur is known to be more effective at higher temperatures (e.g., Yarwood, 1950).

The effect of light on fungicide residues has precluded the use on foliage of at least one material—tetrachlorobenzoquinone, which undergoes photochemical decomposition to chloranilic acid.

2. Hydrogen-ion Concentration

The activity of a number of fungicides, in common with other toxic substances, is modified by the pH level. In general this is true of electrolytes, and usually the undissociated molecule is more toxic than the ion. This arises from the greater lipoid solubility of the undissociated molecule compared with the hydrated ions, which enables more rapid penetration of the cell. The phenomenon has been discussed at length for toxic substances in general by Albert (1960); examples relating to fungi are afforded by

the work of Simon and Blackman (1949) with dinitro-*o*-cresol, and of Block (1955) with 8-hydroxyquinoline. The toxicity of copper sulfate and mercuric chloride have been shown to decrease with decreasing pH (Horsfall, 1956).

3. The "Host"

In most applications of fungicides in agriculture, medicine, or industry, a third factor is involved besides the compound and the fungus—i.e., the "host." The host may be a higher plant or an animal, and the term is being extended for convenience to include nonliving material undergoing fungicide treatment, such as timber or fabric. The host factor may profoundly modify the properties of a fungicide, and some aspects of this interaction are quoted below.

a. In Agriculture. Fungicides used in crop protection are generally used on the living plant, and this fact gives rise to special problems in their use. The application of many very potent antifungal materials is precluded by considerations of toxicity either to the host plant itself, or to the spray operator, or, in the case of food crops, to the consumer. Mercuric chloride, for example, would be eliminated on all three considerations.

A knowledge of the life history of the pathogenic fungus enables fungicide applications to be so timed as to give the chemical an optimal chance of dealing with the fungus. In crop protection much reliance is placed on the use of *"residual"* (or *"protectant"*) fungicides applied to the surface of a healthy plant, the residues of which are able to prevent the germination of, and subsequent infection by, fungal spores alighting on the surface. The success of this form of protection clearly rests on good distribution of the fungicide and on the persistence of a toxic residue under the eroding influences of weather and growth.

In some instances, however, the fungicide may be applied after infection has occurred, as a *"contact"* or *"eradicant"* fungicide, with the object of destroying the established invasion. A more limited range of compounds is useful for this form of action (Byrde, 1961). Much research has been carried out on the possible use of *systemic* fungicides, which act within the plant tissues. So far only limited success has been achieved, and no commercial fungicide appears to possess more than very limited systemic properties.

b. In Medicine. Some of the considerations already mentioned apply to the use of fungicides in medicine: the need for low mammalian toxicity is particularly obvious, and this explains in part the trend toward highly selective antifungal antibiotics in medicine. The object in medicine is to protect or cure the individual (whereas in agriculture it is the crop as a

whole, not the individual plant, which is at stake), and so cost is less limiting.

Medical application of fungicides is often hampered by the inaccessibility of the pathogen in host tissue, particularly in deep-seated infections. In some instances a further complication is that the pathogen exhibits dimorphism, and its granular cells may be surrounded by thick capsular material, rendering them particularly difficult of access. These and other factors in the treatment of mycoses have been summarized by Byrde and Ainsworth (1958).

c. In Industry. Purposes for which fungicides are used include the preservation of wood, of cotton fabrics, and of paint surfaces. In general, the considerations of toxicity to other forms of life are less relevant, but the problem of access is particularly important in wood preservation, where treatment under pressure is now often used to ensure thorough impregnation.

E. Methods of Fungicidal Evaluation

Broadly speaking, tests of fungicides may be divided into three categories: laboratory tests *in vitro;* an intermediate scale test on small samples of animals, plants, or fabrics; and full-scale field testing under practical conditions in which the material might find eventual use.

For *in vitro* tests, such as are frequently used for "screening" large numbers of potential fungicides, a spore germination method may be used. The methods, developed largely at the Boyce Thompson Institute by McCallan and his colleagues, were standardized in two publications by the American Phytopathological Society (1943, 1947), and these methods, or variants of them, are widely used. They involve the germination of fungal spores in the presence of the chemical on glass slides and are particularly relevant where, as in the case of residual fungicides on plants, the inhibition of spore germination is the primary object. When, however, it is desired to test compounds against fungal mycelium, it is common to incorporate the test compound in an agar medium and measure linear growth (Horsfall, 1956) or to incorporate it in a liquid medium and assay growth gravimetrically. Cochrane (1958), in advocating the latter method for nutritional studies, sharply criticized weaknesses inherent in the agar-plate method, which, for example, takes account only of radial growth and not of mycelial density.

For intermediate scale testing of agricultural fungicides, generally under glasshouse conditions, a series of carefully standardized methods has been evolved at the Boyce Thompson Institute (e.g., McCallan and Wellman,

1943). Similar considerations as to the need for standardization of treatment, inoculation, and assessment apply equally to fungicide assays for any purpose: clinical medical trials are often handicapped by a restricted number of test subjects. Timber fungicides are often tested by the wood block method, in which wood samples are impregnated with the test fungicide and exposed to wood-rotting fungi (Horsfall, 1956, p. 23).

Field trials are generally carried out on a statistically designed basis, and fungal development is assessed by standardized methods as in any form of field experimentation.

II. PRINCIPAL GROUPS OF FUNGICIDES

A complete review of all the fungicides introduced is beyond the scope of this review, but fuller accounts have been published by Horsfall (1956), Woodcock (1959), and Martin (1959). Table II summarizes the development of some of the fungicides in current use, and it will be seen that until about 1930 reliance was placed exclusively on inorganic fungicides.

A. Inorganic Fungicides

The two most important groups of inorganic fungicides are those based on copper and on sulfur, and even in 1958 these two groups together accounted for 96% by weight and 74% in monetary value of fungicide applications in world agriculture (from data of Gayner, 1961). Other metal salts are known to be toxic, but they are of negligible importance by comparison.

The inorganic fungicides have the advantages of low cost and, between them, the ability to control a very wide range of fungal pathogens in agriculture: thus, sulfurs are highly effective against powdery mildews, while copper is an excellent protectant against most other plant pathogens.

1. Copper

As shown in the Table, copper is generally used in the form of Bordeaux mixture, or as one of the "fixed coppers." The former owed its introduction to a chance observation by Millardet (1885), and still finds frequent use because of its low cost and excellent persistence on foliage. Formed by a reaction mixture of copper sulfate solution and calcium hydroxide suspension, there has been much speculation as to the active ingredient in its residues on the leaf, which is presented in detail by Martin (1959). It seems likely that the precipitate of freshly prepared Bordeaux mixture is cupric hydroxide which is stabilized in a gelatinous form by adsorbed calcium sulfate. Further evidence on the importance of a gel structure has been presented by Evans *et al.* (1962). The ratio of the two components can be varied, giving mixtures with differing properties. There is good evi-

TABLE II

HISTORICAL DEVELOPMENT OF FUNGICIDES[a]

Common name	Chemical details	Fungitoxicity recorded by
Sulfur	Sulfur	Classical writers (B.C.)
Lime sulfur	Solution of sulfur in calcium hydroxide suspension	Forsyth (1803)
Bordeaux mixture	Mixture of copper sulfate solution and calcium hydroxide suspension	Millardet (1885)
Whitfield's ointment	Mixture of salicylic and benzoic acids	Whitfield (1912)
Organomercury compounds	Usually alkyl or aryl mercury salts	Riehm (1913)
"Fixed" copper compounds	Copper oxychloride, cuprous oxide, basic copper carbonate	A. Wacker and Co. (1927–1930) (see Large, 1940)
—	Salicylanilide	Fargher *et al.* (1930)
Thiram, ferbam, ziram	Dialkyl dithiocarbamates	Tisdale and Williams (1934)
Nabam, zineb, maneb	Alkylene bisdithiocarbamates	Dimond *et al.* (1943)
Dichlone	2,3-Dichloro-1,4-naphthoquinone	ter Horst and Felix (1943)
Glyodin	2-Heptadecyl-2-imidazoline	Wellman and McCallan (1946)
Griseofulvin	Complex spiran structure (Grove *et al.*, 1952)	Grove and McGowan (1947)
Dinocap	2,1′-Methylheptyl-4,6-dinitrophenyl crotonate	Sprague (1949)
Nystatin	See Hazen and Brown (1960)	Hazen and Brown (1951)
Dodine acetate	*n*-Dodecylguanidine acetate	American Cyanamid Co. (1951)
Captan	*N*-Trichloromethylthiocyclohex-4-ene-1,2-dicarboxyimide	Kittleson (1952)

[a] Based on Horsfall (1956) and Somers (1962).

dence to suggest that soluble copper may be liberated from the deposits in one of three ways (1) atmospheric CO_2 and ammonium salts dissolved in rain, (2) fungal secretions, (3) secretions from the host plant (Martin, 1959).

The "fixed" coppers are being increasingly used owing to the ease of preparation of the spray and the fact that they are less wearing to spray machinery.

2. *Sulfur Fungicides*

The inorganic sulfur fungicides most frequently used are lime sulfur and elemental sulfur, the latter in a dispersible formulation. Sulfurs have been

particularly valuable against powdery mildew diseases, which are unaffected by most fungicides.

Lime sulfur is prepared by boiling lime and sulfur and consists of a mixture of, primarily, calcium polysulfide and calcium thiosulfate: the polysulfide sulfur becomes precipitated as elemental sulfur on the plant surface in a form which appears more tenacious than that produced from dispersible sulfur applications.

The mode of action of inorganic sulfur is still in doubt. For many years it was held that its activity lay in the toxicity of hydrogen sulfide, to which sulfur is reduced by fungi (Martin, 1959). Following the work of Miller *et al.* (1953a) the action of sulfur is now attributed to the effect of elemental sulfur itself interfering in normal hydrogenation and dehydrogenation processes by virtue of its role as a hydrogen acceptor. These views are in accord with those initially postulated by Sempio (1932) that sulfur itself is the active toxic substance. Martin (1959) has pointed out that no explanation of the toxic action of sulfur can be satisfying until it provides for the remarkable selectivity of action shown by sulfur, for this chemical is fatal only to fungi and to a limited range of higher plants and of the Arachnida.

B. Organometal Fungicides

1. Organomercury Compounds

As has already been mentioned, mercury is extremely toxic to fungi, but its use on both plants and animals is severely restricted by its high toxicity to all forms of life. This high general toxicity has been overcome to some extent by the introduction of organomercury derivatives. Particularly is this true of phenyl mercury compounds (first used in agriculture by Montgomery *et al.*, 1943), which have found extensive use in agriculture and for topical application in medicine to superficial infections whereas a large number of alkyl mercury compounds have been developed commercially for seed disinfection, where phytotoxic properties are less liable to manifest themselves. Mercury compounds in general are outstanding eradicant fungicides, able to kill established fungal mycelium. Nevertheless even with phenyl mercury compounds, their mercury content must always be borne in mind when use on edible crops is considered.

2. Organotin Derivatives

Rather a similar position is true of the organotin derivatives. Alkyl tin compounds suffered from the disadvantages of high mammalian toxicity and high phytotoxicity, but the corresponding triphenyl compounds are considerably safer while retaining fungitoxicity (van der Kerk, 1961).

C. Organic Fungicides

The year 1934 is often regarded as the opening of the era of organic fungicides, marking as it did the introduction of the dithiocarbamates. However, such a view overlooks the fact that salicylanilide, an organic fungicide which has found some application in agriculture and industry, was developed some years earlier following a survey of the fungicidal properties of synthetic organic compounds at the Shirley Institute (e.g., Fargher *et al.*, 1930). Since this period organic fungicides have assumed an increasing, though still not completely predominant, importance in fungicide usage. They offer the advantages of a greater specificity of action and, in many instances, lower toxicity to other forms of life. In agriculture they are thus of particular value on high quality crops, where the added expense of their use is more than recompensed by the superior quality, and often quantity, of the final product. They have also found wide application in industry. Full reviews of the chemical groups involved have been given by Woodcock (1959) and by Rich (1960).

1. Phenols

The use of phenolic compounds as fungicides in industry and agriculture has been reviewed by Gruenhagen *et al.* (1951). Phenols owe their activity to the active –OH group, and normally much of their toxicity is lost if this is "masked" chemically. Strongly acidic phenols such as dinitro-*o*-cresol and pentachlorophenol are powerful fungicides with eradicant properties, but also high toxicity to other forms of life. Certain bisphenols, however, combine high fungitoxicity with a much lower toxicity to mammals and plants, and these are finding use in industry, medicine, and agriculture (Corey and Shirk, 1955; Corke, 1962).

Derivatives of dinitrophenols have assumed prominence by virtue of a highly specific action against powdery mildew fungi. Normally to reduce phytotoxicity they are used in ester form, as in dinocap (2,1′-methylheptyl-4,6-dinitrophenyl crotonate) (Sprague, 1949) or in binapacryl (2-*sec*-butyl-4,6-dinitrophenyl-3′,3′-dimethylacrylate) (Härtel, 1961).

2. Quinones

The fungitoxicity and biological activity of quinones was reviewed by McNew and Burchfield (1951). Tetrachloro-*p*-benzoquinone, developed in 1938, proved to have limited uses because of its photochemical decomposition, but 2,3-dichloro-1,4-naphthoquinone (ter Horst and Felix, 1943) has found extensive use as a more stable agricultural fungicide, limited only

by its phytotoxicity. Mention was made earlier of studies on the mechanism of fungicidal action of quinones.

3. *Dithiocarbamates*

These constitute a very important class of organic fungicides, which may be subdivided into two groups: the metallic dithiocarbamates, such as ziram and ferbam, together with the closely allied thiram (tetramethylthiuram disulfide), and the bisdithiocarbamates such as zineb, nabam, and maneb, which are chemically more unstable. Both groups have found extensive application in agriculture, largely owing to the versatile range of compounds available together with the fact that they are cheap in comparison with many organic fungicides. Full details of current knowledge of the properties, mode of action, and use of these compounds may be found in a review by van der Kerk (1959) and the book by Thorn and Ludwig (1962).

4. *Heterocyclic Compounds*

This group includes several diverse compounds which are conveniently grouped under this heading. Outstanding among them is captan (*N*-trichloromethylthiocyclohex-4-ene-1,2-dicarboxyimide), which is highly effective against a number of diseases although of very low phytotoxicity to foliage and fruit, and of low mammalian toxicity. Studies on its effect on fungal enzymes have already been cited: others have speculated on the role of the —$SCCl_3$ group in the molecule, variously regarded as a "shaped charge" aiding penetration of the fungal spore (Horsfall, 1956), or as being the basis of the active toxic group following degradation (Lukens and Sisler, 1958).

Glyodin (2-heptadecyl-2-imidazoline) is another heterocyclic compound of relatively low phytotoxicity used in agriculture (Wellman and McCallan, 1946). 8-Hydroxyquinoline and, in particular, its copper complex, have found use in textile protection (Block, 1955), and afford an example of compounds where the ability to form chelate complexes with metals plays an important role in toxicity (see Albert, 1960, for a detailed discussion of the principles involved). Derivatives of 2,4-dichloro-*s*-triazine (Schuldt and Wolf, 1957) have also proved to be useful fungicides in agriculture and industry.

5. *Antibiotics*

Antifungal antibiotics have been used particularly in the medical field, where their relatively high cost is a less important consideration. The use of griseofulvin, in particular, has given rise to striking advances in the treat-

ment of many fungal pathogens of man and higher animals, and a full account of its origin and development has been given by Brian (1960). Another antibiotic which has been used extensively is nystatin (Hazen and Brown, 1960).

6. *Miscellaneous Compounds*

The broad classification of fungicides used in this review necessitates the grouping together of some chemically unrelated compounds in a final section.

Dodine acetate (*n*-dodecylguanidine acetate) is a cationic surface-active material with the rather unexpected properties of a comparatively high water solubility together with considerable persistence on foliage. It appears to have a rather narrower disease spectrum than, for example, captan, but is highly effective against the economically important disease apple scab (Hamilton and Szkolnik, 1958).

Dicloran (2,6-dichloro-4-nitroaniline) has found application in the control of *Botrytis* diseases, and both on a chemical basis and by considerations of patterns of induced resistance (Priest and Wood, 1961) appears to be similar in action to the chloronitrobenzenes (Reavill, 1954).

In conclusion, it is clear that a large and increasing number of organic fungicides have been developed over the past thirty years for the chemical inhibition of fungal growth. This process may be expected to continue, effort being particularly directed to obtaining compounds of low toxicity to other forms of life.

REFERENCES

Albert, A. (1960). "Selective Toxicity with Special Reference to Chemotherapy," 2nd ed., 233 pp. Methuen, London; Wiley, New York.

American Cyanamid Co. (1951). Allylol-substituted alkyl or alkaryl guanidines. British Patent 650, 820 (*Chem. Abstr.* **45**, 10626, 1951).

American Phytopathological Society. Committee on Standardisation of Fungicide Tests (1943). The slide-germination method of evaluating protectant fungicides. *Phytopathology* **33**:627–632.

American Phytopathological Society. Committee on Standardisation of Fungicide Tests (1947). Test-tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. *Phytopathology* **37**:354–356.

Block, S. S. (1955). Fungitoxicity of the 8-quinolinols. *J. Agr. Food Chem.* **3**: 229–234.

Brian, P. W. (1960). Griseofulvin. *Brit. Mycol. Soc. Trans.* **43**:1–13.

Brown, I. F., and H. D. Sisler. (1960). Mechanisms of fungitoxic action of *n*-dodecylguanidine acetate. *Phytopathology* **50**:830–839.

Burchfield, H. P., and E. E. Storrs. (1957). Effects of chlorine substitution and

isomerism on the interactions of *s*-triazine derivatives with conidia of *Neurospora sitophila*. *Contrib. Boyce Thompson Inst.* 18:429–452.

Byrde, R. J. W. (1961). Eradicant fungicides. *In* "Recent Advances in Botany," Proc. 9th Intern. Botan. Congr., Montreal, 1959, pp. 461–466. Univ. Toronto Press, Toronto, Canada.

Byrde, R. J. W., and G. C. Ainsworth. (1958). The chemotherapy of fungal diseases. *In* "The Strategy of Chemotherapy" (S. T. Cowan and E. Rowatt, eds.), pp. 309–335. Cambridge Univ. Press, London and New York.

Byrde, R. J. W., A. H. Fielding, and A. H. Williams. (1960). The role of oxidized polyphenols in the varietal resistance of apples to brown rot. *In* "Phenolics in Plants in Health and Disease" (J. B. Pridham, ed.), pp. 95–99. Macmillan (Pergamon), New York.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York; Chapman & Hall, London.

Corey, R. R., and H. G. Shirk. (1955). The influence of chemical structure on fungal activity IV. The effect of bisphenolic-type compounds. *Arch. Biochem Biophys.* 56:196–203.

Corke, A. T. K. (1962). Tests of spray materials for use against *Gloeosporium* spp. in apple orchards. *Ann. Appl. Biol.* 50:735–747.

Davson, H., and J. F. Danielli. (1943). "The Permeability of Natural Membranes," 361 pp. Cambridge Univ. Press, London and New York.

Dimond, A. E., J. W. Heuberger, and J. G. Horsfall. (1943). A water soluble protectant fungicide with tenacity. *Phytopathology* 33:1095–1097.

Evans, E., J. W. H. Taylor, R. Runham, and M. McLain. (1962). A comparison of certain physical characteristics of Bordeaux mixture and a copper oxychloride wettable powder and their possible significance in relation to biological performance. *Brit. Mycol. Soc. Trans.* 45:81–92.

Fargher, R. G., L. D. Galloway, and M. E. Probert. (1930). The inhibitory action of certain substances on the growth of mould fungi. *Mem. Shirley Inst.* 9:37–52. (*Rev. Appl. Mycol.* 9:783–784.)

Finholt, R. W., M. Weeks, and C. Hathaway. (1952). New theory on wood preservation. *Ind. Eng. Chem.* 44:101–105.

Forsyth, W. (1803). "A Treatise on the Culture and Management of Fruit Trees," 523 pp. London.

Gayner, F. C. H. (1961). Fungicide usage in world agriculture and horticulture. *Soc. chem. Ind.* Monograph No. 15, pp. 23–34.

Grove, J. F., and J. C. McGowan. (1947). Identity of griseofulvin and "curling-factor." *Nature* 160:574.

Grove, J. F., J. MacMillan, T. P. C. Mulholland, and M. A. T. Rogers. (1952). Griseofulvin. Part IV Structure. *J. Chem. Soc.* pp. 3977–3987.

Gruenhagen, R. H., P. A. Wolf, and E. E. Dunn. (1951). Phenolic fungicides in agriculture and industry. *Contrib. Boyce Thompson Inst.* 16:349–356.

Hamilton, J. M., and M. Szkolnik. (1958). Standard and new fungicides for the control of apple scab and cherry leaf spot. *Proc. N. Y. State Hort. Soc., 1958* pp. 72–78.

Härtel, K. (1961). Fungicidal activity of a selective acaricide based on dinitroalkylphenylacrylate. *Meded. LandbHoogesch. Gent* 26:1331–1342.

Hazen, E. L. and R. Brown. (1951). Fungicidin, an antibiotic produced by a soil actinomycete. *Proc. Soc. Exptl. Biol. Med.* 76:93–97.

Hazen, E. L., and R. Brown. (1960). Nystatin. *Ann. N. Y. Acad. Sci.* 89:258–266.

Horsfall, J. G. (1956). "Principles of Fungicidal Action," 279 pp. Chronica Botanica, Waltham, Massachusetts.

Horsfall, J. G., and S. Rich. (1959). Antisporulant action of 2-(trichloropropyl)-benzothiazole. *Phytopathology* **49**:541.

Horsfall, J. G., and S. Rich. (1960). Antisporulant action of hexachloro-2-propanol. *Phytopathology* **50**:640.

Kittleson, A. R. (1952). A new class of organic fungicides. *Science* **115**:84–86.

Large, E. C. (1940). "The Advance of the Fungi," 488 pp. Cape, London.

Lukens, R. J., and H. D. Sisler. (1958). Chemical reactions involved in the fungitoxicity of captan. *Phytopathology* **48**:235–244.

McCallan, S. E. A., and R. H. Wellman. (1943). A greenhouse method of evaluating fungicides by means of tomato foliage diseases. *Contrib. Boyce Thompson Inst.* **13**:93–134.

McIntosh, A. H. (1961). Effects of time and temperature on the fungistatic action of mercury-containing and other compounds on conidia of *Botrytis fabae* Sardiña. *Ann. Appl. Biol.* **49**:433–444.

McNew, G. L., and H. P. Burchfield. (1951). Fungitoxicity and biological activity of quinones. *Contrib. Boyce Thompson Inst.* **16**:357–374.

Martin, H. (1959). "The Scientific Principles of Crop Protection," 4th ed., 359 pp. Arnold, London.

Millardet, P. M. A. (1885). Traitement du mildiou par le mélange de sulphate de cuivre et de chaux. *J. Agr. Prat.* **2**:707–710 (cited by Horsfall, 1956).

Miller, L. P., and S. E. A. McCallan. (1956). Use of radioisotopes in tracing fungicidal action. *Proc. Intern. Conf. Peaceful Uses Atomic Energy, Geneva, 1955* Vol. 12. pp. 170–176. Columbia Univ. Press, New York.

Miller, L. P., S. E. A. McCallan, and R. M. Weed. (1953a). Quantitative studies on the role of hydrogen sulfide formation in the toxic action of sulfur to fungus spores. *Contrib. Boyce Thompson Inst.* **17**:151–171.

Miller, L. P., S. E. A. McCallan, and R. M. Weed. (1953b). Rate of uptake and toxic dose on a spore weight basis of various fungicides. *Contrib. Boyce Thompson Inst.* **17**:173–195.

Montgomery, H. B. S., M. H. Moore, H. Shaw, and W. Steer. (1943). Insecticides and fungicides. *Ann. Rept., East Malling Res. Sta., Kent, 1942* pp. 25–27.

Owens, R. G. (1953a). Studies on the nature of fungicidal action. I. Inhibition of sulfhydryl-,amino-,iron- and copper-dependent enzymes *in vitro* by fungicides and related compounds. *Contrib. Boyce Thompson Inst.* **17**:221–242.

Owens, R. G. (1953b). Studies on the nature of fungicidal action. II. Chemical studies of benzenoid and quinonoid compounds in relation to fungitoxicity and inhibition of amino- and sulfhydryl-dependent enzymes. *Contrib. Boyce Thompson Inst.* **17**:273–282.

Owens, R. G., and G. Blaak. (1960). Site of action of captan and dichlone in the pathway between acetate and citrate in fungus spores. *Contrib. Boyce Thompson Inst.* **20**:459–497.

Owens, R. G., and H. M. Novotny. (1958). Mechanism of action of the fungicide dichlone (2,3-dichloro-1,4-naphthoquinone). *Contrib. Boyce Thompson Inst.* **19**:463–482.

Owens, R. G., and H. M. Novotny. (1959). Mechanism of action of the fungicide captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboxyimide]. *Contrib. Boyce Thompson Inst.* **20**:171–190.

Priest, D., and R. K. S. Wood. (1961). Strains of *Botrytis allii* resistant to chlorinated nitrobenzenes. *Ann. Appl. Biol.* **49**:445–460.

Reavill, M. J. (1954). Effect of certain chloronitrobenzenes on germination, growth and sporulation of some fungi. *Ann. Appl. Biol.* **41**:448–460.

Reese, E. T., and M. Mandels. (1957). Chemical inhibition of cellulases and β-glucosidases. *Res. Rept. (Microbiol.), Quartermaster Res. Develop. Center, Natick, Mass. No.* 17.

Rich, S. (1960). Fungicidal chemistry. *In* "Plant Pathology" (J. G. Horsfall and A. E. Dimond, eds.), Vol. 2, pp. 553–602. Academic Press, New York.

Richmond, D. V., and E. Somers. (1962). Studies on the fungitoxicity of captan. II. The uptake of captan by conidia of *Neurospora crassa*. *Ann. Appl. Biol.* **50**:45–56.

Riehm, E. (1913). Prüfung einiger Mittel zur Bekämpfung des Steinbrandes. *Mitt. K. Biol. Anst.* [Abstr. in *Zentr Bakteriol., Parasitenk., Abt. II* **40**:424 (1914).]

Schuldt, P. H., and C. N. Wolf. (1957). Fungitoxicity of substituted *s*-triazines. *Contrib. Boyce Thompson Inst.* **18**:377–393.

Sempio, C. (1932). Sulla interpretazione del meccanismo intimo di azione dello zolfo come anticrittogamico. *Mem. Reale Accad. Italia, Biol.* 3:1–30 (cited by Martin, 1959).

Simon, E. W., and G. E. Blackman. (1949). The significance of hydrogen-ion concentration in the study of toxicity. *Symp. Soc. Exptl. Biol.* 3:253–264.

Somers, E. (1958). Uptake of a homologous series of fungicides by plant tissue and fungal spores. *Can. J. Botany* **36**:997–1009.

Somers, E. (1961). The fungitoxicity of metal ions. *Ann. Appl. Biol.* **49**:246–253.

Somers, E. (1962). Mechanisms of toxicity of agricultural fungicides. *Sci. Progr. (London)* **50**:218–234.

Sprague, R. (1949). Apple powdery mildew spray trials at the tree fruit station in 1949. *Proc. Wash. State Hort. Assoc.* **45**:47–50.

ter Horst, W. P., and E. L. Felix. (1943). 2,3-Dichloro-1,4-naphthoquinone, a potent organic fungicide. *Ind. Eng. Chem.* **35**:1255–1259.

Thorn, G. D., and R. A. Ludwig. (1962). "The Dithiocarbamates and Related Compounds," 298 pp. Elsevier, Amsterdam.

Tisdale, W. H., and I. Williams. (1934). "Disinfectant." U.S. Patent 1,972,961 (cited by Horsfall, 1956).

van der Kerk, G. J. M. (1959). Chemical structure and fungicidal activity of dithiocarbamic acid derivatives. *In* "Plant Pathology: Problems and Progress, 1908–1958" (C. S. Holton *et al.*, eds.), pp. 280–290. Univ. Wisconsin Press, Madison, Wisconsin.

van der Kerk, G. J. M. (1961). New developments in organic fungicides. *Soc. Chem. Ind.* Monograph No. 15, pp. 67–91.

Wellman, R. H., and S. E. A. McCallan. (1946). Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. *Contrib. Boyce Thompson Inst.* **14**:151–160.

West, W., and F. T. Wolf. (1955). The mechanism of action of the fungicide 2-heptadecyl-2-imidazoline. *J. Gen. Microbiol.* **12**:396–401.

Whitfield, A. (1912). Eczematoid ringworm of the extremities and groins. *Proc. Roy. Soc. Med.* **5**:Part 1, 36–62.

Woodcock, D. (1959). The relation of chemical structure to fungicidal activity. *In* "Plant Pathology: Problems and Progress, 1908–1958" (C. S. Holton *et al.*, eds.), pp. 267–279. Univ. Wisconsin Press, Madison, Wisconsin.

Woolley, D. W. (1945). Observations on antimicrobial action of 2,3-dichloro-1,4-naphthoquinone and its reversal by vitamine K. *Proc. Soc. Exptl. Biol. Med.* **60**:225.

Yarwood, C. E. (1950). Effect of temperature on the fungicidal action of sulfur. *Phytopathology* **40**:173–180.

CHAPTER 21

The Physical Environment for Fungal Growth

1. Temperature

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I. INTRODUCTION

A. How Temperature Affects Reactions

Temperature has a major effect on all cellular activities. Below 0°C, fungal cells may survive but rarely grow, and above 40°C most cells stop growing and soon die. Between these temperatures, fungal activities increase and decrease with the degree of temperature, for the reasons set out below.

In ordinary chemical reactions, the rate increases as the temperature is increased. Enzymatic reactions behave in a similar way, and it is frequently found that, within certain temperature limits, a 10°C rise in temperature causes a doubling or tripling of the reaction rate. However, enzymes begin to suffer thermal inactivation at higher temperatures, often as low as 30°C and frequently above 60°C. Under a given set of conditions, an enzymatic reaction achieves a maximum rate at a certain temperature, known as the optimum. The decline in activity above the optimum is caused by the denaturation of the enzyme, which is a time-consuming process. Therefore, the apparent rate of a reaction at higher temperatures is a function of the time required to measure the rate. If the initial reaction rate can be recorded over seconds, then the rate will be higher than if measured over minutes.

B. The Concept of Cardinal Points

The growth of a fungus or the production of a fungal metabolite is the result of the interactions of numerous enzymatically controlled processes, each of which may have different temperature coefficients and optima. A fungal activity will start at a minimum temperature, increase to an optimum, and then decline and stop at a maximum temperature. These are known as the cardinal temperatures for that activity, but they are dependent upon other factors, which include time of exposure to any temperature.

The difficulty of defining the optimum temperature for biological processes in general was discussed by Blackman (1905), and for fungal growth in particular by Fawcett (1921). Fawcett measured daily increments of mycelial growth of four fungi on agar and found that there was a shifting of the optimum temperature downward with each successive observation period, with the exception of one fungus. The apparent optimum for *Pythiacystis* [*Phytophthora*] *citrophthora* shifted from 27.5°C for the first day to 24°C for the fifth day, for *Phytophthora terrestris* from 34° to 28°C, and for *Diplodia natalensis* from 31°C for the first day to 27°C for the third day. Furthermore at the highest temperature for growth, the rate decreased throughout the period of culture so that it soon reached zero. These facts illustrate that changes are brought about by higher temperatures, in time resulting in limitation of growth. Using *P. citrophthora*, Fawcett suggested that these changes were internal and were not caused by staling of the medium because the placing of new medium at the advancing edges of parts of colonies did not affect the growth rate.

That higher temperatures can affect growth and form of fungal cells indirectly as a result of stimulating the production of metabolites, which stale the medium, was shown by Brown and Horne (1926).

II. EFFECT ON METABOLISM

Those concerned with the industrial production of useful microbial metabolites will be aware that the optimum temperature may not be the same as that for fungal growth. Thus Arcamone *et al.* (1961) noted that in submerged cultures of *Claviceps paspali*, the concentration of a lysergic acid derivative after 9 days was 530 μg/ml at 21°C, but only 20 μg/ml at 30°C although the dry weight of mycelium differed but little over this temperature range.

However, it may be desirable to grow a fungus at one temperature to produce abundant mycelium and then to change the temperature to obtain a maximum yield of a metabolite. This approach was emphasized by Owen

and Johnson (1955) when they showed that *Penicillium chrysogenum* produced significantly more penicillin if started at 30°C, the growth optimum, and transferred to 20°C after 42 hours, than if kept at any constant temperature throughout.

Some instances of the failure of fungi to grow at certain temperatures have been traced to an inability to synthesize essential vitamins or amino acids at those temperatures. Mitchell and Houlahan (1946) showed that a mutant of *Neurospora* failed both to grow and to produce riboflavine at temperatures above 28°C. The mutant did grow and produce riboflavine above 28°C if supplied with trace quantities of riboflavine. Apparently the mutation had caused a defect in the synthetic pathway to riboflavine, and this was revealed at higher temperatures.

Barnett and Lilly (1948) found that *Sclerotinia camelliae* had no inositol deficiency at 10°C, but that a requirement became apparent as the temperature was increased toward 26°C, although the concentration of inositol which stimulated growth at this temperature was inhibitory at 27°C.

Fries (1953) studied a strain of *Coprinus fimetarius* which had a growth optimum at 40°C and grew strongly at 44°C only if provided with a casein hydrolyzate. The active component of the latter was traced to methionine, and it was suggested that a block in methionine synthesis above 40°C was responsible for limiting growth.

These are illustrations that the many processes associated with fungal activities can be affected in different ways by certain conditions, and that consequently growth can be limited by the failure of a single process which is particularly temperature sensitive.

III. EFFECT ON GROWTH

Much of the literature describing the effect of temperature on the growth of mycelium has been reviewed by Wolf and Wolf (1947), Hawker (1950), and Cochrane (1958).

A. Cardinal Temperatures

The extensive survey by Cartwright and Findlay (1934) of many wood-destroying fungi contains examples of most characteristics revealed in studies of growth at different temperatures. Thus *Merulius sylvester* grows slowly at 10° and 35°C and most rapidly at 23°C, the decline in growth on each side of the optimum temperature being approximately equal. *Merulius lacrymans* [*Serpula lacrimans*], which causes dry rot of timbers in buildings in Britain, grows slowly at 8°C and has an optimum at 23°C, but it fails to grow above 27°C. This very rapid decline in ability to grow

as temperature is increased above the optimum is frequently found in fungi. The most frequent optima were in the range 23–30°C, but *Stereum rugosum* grew fastest at 20 C and *Lenzites trabea* at 35 C. An unusually high maximum temperature for growth was that of *Schizophyllum commune* at 44°C, most maxima being between 35° and 40°C.

Ryan *et al.* (1943) used glass tubes for studying mycelial growth of *Neurospora crassa* on agar; they found that the rate of growth increased with temperature in a linear fashion from 0.07 mm/hour at 4.5°C to 5.2 mm/hour at 35.7°C. Rates of growth were constant at a given temperature for at least 200 hours, except above 40°C, where growth progressively slowed and soon stopped. This type of effect is similar to those described by Cartwright and Findlay (1934) and by Fawcett (1921).

The observed cardinal points of species within the same genus differ greatly and probably reflect the temperatures in the natural environments of the fungi. Weimer and Harter (1923) studied eleven species of *Rhizopus* and divided them into three groups by their temperature responses. *Rhizopus chinensis* had an optimum of 40°C, a maximum of 45–50°C and a minimum of 10°C. Another group of six species had optima of 30–35°C and maxima toward 45°C. A third group, including *R. stolonifer,* which incites a rot of sweet potato optimally at 15°C to 23°C, had growth optima of 25–30°C and maxima of 30–35 C. Similarly, Edson and Shapolov (1919) showed that there were different optima among species of *Fusarium* and that there were good correlations between the optima for growth of *F. oxysporum* and *Verticillium alboatrum* and the temperatures at which they were serious plant pathogens.

B. Temperature Coefficients

The temperature coefficient of a reaction is the ratio of its rate at one temperature to its rate at another temperature; e.g., it is termed the Q_{10} when there is a 10°C difference between these two temperatures. Fawcett (1921) estimated the coefficients for the growth of four fungi for each whole interval of 10°C on each successive daily period of his experiments. In general, the Q_{10} for mycelial growth was greatest for the ranges over the lowest temperatures and decreased toward higher temperatures, and the Q_{10} for a particular range of temperatures was always largest on the first day of growth and declined on successive days. Thus the Q_{10} for *Phytophthora terrestris* at 8–18°C was 30 on the first day of growth, 26 on the second, 15 on the third, and 6.6 on the fourth day. The Q_{10} at 20–30°C was 2.2 on the first day and it declined to 1.7 on the fourth day. The highest Q_{10} for each of the other fungi was between 12 and 17 on the first day for 8–18°C. Coefficients of about 2–3, which are frequently re-

corded for other biological processes at "physiological" temperatures, were obtained for the growth of all four fungi at different 10°C ranges between about 13°C and 30°C.

The growth of fungi is affected by temperature in much the same way as other biological activities, although some fungi will tolerate more extreme temperatures than any other organisms but bacteria and algae.

C. Growth at Extreme Temperatures

Most fungi have minimum temperatures for growth of 5–10°C, but there are some that will grow at and below freezing point. For example, Brooks and Hansford (1923) found that some strains of *Cladosporium herbarum,* which grow on meat in cold storage, would grow at −6°C.

Conversely, a few fungi that have growth maxima between 40°C and 50°C have been mentioned, and there are others that grow optimally at above 40°C. Thus *Penicillium dupontii* grows well at 40–47°C, but poorly below 40°C and not at all at room temperature (Raper and Thom, 1949). La Touche (1950) described a species of *Chaetomium,* which was isolated from fermenting straw, with an optimum at 50°C and an ability to grow at 60°C if transferred to that temperature through a gradual sequence of rising temperatures.

IV. SURVIVAL AT EXTREME TEMPERATURES

A. Low Temperatures

Undoubtedly some fungal cells will survive exposure to subzero conditions, but there are examples of both survival and death in the literature. Thus Forbes (1939) found that spores of *Puccinia coronata* did not survive exposure to −18°C although they retained their viability when kept at 0°C. Ward (1902) showed that urediospores of *P. dispersa* [*P. recondita*] withstood 10 minutes, but not 4 hours, at −5°C, Harter and Zaumeyer (1941) noted that urediospores of *Uromyces phaseoli* [*U. appendiculata*] can be kept viable for several years if dried for a few days at room temperature before transfer to −20°C, and Melander (1935) found that spores of *P. graminis* survived temperatures below −29°C better if previously hardened by exposure to temperatures of 0 to 1°C for 10 days. The author has kept viable cultures of *Botrytis* spp. at −20°C for a year.

Cochrane (1958) points out that two important factors which determine the fate of cells at low temperatures are the rates of freezing and of thawing. During slow freezing, crystals of ice form around cells causing dehydration and injury to the cells, but the damage can be reduced if ethylene

glycol is added. Rapid freezing may also reduce cellular injury. Conversely the rate of thawing is most important in permitting survival. Mazur (1956) cooled conidia of *Aspergillus flavus* to −70°C, and found that only 7% germinated when they were warmed at 0.9°C per minute, but that 75% germinated when they were warmed at 700°C per minute. The lethal effects of slow warming were most pronounced between −20°C and 0°C.

Under natural conditions, repeated slow freezing and thawing may be most damaging to fungal cells, but survival is probably modified by the type of cell, spore or mycelium, and its dryness. Low temperatures may be used to preserve viable cultures or spores, but it would be advisable first to make tests with the organism in question.

B. High Temperatures

Most fungal cells are killed by brief exposure to temperatures of 60°C. The simple relationship between time and temperature was well shown by Henderson Smith (1923), who examined the germination of *Botrytis cinerea* spores after they had been exposed for different intervals to different temperatures: 50% killing of the spores at 38°C required eight times the exposure needed at 50°C.

Thermal death point may be defined as the lowest temperature required to kill all cells in 10 minutes. Ames (1915) compared the death points of six fungi responsible for storage rots and found that they ranged from 47°C for *Cephalothecium roseum* to 60°C for *Rhizopus stolonifer*. These results are typical for many fungi, but there are some exceptions, for example, *Byssochlamys fulva* with 20% survival of ascospores after 10 minutes at 85°C (Hull, 1939).

Moist heat is often more effective in killing cells than dry heat, as shown by Snell (1923), who subjected test blocks of Sitka spruce containing five fungi to various times and temperatures of moist and dry heat. In moist heat, the most resistant fungus was killed in 12 hours at 55°C, but in dry heat this species was not killed by 20 days at 70°C although it did succumb in 12 hours at 105°C.

Laboratory sterilization of media and glassware is usually effected by exposure to steam at 15 pounds pressure per square inch for 20 minutes, but these conditions are primarily concerned with killing bacterial spores rather than fungal spores and mycelium, which are highly susceptible under less severe conditions, as described above.

REFERENCES

Ames, A. (1915). The temperature relations of some fungi causing storage rots. *Phytopathology* 5:11–19.

Arcamone, F., E. B. Chain, A. Ferretti, A. Minghetti, P. Pennella, A. Tonolo, and L. Vero. (1961). Production of a new lysergic acid derivative in submerged culture by a strain of *Claviceps paspali* Stevens and Hall. *Proc. Roy. Soc.* **B155**:26–54.

Barnett, H. L., and V. G. Lilly. (1948). The interrelated effects of vitamins, temperature and pH upon vegetative growth of *Sclerotinia camelliae*. *Am. J. Botany* **35**:297–302.

Blackman, F. F. (1905). Optima and limiting factors. *Ann. Botany (London)* **19**: 281–295.

Brooks, F. T., and C. G. Hansford. (1923). Mould growth upon cold store meat. *Brit. Mycol. Soc. Trans.* **8**:113–141.

Brown, W., and A. S. Horne. (1926). Studies in the genus *Fusarium*. III. An analysis of factors which determine microscopic features of *Fusarium* strains. *Ann. Botany (London)* **40**:203–222.

Cartwright, K. St. G., and W. P. K. Findlay. (1934). Studies on the physiology of wood-destroying fungi. II Temperature and rate of growth. *Ann. Botany (London)* **48**:481–495.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Edson, H. A., and M. Shapolov. (1919). Temperature relations of certain potato-rot and wilt-producing fungi. *J. Agr. Res.* **18**:511–524.

Fawcett, H. S. (1921). The temperature relations of growth in certain parasitic fungi. *Univ. Calif. (Berkeley) Publ. Agr. Sci.* **4**:183–232.

Forbes, J. L. (1939). Factors affecting the development of *Puccinia coronata* in Louisiana. *Phytopathology* **29**:659–684.

Fries, L. (1953). Factors promoting growth of *Coprinus fimetarius* under high temperature conditions. *Physiol. Plantarum* **6**:551–563.

Harter, L. L., and W. J. Zaumeyer. (1941). Differentiation of physiologic races of *Uromyces phaseoli typica* on bean. *J. Agr. Res.* **62**:717–731.

Hawker, L. E. (1950). "Physiology of Fungi," 360 pp. Oxford Univ. Press (Athlone), London and New York.

Henderson Smith, J. (1923). The killing of *Botrytis cinerea* by heat, with a note on the determination of temperature coefficients. *Ann. Appl. Biol.* **10**:335–347.

Hull, R. (1939). Study of *Byssochlamys fulva* and control measures in processed fruit. *Ann. Appl. Biol.* **26**:800–822.

La Touche, C. J. (1950). On a thermophile species of *Chaetomium*. *Brit. Mycol. Soc. Trans.* **33**:94–104.

Mazur, P. (1956). Studies on the effect of subzero temperatures on the viability of spores of *Aspergillus flavus*. I. The effect of rate of warming. *J. Gen. Physiol.* **39**: 869–888.

Melander, L. W. (1935). Effect of temperature and light on development of the uredial stage of *Puccinia graminis*. *J. Agr. Res.* **50**:861–880.

Mitchell, H. K., and M. B. Houlahan. (1946). *Neurospora*. IV. A temperature sensitive riboflavin-less mutant. *Am. J. Botany* **33**:31–35.

Owen, S. P., and M. J. Johnson. (1955). The effect of temperature change on the production of penicillin by *Penicillium chrysogenum* W49-133. *Appl. Microbiol.* **3**:375–379.

Raper, K. B., and C. Thom. (1949). "A Manual of the Penicillia," 875 pp. Williams & Wilkins, Baltimore, Maryland.

Ryan, F. J., G. W. Beadle, and E. L. Tatum. (1943). The tube method of measuring the growth rate of *Neurospora*. *Am. J. Botany* **30**:784–799.

Snell, W. H. (1923). The effect of heat upon the mycelium of certain structural timber-destroying fungi within wood. *Am. J. Botany* **10**:399–411.

Ward, H. M. (1902). On the relations between host and parasite in the bromes and their brown rust, *Puccinia dispersa* (Erikss). *Ann. Botany* (*London*) **16**:233–315.

Weimer, J. L., and J. L. Harter. (1923). Temperature relations of eleven species of *Rhizopus*. *J. Agr. Res.* **24**:1–40.

Wolf, F. A., and F. T. Wolf. (1947). "The Fungi," Vol. II, 538 pp. Wiley, New York.

CHAPTER 22

The Physical Environment for Fungal Growth

2. Hydrostatic Pressure

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I. INTRODUCTION

The parameter of hydrostatic pressure in relation to life processes has generally been neglected. Yet when one considers that a vast portion of the biosphere is under hydrostatic pressure, one realizes that pressure must be an important factor. Generally speaking, physiological and biochemical studies are made at 1 atmosphere (atm) (which is not even mentioned, but assumed) whereas temperature and time are rigidly controlled. Even the marine biologist, although he recognizes the problem, does not deal with this aspect to any great degree. The "perfect gas law" ($PV = nRT$) and Le Chatelier's principle when applicable to biological systems should not be neglected. This section will deal only with hydrostatic pressures from 1 to 1000 atm.

The Talisman Expedition in 1882–1883 established the fact that life can exist under the hydrostatic pressure at the bottom of the sea. The vertical migration of some marine plankton as a result of changes in hydrostatic pressure (Knight-Jones and Qasim, 1955), activation of prophage in *Escherichia coli* (Rutberg and Hedén, 1960), malic dehydrogenase activity at 101°C under hydrostatic pressure (Morita and Haight, 1962), sol-gel reactions in cell division (Marsland, 1951), occurrence of barophilic bacteria (ZoBell and Morita, 1957), and the dredging of life from the various hadal portions of the sea (Bruun, 1955) are examples where hydrostatic pressure plays an important role in biology. Numerous other investigations

dealing with hydrostatic pressure effects in relation to metabolic processes and physiology are cited by Johnson *et al.* (1954). However, fungi have been little used for studies in relation to the parameter of pressure.

II. OCCURRENCE OF FUNGI IN RELATION TO DEPTH OF THE BIOSPHERE

Hydrostatic pressure increases approximately 1 atm for every 10 meters (*m*) in depth of the ocean. Within the biosphere, especially in the ocean, hydrostatic pressures over 1000 atm exist. The distribution of fungi in relation to depth in the biosphere has not been investigated. Most of the ecological investigations have been confined to the nearshore environment, such as inland water, bays, and estuaries, with a few investigations going beyond the continental shelf. Whether or not pressure plays an important part in the vertical distribution of fungi is not known. Saprophytic fungi were noted by Sparrow (1937) at depths from 18 to 1127 m while Höhnk (1956) was able to demonstrate the occurrence of fungi in North Sea sediment cores from depths of 1150 and 1250 m. Höhnk (1959) also recovered fungi from water obtained from a depth of 4610 m and from sediment at 3425 m. Numerous cores taken at depths of 1700–6000 m were examined for bacteria during the Mid-Pacific Expedition by Morita and ZoBell (1955), and quite frequently elongated filamentous fungi were observed. These filamentous forms were believed to have germinated from spores that had settled to the sea floor. It should also be mentioned that no filamentous fungi were detected from material obtained from the bottom of the various hadal portions of the Indian and Pacific Oceans (ZoBell and Morita, 1957); however, in the last two studies media for fungi were not employed. Both baroduric and barophilic bacteria were found in sediment samples from the hadal portions of the sea.

Kriss (1959) reported that yeasts have been found in the hydrogen sulfide zones 50 miles from shore at 800 and 1250 m in the Black Sea and at 4000 *m* in the Pacific Ocean.

III. EFFECT OF HYDROSTATIC PRESSURE ON PHYSIOLOGICAL ACTIVITIES

As early as 1884, Certes and Cochin reported that yeast cells held at 300–400 atm for several days remained viable and that fermentation resulted under these conditions. Verification of these results is presented in Table I.

Studies on fungi in relation to pressure have been neglected. Hill (1962) found that 600 atm or above at 27°C for 72 hours was sufficient to retard

TABLE I

EFFECT OF HYDROSTATIC PRESSURE ON THE PRODUCTION OF ETHANOL BY *Saccharomyces cerevisiae*[a]

Atmospheres	Ethanol[b] (mg/6 ml)
1	4.12
200	3.16
400	1.77
600	0.95
800	0.41
1000	0.15

[a] The reaction mixture contained equal volumes of 0.278 *M* glucose in 0.067 *M* phosphate buffer, pH 5.6 and washed cells in 0.067 *M* phosphate buffer, pH 5.6. The cells were harvested from an 18-hour culture, washed twice in 0.067 *M* phosphate buffer, pH 5.6, and adjusted to give a transmittance of 30% at 600 mμ in a spectrophotometer (Bausch and Lomb Spectronic 20). The reaction mixture was incubated for 3 hours at 27°C. The values are corrected for controls (Morita *et al.*, 1960).

[b] Milligrams of ethanol produced per 6 ml of reaction mixture.

all growth of the aquatic phycomycete *Allomyces macrogynus*. When mycelia and zoospores were held under the above conditions and then transplanted to appropriate medium, no growth could be demonstrated, nor did the zoospores germinate. Hill also observed by use of electron micrographs of ultrathin sections that the mitochondria of *Allomyces* showed no visible cytological difference when held at 1 or 600 atm although metabolic function, in terms of the dehydrogenase activities (malic, succinic, oxalosuccinic, and α-ketoglutaric acids), decreased with increased pressure. No activity of isocitric and α-ketoglutaric dehydrogenases could be detected at 1000 atm. However, it should be emphasized that various organisms differ in their response to pressure.

The theory of absolute reaction rates, as applied to biological phenomena, is adequately reviewed by Johnson *et al.* (1954) and Johnson (1957). According to Lamanna and Mallette (1959) the absolute reaction theory has not been as successful when applied to other biological studies as in the case of bioluminescence; this may reflect deficiencies in the theory, or the experimental difficulties in making the required necessary precise observations. This concept can be applied to certain reactions (i.e., enzyme reactions), but when it involves the "biological whole" where the cell is an organized growing and metabolizing structure, the interaction of all factors, including environments, must be taken into consideration. The studies on growth, reproduction, and death in bacteria under various hydrostatic pressure are perhaps the best type of experiments performed to date reflecting the sum of all factors (cellular, environmental, physical, and chemical).

Because of the lack of data involving pressure studies with fungi, the subject matter will have to be discussed using results of studies done with other organisms.

Molecular volume changes due to temperature and pressure play an important role in the ability of the cell to metabolize. If these changes are too drastic the cell will die. Generally speaking, those reactions that increase in molecular volume are inhibited by pressure while those that decrease in molecular volume are enhanced. ZoBell and Johnson (1949) have shown that moderate pressures enable many common species of terrestrial bacteria to grow at temperatures above the normal optimum growth temperatures at 1 atm. An extreme example of the pressure-temperature relationship was demonstrated by Morita and Haight (1962) when they found that the molecular volume increase in malic dehydrogenase due to a temperature of 101 C was counteracted by pressures from 700 to 1500 atm (highest employed). Because pressure was able to keep the enzyme from undergoing complete denaturation, which is due to an unfolding and an increase in molecular volume of the enzyme molecule, malic dehydrogenase activity could be demonstrated at 101°C.

Changes in the organism's environment can take place in the ocean. It is well known that the pH of the sea water decreases with increased pressure (Buch and Gripenberg, 1932). When working with reaction mixtures in the laboratory one must take into consideration the effect of various types of buffers. Those buffers with a large ΔV are influenced by pressure more than those with a small ΔV. Kauzmann (1954) states that the pH of a phosphate buffer solution can be expected to change 0.4 units in acidity under a pressure of 10,000 pounds per square inch (psi). Since the pH can be changed in a natural or artificial environment, other environmental changes undoubtedly occur.

Marsland and Brown (1936, 1942) demonstrated that pressure decreased the ability of amoeba to produce pseudopodia. This observation was interpreted in terms of the sol-gel equilibrium in which pressure and temperature have opposite effects. Intracellular gels are endothermic and usually of the type to increase in volume on gelation. Increases in pressure shifts the sol-gel equilibrium to the right. Marsland *et al.* (1953) were able to demonstrate that intracellular gels require energy to form the gel state and that adenosine triphosphate (ATP) helps the cell to form the gel state under pressure.

Cells of *Serratia marinorubra* (ZoBell and Oppenheimer, 1950) and *Escherichia coli* (ZoBell and Cobet, 1962), when placed under conditions of temperature and pressure which prevented reproduction, demonstrated the ability to grow into long filaments. In *S. marinorubra* the filaments were best formed at 600 atm, but, when the pressure was released, the filaments

began dividing shortly thereafter into normal-sized cells of *S. marinorubra.* In *E. coli,* pressure retarded cell division more than growth or increase in biomass, but at 525 atm no growth could be detected. Furthermore, the lag phase is extended by increased pressure. Pressures of 400 atm or more increased the death rate of *E. coli* in nutrient medium over that at 1 atm, while at 1000 atm the death rate increased with temperature. The variability of response to pressure by various species of bacteria is well demonstrated in the work of Oppenheimer and ZoBell (1952).

Pseudomonas perfectomarinus and *E. coli* exhibit abnormal morphology when grown under pressure (Berger, 1959). At about 50–150 atm cross-wall formation and cell division were inhibited so that the cells grew into long filaments. The larger cells often lysed and released into solution material that absorbed at 260 mμ. At 300 atm diaminopimelic acid increased the release of the material absorbing at 260 mμ and also decreased the average size of the cells.

Morita and ZoBell (1956) demonstrated that the succinic dehydrogenase activity of *E. coli* cells was inhibited by pressures of 200–600 atm. Malic and formic dehydrogenases in cells of *E. coli* were also inactivated by moderate pressure (Morita, 1957a). *Escherichia coli* cells previously held at 600 atm at 30°C for 3 hours produced more ammonia from aspartic acid, alanine, and glutamic acid than cells held at 1 atm at 30°C for 3 hours (Morita, 1957b), but the reason for this effect is not known. Haight and Morita (1962) found that the effects of pressure and temperature were not exactly the same when working with cells and cell-free preparations of *E. coli.* Pressure was found to decrease aspartase activity at 45°C or lower when a cell-free preparation was used, and at 53°C or lower when cells were employed. This is further evidence that when the enzyme reaction is studied under pressure the results cannot necessarily be correlated with the cellular activity. Aspartase activity of the cell-free preparation was greater under pressures of 700 or 1000 atm than at 1 atm when the incubation temperature was 56°C. When the enzyme preparation was held at 1 atm for 35 minutes at 56° C. it underwent a small amount of thermal denaturation, but no thermal denaturation occurred under the same conditions under 700 or 1000 atm (molecular volume increase opposed by pressure). Haight and Morita (1962) also demonstrated that, in the case of aspartase, pressure alone does not protect the enzyme from thermal denaturation. Substrate must be present in order for the protective effect of pressure to be manifested.

In studying the rate of hydrolysis of a phenylglycoside by a glycosidase (20–50°C), Berger (1958) found that increased pressure inhibits both the rate of hydrolysis and the rate of thermal denaturation. When substrate is absent, thermal inactivation of the enzyme is accelerated by increased

pressure. He warns that the finding of apparent first-order kinetics for an inactivation process may lead one to conclude erroneously that the process is simple.

From the foregoing presentation, it can be readily seen that the parameters of pressure and temperature need further investigation.

REFERENCES

Berger, L. R. (1958). Some effects of pressure on a phenylglycosidase. *Biochim. Biophys. Acta* 30:522–528.

Berger, L. R. (1959). The effect of hydrostatic pressure on cell wall formation. *Bacteriol. Proc.* p. 129.

Bruun, A. F. (1955). Life and life conditions of the deepest deep sea. *Proc. 8th Pacific Sci. Congr. Pacific Sci. Assoc., Quezon City, 1953* Vol. 1, pp. 399–409.

Buch, K., and S. Gripenberg. (1932). Über den Einfluss des Wasserdruckes auf pH und das Kohlensäuregleichgewicht in grösseren Meerestiefen. *J. Conseil, Conseil Perm. Intern. Exploration Mer* 7:233–245.

Certes, A., and D. Cochin. (1884). Action des hautes pressions sur la vitalité de la levure et sur les phenomènes de la fermentation. *Compt. Rend. Soc. Biol.* 36:639–640.

Haight, R. D., and R. Y. Morita. (1962). Interaction between the parameters of hydrostatic pressure and temperature on aspartase of *Escherichia coli*. *J. Bacteriol.* 83:112–120.

Hill, E. P. (1962). Some effects of hydrostatic pressure on growth and mitochrondria of *Allomyces macrogynus*. Ph.D. Thesis, Univ. of Nebraska.

Höhnk, W. (1956). Studien zur Brack- und Seewassenmykologie VI. Über die pilzliche Besiedlung verschieden salziger suhmerser Standorte. *Veroeffentl. Inst. Meeresforsch. Bremerhaven* 4:195–213.

Höhnk, W. (1959). Ein Beitrag zur ozeanischen Mykologie. *Deut. Hydrograph. Z. Suppl.* B4, No. 3, 81–87.

Johnson, F. H. (1957). *In* "Microbial Ecology" (R. E. O. Williams and C. C. Spicer, eds.), pp. 134–167. Cambridge Univ. Press, London and New York.

Johnson, F. H., H. Eyring, and M. J. Polissar, eds. (1954). "The Kinetic Basis of Molecular Biology," 874 pp. Wiley, New York.

Kauzmann, W. J. (1954). *In* "The Kinetic Basis of Molecular Biology" (F. H. Johnson, H. Eyring, and M. J. Polissar, eds.), p. 302. New York.

Knight-Jones, E. W., and S. Z. Qasim. (1955). Responses of some marine plankton animals to changes in hydrostatic pressure. *Nature* 175:941–942.

Kriss, A. E. (1959). "Marine Microbiology," 536 pp. Oliver & Boyd, Edinburgh and London. (Translated from Russian by J. M. Shewan and Z. Kabata, 1962.)

Lamanna, C., and M. F. Mallette. (1959). "Basic Bacteriology," 2nd ed., 853 pp. Williams & Wilkins, Baltimore, Maryland.

Marsland, D. A. (1951). The action of hydrostatic pressure on cell division. *Ann. N.Y. Acad. Sci.* 51:1327–1335.

Marsland, D. A., and D. E. Brown. (1936). Amoeboid movement at high hydrostatic pressure. *J. Cellular Comp. Physiol.* 8:167–178.

Marsland, D. A., and D. E. Brown. (1942). The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. *J. Cellular Comp. Physiol.* 20:295–305.

Marsland, D. A., J. Landau, and A. Zimmerman. (1953). Adenosine triphosphate as an energy source in cell division; pressure temperature experiments on the cleaving eggs of *Arbacia* and *Chaetopterus*. *Biol. Bull.* **105**:366–367.

Morita, R. Y. (1957a). Effect of hydrostatic pressure on succinic, formic, and malic dehydrogenases in *Escherichia coli*. *J. Bacteriol.* **74**:251–255.

Morita, R. Y. (1957b). Ammonia production from various substrates by previously pressurized cells of *Escherichia coli*. *J. Bacteriol.* **74**:231–233.

Morita, R. Y., and R. D. Haight. (1962). Malic dehydrogenase activity at 101°C under hydrostatic pressure. *J. Bacteriol.* **83**:1341–1346.

Morita, R. Y., and C. E. ZoBell. (1955). Occurrence of bacteria in pelagic sediments collected during the Mid-Pacific Expedition. *Deep-Sea Res.* **3**:66–73.

Morita, R. Y., and C. E. ZoBell. (1956). Effect of hydrostatic pressure on the succinic dehydrogenase system in *Escherichia coli*. *J. Bacteriol.* **71**:668–672.

Morita, R. Y., P. W. Stave, and R. D. Haight. (1960). Unpublished data.

Oppenheimer, C. E., and C. E. ZoBell. (1952). The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. *J. Marine Res.* (*Sears Found. Marine Res.*) **11**:10–18.

Rutberg, L., and C. G. Hedén. (1960). The activation of prophage in *Escherichia coli* by high pressure. *Biochem. Biophys. Res. Commun.* **2**:114–120.

Sparrow, F. K. (1937). The occurrence of saprophytic fungi in marine muds. *Biol. Bull.* **73**:242–248.

ZoBell, C. E., and A. B. Cobet. (1962). Growth, reproduction, and death rates of *Escherichia coli* at increased hydrostatic pressures. *J. Bacteriol.* **84**:1228–1236.

ZoBell, C. E., and F. H. Johnson. (1949). The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. *J. Bacteriol.* **57**:179–189.

ZoBell, C. E., and R. Y. Morita. (1957). Barophilic bacteria in some deep sea sediments. *J. Bacteriol.* **73**:563–568.

ZoBell, C. E., and C. H. Oppenheimer. (1950). Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. *J. Bacteriol.* **60**:771–781.

CHAPTER 23

The Physical Environment for Fungal Growth

3. Light

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I. INTRODUCTION

Many fungi are affected by light. The effects of light on fungi may be divided roughly into two categories: morphogenetic effects—in which light induces or inhibits the formation of a structure—and nonmorphogenetic effects—in which light influences the rate or the direction of movement or growth of a structure or the synthesis of a compound. References to much of the literature on this topic may be found in the extensive bibliography of Marsh *et al.* (1959). This chapter is concerned only with nonmorphogenetic responses; the morphogenetic effects of light are considered elsewhere in this treatise (Volume II).

For purposes of discussion, the nonmorphogenetic responses of fungi to light may be classified on a basis of the type of responding structure and the nature of the response. Both vegetative hyphae and reproductive structures respond to light; the response may be a *nonoriented one*—either a stimulation or an inhibition of the rate of growth or the synthesis of a compound—or an *oriented one*, in which the response bears a spatial relationship to the source of illumination. If an oriented response to light is by movement, it is *phototaxis;* if it is by growth, it is *phototropism.*

II. NONORIENTED RESPONSES

A. Vegetative Structures

Examples of effects of light on the vegetative growth of fungi are rare, and each seems to present a special case. For instance, the dry weight of thalli of *Blastocladiella emersonii* harvested from cultures grown in light (60–80 foot-candles) on a complex medium was as much as 141% of that of cultures grown in darkness (Cantino and Horenstein, 1956). On synthetic media, growth was also stimulated by light, but the amount of stimulation depended on the composition of the medium (Cantino, 1959). The effect of light on *Blastocladiella* appears to be a very general one, because it is associated with increased CO_2 fixation, stimulation of the succinate-ketoglutarate-isocitrate (SKI) cycle, and nucleic acid synthesis (Turian and Cantino, 1959; Cantino and Turian, 1961). Only blue light is stimulatory, but the nature of the photoreceptor remains unknown (Cantino and Horenstein, 1959). Stimulation of vegetative growth by light has also been reported to occur in *Thraustochytrium roseum* (Goldstein, 1963).

Another example of the effect of light on vegetative growth is the inhibition of the elongation of hyphae of *Pilobolus kleinii* (Page, 1952a). When *P. kleinii* was grown in growth tubes on synthetic media containing hemin, the hyphae elongated at a constant rate in darkness. In light, however, the growth rate began to decline after several days, and finally the hyphae stopped elongating. This inhibition appears to be related to the medium, because growth on media containing dung extract instead of hemin was not inhibited by light. Since inhibition occurred when filters containing dung extract were interposed between the light source and cultures growing on media with hemin, it is evident that the effect of dung extract was not merely to shade the hyphae (Willoughby, 1961). Mycelia whose growth had ceased in light resumed their growth when cultures were transferred to darkness, but usually the hyphae did not attain a rate of elongation equal to that previous to illumination (Page, 1952b). Some other features of the inhibition, in addition to its occurrence on media containing hemin, suggest that it might be attributable to a photodynamic effect (Willoughby, 1961). As such, it must be considered a special case rather than one of general significance.

Inhibition of hyphal growth by light has been demonstrated in two basidiomycetes, and the response appears to be rapid. When haplophasic hyphae of *Coprinus lagopus* were exposed to light, they continued to elongate for 5–10 minutes; then the growth rate fell rapidly until growth

ceased 25–35 minutes after the beginning of exposure (Borriss, 1934). When hyphae were returned to darkness, they resumed growth, and if they were resubjected to light, inhibition began after a shorter lag period than when they were fully dark-adapted. Hyphae that were kept in light after they had stopped growing did not begin to grow again, at least during the 1-hour period of observation. A similar inhibition of growth by light was shown by germ tubes from urediospores of *Puccinia triticina* [*P. recondita*] (Gettkandt, 1954). Growth of germ tubes of this rust slowed 4–10 minutes after the beginning of illumination.

B. Sporangiophores

1. Thamnidium

A slow negative growth response is shown by sporangiophores of *Thamnidium elegans* (Lythgoe, 1961). If young (stage I) sporangiophores of this member of the Mucorales are illuminated, the growth rate decreases and reaches a minimum about 35 minutes after the beginning of the exposure. The rate then increases until the original rate is regained about 30 minutes later. More mature sporangiophores (stage IV) respond more quickly, and for both stages, the log of the rate of onset of inhibition is proportional to the absolute temperature. The action spectrum for this response has not yet been determined, nor is it known whether adaptation occurs.

2. Phycomyces

The nonoriented response of a fungus to light that has been studied in by far the greatest detail is the transient light-growth response of the sporangiophore of *Phycomyces*. This has been the subject of a series of analytical papers published by Castle and by Delbrück and co-workers (cf. Delbrück, 1963; Shropshire, 1963). The development of the sporangiophore has been described and divided into stages by Castle (1942). The sporangiophore arises from the mycelium, emerges from the medium, and elongates in the air (stage 1). Elongation stops while the globose sporangium is being formed (stages II and III), and then growth is resumed (stage IVa). After a short time, the direction of rotation of the tip of the sporangiophore reverses, and the sporangiophore continues to elongate for many hours (stage IVb). The rate of elongation during stage IVb is constant in darkness or in light of constant intensity as long as the temperature remains the same (Castle, 1928). If, however, the level of illumination is changed, the sporangiophore responds by a transient change in growth rate.

Various aspects of this transient growth response of the *Phycomyces*

sporangiophore have been investigated. First, a response may be evoked either by an increase in the level of illumination which is relatively permanent (step-up) or brief (pulse-up), or by a decrease in the level of illumination which may be of long (step-down) or short (pulse-down) duration (Delbrück and Reichardt, 1956). After a stimulus of the pulse-up type, the growth rate of the sporangiophore remains unchanged for about 2.5 minutes. It then increases to a maximum after 5 minutes, decreases rapidly to a minimum about 8 minutes after the exposure, and finally returns to the initial growth rate after another 12 minutes. The response to a stimulus of the step-up type follows a similar course, except that the growth rate never falls below the initial rate. With either a pulse-down or a step-down in illumination, the rate of elongation first decreases and then increases until it regains the initial rate, so that the sign of the response is opposite to that obtained with an increase in the level of illumination. The average rate of elongation over the course of the response is the same as the rate of steady-state growth, so that the response may be said to consist of a redistribution of growth in time. Within limits, the magnitude of the response depends on the dose, and reciprocity is followed over a considerable range of stimuli, provided that the duration of the exposure does not exceed 1 minute. The action spectrum for this response has been determined accurately by Delbrück and Shropshire (1960). Wavelengths in the blue and ultraviolet are effective, and the action spectrum shows peaks at 485, 455, 385, and 280 mμ.

One of the interesting features of the transient response of *Phycomyces* is that the sporangiophores show adaptation to levels of illumination that cover a billionfold range of intensity. Delbrück and Reichardt (1956) defined the level of adaptation in mathematical terms. The response appears to be a function of the ratio of the intensity to the level of adaptation as thus defined. If the ratio is unity, no response is expected; if it is greater than 1, a positive response should occur, whereas if it is less than 1, a negative response is expected. The responses observed agreed well with those predicted for various programs of illumination, including an ingenious "sunrise" experiment in which an exponential increase in light intensity induced the expected above-normal growth rate for as long as the increase could be maintained.

Whether the transient light-growth response of *Phycomyces* is homologous with the light-induced growth response of *Thamnidium* is questionable. Not only is the initial sign of the response opposite in the two cases—light induces an increase in the rate of growth of *Phycomyces* sporangiophores but a decrease in the rate of elongation of the *Thamnidium* sporangiophore —but also the pace of the response in *Thamnidium* is so leisurely as to suggest a different system of response.

C. Synthetic Reactions

Reference has been made to the work of Cantino and co-workers (Section II, A), with *Blastocladiella,* who have reported that the synthesis of nucleic acids and other chemical reactions is stimulated by light. Moreover, it will be noted below (Section IV, B) that, with the exception of *Blastocladiella,* all the light-induced growth responses studied thus far appear to involve an effect upon wall synthesis.

Another type of response has involved the synthesis of pigments in fungi. Thus, Schaeffer (1953) has reported that blue light depressed melanogenesis and tyrosinase activity in a mutant of *Neurospora crassa.* The opposite effect of light on melanin formation has been described for *Aureobasidium pullulans* (Lingapapa *et al.,* 1963) and *Cladosporium mansonii* (Sussman *et al.,* 1963). The two latter organisms differ in their response to light on certain media, variations which influence melanogenesis drastically. Growth of *A. pullulans* was stimulated markedly when polysaccharides like starch and dextrin were the carbon source. Moreover, light-grown cells of *C. mansonii* were about $3 \times 5\ \mu$ and predominantly mycelial, compared to dark-grown ones which averaged $13 \times 14\ \mu$ and were mainly unicellular. Finally, it is a common observation that cultures of *Neurospora* turn pink in the light. Zalokar (1954) has established that carotenoid synthesis is stimulated under these conditions.

III. ORIENTED RESPONSES

A. Phototaxis

Responses by movement to asymmetrical illumination appear to be rare in fungi. Such responses might be possible in motile zoospores of aquatic phycomycetes or slime molds, but so far they have not been reported. The presence of an eyespot in the swarmers of *Labyrinthula algeriensis* (Hollande and Enjumet, 1955) suggests that these cells might respond to light, but the eyespot also provides an argument for excluding these organisms from the Myxomycophyta.

Perhaps the only authentic case of phototaxis in an organism usually included with the fungi is the positive phototactic response of the migrating pseudoplasmodium of species of *Dictyostelium.* These communal entities are extremely sensitive to light. For example, light from a 1-watt neon lamp sufficed for the routine orientation of pseudoplasmodia of *D. mucoroides* (Bonner and Shaw, 1957), and migrating pseudoplasmodia of *D. discoideum* will move toward the light from the dial of a luminous wrist-

watch (Bonner, 1959). In a more quantitative study (cf. Raper, 1962), almost half the pseudoplasmodia tested responded to a 30-minute exposure to 0.04 ergs/mm^2/second.

The mechanism of the phototactic response of *D. discoideum* is not well understood. The light stimulus is perceived by the anterior portion of the migrating pseudoplasmodium, and a lens effect seems to be involved, because when pseudoplasmodia were illuminated with a small spot of light, they turned out of the beam (cf. Raper, 1962). Although Bonner *et al.* (1950) found that pseudoplasmodia responded to all wavelengths from 380 to 700 mμ, it would appear that the photoreceptor was saturated by the relatively high intensities used, because a more precise determination of the action spectrum with low intensities from a monochromator indicated a major peak of sensitivity near 425 mμ with a lesser peak near 550 mμ (cf. Raper, 1962).

B. Phototropism

Responses by growth toward asymmetrical light stimuli are shown by representatives of all the major groups of filamentous fungi. Much of the literature on phototropism is included in the comprehensive and critical review by Banbury (1959), and the subject is treated by Carlile (1965). In a few cases, phototropic responses of vegetative hyphae have been observed, but in most cases it is reproductive structures that respond to asymmetrical illumination.

1. Vegetative Hyphae

Negative phototropic curvature of germ tubes from urediospores of seven species of *Puccinia* was reported by Gettkandt (1954), but the occurrence of the response appears to be sporadic: germ tubes of three other species of the same genus were unaffected by light. Germ tubes of germinating conidia of *Botrytis cinerea* were also found to be negatively phototropic. By the use of glass filters, Gettkandt determined that blue, violet, and ultraviolet light induced curvatures of germ tubes of *Puccinia recondita,* and wavelengths of 450–480 mμ were most active.

The response of rust germ tubes differs from that of most reproductive structures that have been studied in detail because the curvatures result from inhibition of elongation of the wall farthest from the source of light. As has been mentioned, Gettkandt (1954) showed that light inhibits the elongation of germ tubes. This inhibition was confirmed by the use of Buder's half-lighting technique. When only one longitudinal half of the tip of a germ tube was illuminated, the tip curved toward the illuminated half. Further, Gettkandt considered that positive phototropic curvature of germ

tubes immersed in mineral oil was evidence for a lens effect. Thus, since the germ tube acts as a cylindrical lens, light is focused on the wall farthest from the source when a tip is illuminated unilaterally, so that growth of the wall is inhibited, and a negative phototropic curvature is produced.

2. Reproductive Structures

The reproductive structures of many fungi have been shown to respond to unilateral illumination. In the Phycomycetes, the sporangiophores of many of the Mucorales are positively phototropic; and in the Entomophthorales, the conidia of *Conidiobolus, Entomophthora,* and *Basidiobolus* are discharged toward light. In Ascomycetes, the stipes of the apothecia of such discomycetes as *Aleuria repanda* (Elliott, 1927) are positively phototropic, and the beaks of the perithecia of many pyrenomycetes curve toward a source of light (Backus, 1937; Ingold and Hadland, 1959; Callaghan, 1962). Buller (1934) observed phototropic responses of the asci of some 15 species of Discomycetes. In some, such as *Aleuria vesiculosa* and species of *Ascobolus,* the asci begin to grow in the direction of the light relatively early in their development, so that the upper third of each ascus is curved. In others, such as *Ciliaria scutellata, Melastiza miniata,* and *Cheilymenia vinacea,* the response begins so late that only the pore through which ascospores are discharged is displaced toward the source of light. The paraphyses of *Aleuria vesiculosa* are also positively phototropic.

In Basidiomycetes, the stipes of some hymenomycetes are positively phototropic. For example, when *Lentinus lepideus* is grown in darkness, it develops a fingerlike stipe which is positively phototropic until the pileus begins to develop (Buller, 1905). Similarly, the fruit bodies of *Coprinus niveus* are positively phototropic until the stipes are 3–4 cm long, but sensitivity to light is lost shortly before the pileus begins to expand (Buller, 1909).

Little is known concerning the mechanism of the responses of multicellular structures, such as the stipes of hymenomycetes or the beaks of pyrenomycetes. The spectral limits of the response have been established for *Sordaria* (Ingold and Hadland, 1959), *Pleurage* (Callaghan, 1962), and *Coprinus* (Borriss, 1934), and all these fungi respond to light only at the blue end of the spectrum. Jeffreys and Greulach (1956) were unable to relate the response of *Coprinus* to indoleacetic acid, but the experiments of Gruen (1963) provide convincing evidence that the gills are the source of a substance that promotes the growth of the stipe of *Agaricus bisporus.* Although this species is not itself phototropic, it is tempting to suggest that lateral displacement of some such substance might be involved in the response of phototropically active mushrooms. On the other hand, the suggestion of Banbury (1959) that the response of the stipe might be the

summation of the responses of the individual hyphae is equally plausible at this time.

Morphological and mechanical aspects of the phototropic response have been studied in the sporangiophores of several phycomycetes, particularly *Phycomyces* (cf. Delbrück, 1963; Shropshire, 1963), *Thamnidium* (Lythgoe, 1961), and *Pilobolus* (cf. Page, 1962). Both young (stage I) and older (stage IVb) sporangiophores of *Phycomyces* show positive curvatures under most conditions, but the latter have been studied most intensively. When a sporangiophore is illuminated unilaterally, it begins to bend after a lag of 3–5 minutes, and bending continues at a rate of 5–7 degrees per minute until the sporangiophore is directed either toward the light or somewhat above it. The tendency of sporangiophores to aim somewhat above the source of light at low intensities has been ascribed both to optical effects and to an equilibrium between phototropism and geotropism (Varjú *et al.*, 1961).

Negative phototropic curvatures have been observed in *Phycomyces* under three conditions. First, if sporangiophores are submerged in mineral oil (refractive index 1.47) or fluorochemicals, they bend away from light (Buder, 1918; Shropshire, 1962). Second, sporangiophores respond negatively to ultraviolet light (235–312 mμ) (Curry and Gruen, 1957, 1959). Third, if a sporangiophore that is in the process of responding to unilateral illumination is subjected to a flash of intense light from any direction, it bends away from the first source of light briefly, then reverses and resumes bending toward the first light (Castle, 1961a). The first two types of negative phototropism are explicable in optical terms, but the third type is a puzzling phenomenon which must be taken into account in any general explanation of the phototropic response.

There are two intriguing questions with regard to the phototropic response of *Phycomyces*. First, how is the direction of curvature maintained in a structure that elongates by spiral growth? In order to account for all the features of the responses of sporangiophores, a three-layered system was postulated (Cohen and Delbrück, 1959; Delbrück and Varjú, 1961; Shropshire, 1963) in which the inner layer with the photoreceptor is attached to the sporangium and twists but does not stretch, a middle layer ("inner wall") is attached to the base and stretches but does not twist, and the outer layer twists, stretches, and passively follows the course of the "inner wall." These hypothetical layers have not yet been related to visible structures.

A second question is, what is the relationship between the transient response of *Phycomyces* and the phototropic response? Since both responses occur in the same general region, and since their action spectra are identical, it is reasonable to suppose that they share the same mechanism; how-

ever, they differ in two respects: the tropic response does not show adaptation, and the tropic response is continuous, whereas the transient response is completed in a few minutes. That a tropic response can be induced in the absence of a transient response was shown by the simple but ingenious expedient of changing the direction of illumination without altering its intensity (Castle, 1961b). From this and other experiments, Castle concluded that phototropism is a steady-state process and cannot be founded on differential light–growth responses.

Although it has not yet been studied in detail, the phototropic response of *Thamnidium* (Lythgoe, 1961) appears to differ considerably from that of *Phycomyces*. When a sporangiophore of *Thamnidium* is exposed to unilateral illumination, there is no response for approximately 40 minutes, then the sporangiophore begins to bend, the first curvature appearing about 100 μ below the tip. Thus the curvature does not become apparent until after the sporangiophore has passed the growth rate minimum described previously (Section II, B, 1). If unilateral illumination is stopped when the growth rate reaches its minimum, the sporangiophore does not bend. If a sporangiophore is illuminated from one direction until the minimum is reached, then allowed to remain in darkness for 10 minutes, and finally illuminated from a second direction, it bends toward the second light.

From these data, Lythgoe (1961) concluded that in *Thamnidium* "the phototropic response is divided into two stages; (1) an unoriented reaction associated with the slowing of the growth rate, which causes the cell to become sensitive to laterally asymmetric illumination; (2) the phototropic response proper where the cell has become sensitive to laterally asymmetric illumination and which is associated with an increase in growth rate." Further evidence that light stimulates growth of the wall farthest from the light during phototropic curvature was the reversal of the sign of the response when sporangiophores were immersed in mineral oil. Whether a similar two-stage response occurs in other genera of mucors is not known.

The morphology of the response of sporangiophores of *Pilobolus* differs in young and mature structures. Mature sporangiophores respond to unilateral illumination by bending at a point basal to the subsporangial swelling. After a lag period of about 20 minutes, the sporangiophore begins to bend and continues to do so until it passes the direction of light; it then reverses direction until the sporangiophore is aimed directly at the light. If curvature of the sporangiophore is plotted against time, the resulting curve has the form of a damped oscillation (Page, 1962). At least under certain conditions, young sporangiophores respond in quite a different manner. If young sporangiophores in which the sporangium has not begun to develop and which have been in darkness are exposed to uni-

lateral illumination, they respond by growing in the direction of the light after a latent period of about 1 hour. In this case the response consists of a relocation of the growing zone rather than of differential growth. Whether young sporangiophores which have been elongating in light respond in this way or in the manner of *Phycomyces* and *Thamnidium* is not known. There is some evidence that both types of response occur, but the matter requires further investigation.

C. Optics and the Location of Photoreceptors

The optical properties of sporangiophores of the Mucorales have been investigated in some detail because of their relation to the location of the photoreceptor and the nature of the response. The photosensitive portion of the *Phycomyces* sporangiophore has been considered to have the properties of a cylindrical lens. This conclusion is supported by the reversal of the response when sporangiophores are immersed in mineral oil (Buder 1918) or fluorochemicals (Shropshire, 1962) with a refractive index higher than that of the cell sap. Buder (1918) assumed that the photoreceptor was located in cytoplasm adjacent to the wall, so that, when the sporangiophore was illuminated from one side in air, light was focused by the cylindrical lens in such a way that the density of the luminous flux was greater near the wall farthest from the light than near the wall closest to the light. Under mineral oil, the situation was reversed. The lens effect does not prove that the photoreceptor is located near the wall, because Castle (1933) pointed out, in a cylindrical lens the integrated path length of the light rays is greater in the half of the cylinder farther from the light than in the half nearer the light, so if the photoreceptor were uniformly distributed, the same result would be obtained in the mineral oil experiment. Further evidence for a lens effect is the fact that the sign of the response of *Phycomyces* sporangiophores is reversed when sporangiophores are exposed to unilateral ultraviolet light (Curry and Gruen, 1957, 1959) because Dennison (1959) found that sporangiophores contain gallic acid which absorbs strongly those wavelengths which induce negative curvatures. This internal screen blocks the lens effect, so that negative curvatures result from the stimulation of elongation of the wall nearest the light (Delbrück and Shropshire, 1960). More direct evidence for the lens effect was reported by Shropshire (1962), who found that curvature could be reversed by interposing a small cylindrical lens between the *Phycomyces* sporangiophore and a light source, so that the sporangiophore was exposed to diverging rays.

It is difficult to reconcile a lens effect with the behavior of young sporangiophores of *Pilobolus,* at least in those cases in which the spo-

giophores respond by relocation of the growing zone. It is of interest in this connection that young sporangiophores of *P. kleinii* respond negatively to unilateral illumination with ultraviolet light (Page and Curry, 1963).

With respect to the longitudinal distribution of the photoreceptor in *Phycomyces*, Castle (1959) proposed that since the transient response was distributed throughout the growing zone, the photoreceptor must be also. This conclusion was supported by the results of Cohen and Delbrück (1959), who illuminated various parts of the growing zone with narrow beams of visible light; however, the reactive zone appeared to be more limited when ultraviolet light was used (Delbrück and Varjú, 1961).

Unlike the other sporangiophores, the mature sporangiophore of *Pilobulus* does not align itself along the resultant when it is exposed to two equal sources of light simultaneously; rather, it aims at either one light or the other if the angle between the lights exceeds about 7 degrees (Jolivette, 1914; van der Wey, 1929). In seeking the explanation for this behavior, Buller (1934) made a thorough study of the optics of the sporangiophore and concluded that the subsporangial swelling acts as a lens which focuses light on the back wall of the sporangiophore unless it is aimed directly at a light, in which case the orange ring at the base of the subsporangial swelling is illuminated symmetrically. Van der Wey (1929) reached a similar conclusion at about the time that Buller was making his observations. That the orange ring does not actually contain the photoreceptor is indicated by the results of Schneider and of Paul (cf. Banbury, 1959), who probed the area with narrow beams of light and found the region with the orange pigment to be insensitive. It would appear from these results that the photosensitive region is below the orange ring.

Additional information on the location and condition of fungal photoreceptors has been obtained by the use of polarized light. Since spores of *Botrytis cinerea* tend to germinate parallel to the vibration planes, and since partially illuminated spores germinate from their brightest parts, Jaffe and Etzold (1962), concluded that the photoreceptor molecules are oriented with their axes of maximal absorption perpendicular to the cell surface. On the basis of an extensive survey of the literature, these authors suggested that photoreceptor molecules are generally highly oriented, but there is no rule as to the plane of orientation. From their own data, Jaffe and Etzold deduced that the photoreceptors in *Botrytis* lie about 0.5 μ from the surface either in the inner half of the wall or in the boundary between the wall and cytoplasm.

IV. MECHANISM OF LIGHT RESPONSES

A. Photoreceptors

Not only is the precise location of photoreceptors in fungi uncertain, but also there is controversy with regard to their identity. From the fact that the action spectrum of the phototropic response of mature sporangiophores of *Pilobolus*, as determined by the use of filters, resembled the absorption spectrum of β-carotene, Bünning (1937, 1938a,b) concluded that the photoreceptor in both fungi and *Avena* was a carotenoid. The conclusion drawn from this pioneering work went unchallenged until Galston and Baker (1949) reported that indoleacetic acid is destroyed by riboflavine *in vitro* and suggested that the photoreceptor involved in higher plant phototropism might be a flavine or a flavoprotein. The absorption spectra of flavines and carotenoids are so similar in the visible part of the spectrum that even detailed action spectra, such as those for phototropism (Curry and Gruen, 1959) and the transient growth response (Delbrück and Shropshire, 1960) of *Phycomyces*, do not permit a clear decision to be made between the two classes of pigments. The peak at 365–380 mμ shown by these action spectra as well as a less detailed one for the phototropic response of young sporangiophores of *Pilobolus kleinii* (Page and Curry, 1963) favors the flavinoid hypothesis, but until the condition of the photoreceptor—whether it is attached to a protein or whether it is dissolved, and in what—is known, further speculation is idle.

The similarity of the detailed action spectra as well as of the spectral limits determined for other oriented and nonoriented responses suggests that one pigment (or class of pigments) functions as the photoreceptor in the vast majority of cases. A possible exception is *Entomophthora coronata* (= *Conidiobolus villosus*). This fungus projects its conidia toward light, and it responds to wavelengths as long as 630 mμ (Page and Brungard, 1961). These limits were obtained by projecting a spectrum on a culture and were confirmed by the use of glass filters. Further trials with "monochromatic" glass filter combinations have confirmed the published results and have indicated that the fungus is about fifty times more sensitive to light at 405 mμ as to that at 630 mμ. This strain of *Conidiobolus* contains no easily extractable carotenoids, but it does contain an unidentified pigment that can be extracted with acetone or dioxane and has the characteristics of a porphyrin with absorption peaks at 412, 506, 543, 580, and 630 mμ.

B. Biochemistry

At the present time there is little information on the biochemical mechanisms that underlie either the oriented or the nonoriented responses. With the exception of the stimulation of growth of *Blastocladiella* by light (Section II, A), all the responses that have been studied in detail seem to involve synthesis of wall material. In the case of nonoriented responses only a change in the rate of synthesis is required, but in the oriented responses a change in the site of synthesis may be required as well. How light induces these changes is unknown. Although *Blastocladiella* appears to be exceptional, the studies of Cantino and co-workers are valuable because they constitute the only detailed information available on the relationship between a response to light and the biochemical activities of a fungus cell. Even more pertinent to an understanding of the general pattern is the report by Gettens and Shropshire (1963) that the concentration of reducing sugar and other metabolites parallels the course of the transient response of *Phycomyces;* and in their oral presentation, these workers reported that the concentration of ATP increased after sporangiophores had been exposed to a saturating pulse-up exposure. If this ATP could be shown to provide the energy for wall synthesis, a link between the reception of light energy and a visible response would be established.

REFERENCES

Backus, M. P. (1937). Phototropic response of perithecial necks in *Neurospora*. *Mycologia* 29:383–386.

Banbury, G. H. (1959). *In* "Handbuch der Pflanzenphysiologie" (W. Ruhland, ed.), Vol. XVII, pp. 530–578. Springer, Berlin.

Bonner, J. T. (1959). Differentiation in social amoebae. *Sci. Am.* 201(6):152–162.

Bonner, J. T., and M. J. Shaw. (1957). The role of humidity in the differentiation of the cellular slime molds. *J. Cellular Comp. Physiol.* 50:145–154.

Bonner, J. T., W. W. Clarke, C. L. Neely, and M. K. Slifkin. (1950). The orientation to light and the extremely sensitive orientation to temperature gradients in the slime mold *Dictyostelium discoideum*. *J. Cellular Comp. Physiol.* 36:149–158.

Borriss, H. (1934). Über den Einfluss äusserer Faktoren auf Wachstum und Entwicklung der Fruchtkörper von *Coprinus lagopus*. *Planta* 22:644–684.

Buder, J. (1918). Die Inversion des Phototropismus bei *Phycomyces*. *Ber. Deut. Botan. Ges.* 36:104–105.

Buller, A. H. R. (1905). The reactions of the fruit bodies of *Lentinus lepideus* to external stimuli. *Ann. Botany (London)* 19:427–435.

Buller, A. H. R. (1909). "Researches on Fungi," Vol. I, 287 pp. Longmans, Green, New York.

Buller, A. H. R. (1934). "Researches on Fungi," Vol. VI, 513 pp. Longmans, Green, New York.

Bünning, E. (1937). Phototropismus und Carotinoide. I. Phototropische Wirksamkeit von Strahlen verschiedener Wellenlänge und Strahlungsabsorption im Pigment bei *Pilobolus*. *Planta* **26**:719–736.

Bünning, E. (1938a). Phototropismus und Carotinoide. II. Das Carotin der Reizaufnahmenzonen von *Pilobolus, Phycomyces,* und *Avena*. *Planta* **27**:148–158.

Bünning, E. (1938b). Phototropismus und Carotinoide. III. Weitere Untersuchungen an Pilzen und höheren Pflanzen. *Planta* **27**:583–610.

Callaghan, A. A. (1962). Observations on perithecium production and spore discharge in *Pleurage setosa*. *Brit. Mycol. Soc. Trans.* **45**:249–254.

Cantino, E. C. (1959). Light-stimulated development and phosphorus metabolism in the mold *Blastocladiella emersonii*. *Develop. Biol.* **1**:396–412.

Cantino, E. C., and E. A. Horenstein. (1956). The stimulatory effect of light upon growth and CO_2 fixation in *Blastocladiella*. I. The S. K. I. cycle. *Mycologia* **48**:777–799.

Cantino, E. C., and E. A. Horenstein. (1959). The stimulatory effect of light upon growth and carbon dioxide fixation in *Blastocladiella* III. Further studies *in vivo* and *in vitro*. *Physiol. Plantarum* **12**:251–263.

Cantino, E. C., and G. Turian. (1961). A role for glycine in light-stimulated nucleic acid synthesis by *Blastocladiella emersonii*. *Arch. Mikrobiol.* **38**:272–282.

Carlile, M. J. (1965). The photobiology of fungi. *Ann. Rev. Plant Physiol.* **16**: in press.

Castle, E. S. (1928). Temperature characteristics and the growth of the sporangiophores of *Phycomyces*. *J. Gen. Physiol.* **11**:407–413.

Castle, E. S. (1933). The physical basis of the positive phototropism of *Phycomyces*. *J. Gen. Physiol.* **17**:49–62.

Castle, E. S. (1942). Spiral growth and reversal of spiraling in *Phycomyces* and their bearing on primary wall structure. *Am. J. Botany* **29**:664–672.

Castle, E. S. (1959). Growth distribution in the light-growth response of *Phycomyces*. *J. Gen. Physiol.* **42**:697–702.

Castle, E. S. (1961a). Phototropic inversion in *Phycomyces*. *Science* **133**:1424–1425.

Castle, E. S. (1961b). Phototropism, adaptation, and the light-growth response of *Phycomyces*. *J. Gen. Physiol.* **45**:39–46.

Cohen, R., and M. Delbrück. (1959). Photoreactions in *Phycomyces*. Growth and tropic responses to the stimulation of narrow test areas. *J. Gen. Physiol.* **42**:677–695.

Curry, G. M., and H. E. Gruen. (1957). Negative phototropism of *Phycomyces* in the ultra-violet. *Nature* **179**:1028–1029.

Curry, G. M., and H. E. Gruen. (1959). Action spectra for the positive and negative phototropism of *Phycomyces* sporangiophores. *Proc. Natl. Acad. Sci. U.S.* **45**:797–804.

Delbrück, M. (1963). Der Lichtsinn von *Phycomyces*. *Ber. Deut. Botan. Ges.* **75**:411–430.

Delbrück, M., and W. Reichardt. (1956). *In* "Cellular Mechanisms in Differentiation and Growth" (D. Rudnick, ed.), pp. 3–44. Princeton Univ. Press, Princeton, New Jersey.

Delbrück, M., and W. Shropshire. (1960). Action and transmission spectra of *Phycomyces*. *Plant Physiol.* **35**:194–204.

Delbrück, M., and D. Varjú. (1961). Photoreactions in *Phycomyces*. Responses to the stimulation of narrow test areas with ultra-violet light. *J. Gen. Physiol.* **44**:1177–1188.

Dennison, D. S. (1959). Gallic acid in *Phycomyces* sporangiophores. *Nature* **184:** Suppl. 26, 2036.

Elliott, J. S. B. (1927). *Aleuria repanda* Pers. *Brit. Mycol. Soc. Trans.* **12:**166–169.

Galston, A. W., and R. S. Baker. (1949). Studies on the physiology of light action II. The photodynamic action of riboflavin. *Am. J. Botany* **36:**773–780.

Gettens, R. H., and W. Shropshire. (1963). Light-induced biochemical changes in *Phycomyces*. *Plant Physiol.* **38:** Supp., iv.

Gettkandt, G. (1954). Zur Kenntnis des Phototropismus der Keimmyzelien einiger parasitischer Pilze. *Wiss. Z. Univ. Halle-Wittenberg, Math.-Nat.* **3:**691–709.

Goldstein, S. (1963). Studies of a new species of *Thraustochytrium* that displays light stimulated growth. *Mycologia* **55:**799–811.

Gruen, H. E. (1963). Endogenous growth regulation in carpophores of *Agaricus bisporus*. *Plant Physiol.* **38:**652–666.

Hollande, A., and M. Enjumet. (1955). Sur l'évolution et la systématique des Labyrinthulidae, étude de *Labyrinthula algeriensis nov. sp. Ann. Sci. Nat. Zool. Biol. Animale* **17:**357–368.

Ingold, C. T., and S. A. Hadland. (1959). Phototropism and pigment production in *Sordaria* in relation to gravity and light. *Ann. Botany* (*London*) [*N.S.*] **23:**425–429.

Jaffe, L., and H. Etzold. (1962). Orientation and locus of tropic photoreceptor molecules in spores of *Botrytis* and *Osmunda*. *J. Cell Biol.* **13:**13–31.

Jeffreys, D. B., and V. A. Greulach. (1956). The nature of tropisms of *Coprinus sterquilinus*. *J. Elisha Mitchell Sci. Soc.* **72:**153–158.

Jolivette, H. D. M. (1914). Studies on the reactions of *Pilobolus* to light stimuli. *Botan. Gaz.* **57:**89–121.

Lingappa, Y., A. S. Sussman, and I. A. Bernstein. (1963). Effect of light and media upon growth and melanin formation in *Aureobasidium pullulans* (De By.) Arn. (=*Pullularia pullulans*). *Mycopathol. Mycol. Appl.* **20:**109–128.

Lythgoe, J. N. (1961). Effect of light and temperature on the growth of *Thamnidium elegans* Link. *Brit. Mycol. Soc. Trans.* **44:**199–213.

Marsh, P. B., E. E. Taylor, and L. M. Bassler. (1959). A guide to the literature on certain effects of light on fungi: reproduction, morphology, pigmentation, and phototropic phenomena. *Plant Disease Reptr.* Suppl. **261:**251–312.

Page, R. M. (1952a). The effect of nutrition on growth and sporulation of *Pilobolus*. *Am. J. Botany* **39:**731–739.

Page, R. M. (1952b). Unpublished data.

Page, R. M. (1962). Light and the asexual reproduction of *Pilobolus*. *Science* **138:**1238–1245.

Page, R. M., and J. Brungard. (1961). Phototropism in *Conidioblus*, some preliminary observations. *Science* **134:**733–734.

Page, R. M., and G. M. Curry. (1963). Unpublished data.

Raper, K. B. (1962). The environment and morphogenesis in cellular slime molds. *Harvey Lectures Ser.* **57:**111–141

Schaeffer, P. (1953). A black mutant of *Neurospora crassa*. Mode of action of the mutant allele and action of light. *Arch. Biochem. Biophys.* **47:**359–379.

Shropshire, W. (1962). The lens effect and phototropism of *Phycomyces*. *J. Gen. Physiol.* **45:**949–958.

Shropshire, W. (1963). Photoresponses of the fungus, *Phycomyces*. *Physiol. Rev.* **43:**38–67.

Sussman, A. S., Y. Lingappa, and I. A. Bernstein. (1963). Effect of light and media

upon growth and melanin formation in *Cladosporium mansoni*. *Mycopathol. Mycol. Appl.* **20**:307–314.

Turian, G., and E. C. Cantino. (1959). The stimulatory effect of light on nucleic acid synthesis in the mould *Blastocladiella emersonii*. *J. Gen. Microbiol.* **21**:721–735.

van der Wey, H. G. (1929). Ueber die phototropische Reaktion von *Pilobolus*. *Koninkl. Ned. Akad. Wetenschap., Proc.* **32**:1–13.

Varjú, D., L. Edgar, and M. Delbrück. (1961). Interplay between the reactions to light and gravity in *Phycomyces*. *J. Gen. Physiol.* **45**:47–58.

Willoughby, R. (1961). "Effects of light on growth of *Pilobolus*." M.A. thesis, Stanford University.

Zalokar, M. (1954). Studies on biosynthesis of carotenoids in *Neurospora crassa*. *Arch. Biochem. Biophys.* **50**:71–80.

CHAPTER 24

The Physical Environment for Fungal Growth

4. Effects of Radiation

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I. INTRODUCTION

Fungi, like all other organisms, are exposed to the natural radiations of our environment, i.e., ultraviolet radiation, visible and infrared radiations, cosmic rays, radioactive decay. These radiations may be damaging, beneficial, or innocuous depending on the nature of the fungus and the nature of the radiation.

In this chapter the effects of radiations on fungi are considered primarily with respect to effect on the fungi and secondarily from the point of view of information derived as to the mechanism of radiation effects. We will not deal with the recent genetic work based on the very important observations that radiations produce mutations in fungi and that these can be used as "markers" for genetics and biochemistry. This subject is discussed in Chapter 29.

If. CHARACTERISTICS OF FUNGI THAT INFLUENCE RADIATION EFFECTS

It might not be obvious that the nature and characteristics of the fungus interact with the character of the radiation to determine the effect of the radiation. A fungus proliferating primarily as a coenocytic mycelium, or sporulating via multinucleate conidia, might be expected to show very little response to a dosage of radiation that might prove extremely damaging

to a fungus sporulating via uninucleate conidia. This is not to say necessarily that similar damage was not wrought in all cases. Rather, it is a matter of the time of expression of the effect. In the case of the uninucleate conidia, the effect of the radiation might appear almost immediately upon germination. In the case of multinucleate mycelia or conidia, the expression might be masked for a considerable period of time, or indefinitely if the affected nuclei do not survive in the intramycelial or conidial competition.

It is implied in the above that the primary site of radiation effects is the nucleus, or a related structure. This is an oversimplification since there are, of course, important cytoplasmic effects of radiation as well, which will be discussed below.

There are other characteristics of fungi that influence the expression of radiation effects. These include the state of nutrition, whether the organism is actively dividing or at rest, the particular strain of organism being irradiated. We will consider these "internal" modifying factors, as well as "external" modifying factors such as temperature, moisture content, oxygen level, etc., later.

It will be obvious at this point that, for experimental irradiation work with fungi, a wide choice of conditions are available. Depending on the specific objective, one might wish to work with a suspension of uninucleate conidia, or with genetically marked multinucleate conidia, or with hyphae, or with haploid or higher-ploid yeast cells, or with an organism that has a sexual cycle, or with one that produces resistant spores, or with a starved suspension of conidia, or with germinating spores, etc. The point is that *there is an interaction between the fungus and the radiation, and that fungi* are excellent experimental objects for radiation research because a variety of such interactions can be set up and controlled. The nature and state of the organism at the time of irradiation, as well as before and after irradiation, can influence the outcome of a radiation treatment. This must be kept in mind when reading the literature on irradiation of fungi, or when designing experiments in this area.

III. EFFECTS OF IONIZING RADIATIONS ON FUNGI

A. Mechanism of Action

The mechanism through which ionizing radiations exert their biological effects is not fully understood. For purposes of this presentation, it will be enough to refer to some of the literature directly pertaining to studies of radiobiological mechanisms (see, for example, the general references listed at the end of the section, as well as Zirkle and Tobias, 1953; Barron, 1954; Dale, 1954; Alper, 1956; Howard-Flanders and Alper, 1957; Gray,

1959) and to restate what appear to be the most productive hypotheses. The direct action theory states that a sensitive (nuclear) site is affected by an ionizing particle; the indirect action theory states that ionization produces chemical changes, such as free radical formation, and that these chemical entities produce the observed radiobiological effects. The indirect action theory allows for somewhat greater flexibility and appears better able to accommodate such observations as effects of chemicals, effects of changes in oxygen level, effects in changes of phase state, ionic yields greater than unity. At present, the most reasonable view, in this author's judgment, retains the feature of the target theory of a sensitive site and combines this with the indirect theory of radiation-induced chemical events which, in turn, affect sensitive sites (Zirkle and Tobias, 1953; Dale, 1954; Gray, 1959). The modifiable direct-action model (Alper, 1956; Howard-Flanders and Alper, 1957; Alexander, 1957) would more explicitly state that the primary chemical change occurred directly in the sensitive site. As active research continues in this field, it seems quite within the realm of possibility that an entirely new synthesis of current theories may emerge. For the present, the theories as stated provide adequate operational models on which to base critical tests (see Wood, 1959).

The literature covering effects of radiation on fungi was summarized by Pomper and Atwood (1955). Among recent reviews of radiation effects on microorganisms, including fungi, are those by de Serres (1961) and Stapleton (1960).

It will help in understanding the subsequent material to define the commonly used units of ionizing radiation (Morgan, 1963):

r: The roentgen (r) is the unit of exposure of X- or γ-radiation such that the associated corpuscular emission per 0.001293 gm of air produces, in air, ions carrying 1 electrostatic unit (esu) of quantity of electricity of either sign. This means that 1 r produces 1.61×10^{12} ion pairs per gram of air, which corresponds to the absorption of 83.8 ergs of energy per gram of air.

rad: The rad is the unit of absorbed dose corresponding to 100 ergs per gram of medium or tissue.

rep: The roentgen-equivalent-physical (rep) is the quantity of ionization produced when 83 ergs are dissipated by the radiation per gram of tissue. It may be used to express ionization in tissues caused by radiations other than X- and γ-rays (electrons, protons, α-particles, neutrons).

B. "Nongenetic" Effects

The majority of studies on radiation effects on fungi have dealt with what might be considered genetic manifestations, i.e., mutational or lethal

effects. There are, obviously, also "nongenetic" effects of ionizing radiation on fungi, and some of these will be considered now.

When cells of *Saccharomyces cerevisiae* are X-irradiated, cell division may be retarded (Holweck and Lacassagne, 1930). There is a substantial increase in cell volume, which at LD_{50} was reported to persist for at least 3 to 4 generations (Brace, 1950). Moderate exposures to X-rays were reported by Burns (1956) to cause little delay in budding of interdivisional cells, but there was about a sixfold delay in the time of appearance of the third generation after irradiation. The fourth-generation division time was little affected. Budding cells were found to be 5–6 times as resistant to radiation-induced delay as nonbudding cells. An actual increase in the rate of growth after an initial delay was observed by James and Müller (1961) with *S. cerevisiae*. After inhibition was ended (second division), the mean generation time for irradiated (5000 r) cells was 1.45 hours as compared to 1.68 hours for control cells. This stimulatory effect was interpreted as a direct stimulation of the rate of mitosis.

A variety of biochemical consequences must be expected to result from exposing a fungus to ionizing radiation. A recent review of biochemical effects of X-radiation in various organs, organisms, and microorganisms (Stocken, 1959) provides a good background of information in this area. Rothstein (1959) reviewed some of the biochemical and physiological changes that occur in irradiated yeast.

Gray (1959) reported that adaptive enzyme formation in yeast was not inhibited by irradiation up to 400 krad. Tai (1962) found an increase in catalase activity in X-irradiated (5400 rad) conidia of *Aspergillus niger*. This was interpreted as induction of catalase formation by hydrogen peroxide produced as a result of irradiation of the medium, but other interpretations could be applied to this observation based on effects known to occur in the internal milieu of irradiated cells. For example, Bair and Stannard (1955) showed a relationship between the effects of X-radiation on metabolism of yeast cells and the electrolyte balance of the cells and/or medium. They demonstrated that the effect of a particular X-ray dosage on respiration or fermentation depends on the composition of the medium during irradiation (particularly the hydrogen and potassium ion concentrations), the time elapsed between irradiation and testing, prior starvation (which sensitizes), and treatment with potassium-free ion exchange resins (Dowex 50) before or after irradiation. Bair and Stannard found that X-radiation increased cell permeability, and hence conditions promoting loss of potassium ions sensitized the cell. Conversely, addition of extra potassium protected against, or partially reversed, X-radiation effects. Very large doses are required for serious damage to respiration or fermentation

of intact cells. Bair and Hungate (1958) found that ethylenediaminetetraacetic acid (EDTA) increased the radiation sensitivity of *S. cerevisiae,* interpreted on the basis of a general change in the electrolyte balance of the cell. Also of interest in this connection is the report (Engelhard *et al.*, 1959) that X-radiation could be used to selectively inactivate the centers controlling ion transport separately from those controlling survival, and that the centers controlling ion transport do not contain fermentation enzymes. It seems likely that use of ionizing radiation as a tool to help characterize the internal organization of a fungal cell presents a worthwhile field of research.

The effect of X-radiation on the phosphorus metabolism of fungi has been shown to be fairly complex. Forssberg and Novak (1960) found that irradiated sporangiophores of *Phycomyces* showed an immediate increase in acid-labile organic phosphate accompanying a decrease in growth rate (period of approximately 3 minutes after irradiation). Then, there was a decrease in acid-labile organic phosphate in the next 5–10 minutes, accompanying an increase in growth rate. A steady state was reached about 15–20 minutes after irradiation. Other biochemical changes were occurring during this period, as inferred from changes in the rate of lactic acid production. Spoerl *et al.* (1959) found an increase in acid-insoluble polyphosphates in X-irradiated *S. cerevisiae* under irradiation conditions such that division was inhibited. They also found an increase in P^{32} incorporation into acid-soluble organic phosphate of irradiated cells. Gal'tsova and Novichkova (1962) made the interesting observation that three strains of *Saccharomyces* responded to low doses of X-rays by increasing their protein content by 10–20%. Higher doses caused large increases in the contents of amino acids found in the protoplasts.

C. "Genetic" Effects—Killing and Mutation

There is a fairly extensive body of literature on killing of fungi and induction of mutations with ionizing radiations of various types and energies (see, for example, Zirkle, 1940; Stapleton *et al.*, 1952; Stapleton and Hollaender, 1952; Zirkle and Tobias, 1953; Sinclair *et al.*, 1959; Sayeg *et al.*, 1959; Käfer, 1963). Although all the data in the literature are not in agreement, it appears that in general more densely ionized radiations kill fungi more effectively, i.e., at lower dosage, than do more dispersed radiations. It should be noted that the reverse was observed for mutation induction in *Aspergillus terreus* by Stapleton *et al.* (1952). Sayeg *et al.* (1959) made an extensive series of irradiations of haploid *Saccharomyces cerevisiae;* they found that radiation sensitivity increased with increasing

linear energy transfer (LET) until it reached a maximum corresponding to a relative biological effectiveness (RBE) of 2 for 3.4 Mev α-particles from Po^{210}. Some of these results are shown in Table I. Possible sources of con-

TABLE I

RADIATION SENSITIVITY OF HAPLOID *Saccharomyces cerevisiae*[a]

(1) Expt. no.	(2) Radiation	(3) Total average LET (Mev cm²/gm)	(4) Slope of survival curve (10^{-4} rad^{-1})	(5) LD_{50} (rads)	(6) Slope of 50-kv X-ray survival curve (10^{-4} rad^{-1})	(7) Cross section for inactivation (10^{-8} cm²/particle)	(8) Approximate dose rate (rads/min)
1a	Alpha (27 Mev)	258 ± 6	2.40 ± 0.18	2890	2.24 ± 0.22	0.0991 ± 0.0076	1100–2300
1b	Alpha (6 Mev)	850 ± 100	3.05 ± 0,41	2270	2.24 ± 0.22	0.416 ± 0.074	3300–10,000
1c	Deuteron (19 Mev)	48 ± 2	1.96 ± 0.11	3550	2.24 ± 0.22	0.0150 ± 0.0010	1100–2100
2a	Carbon (65 Mev)	2680 ± 300	2.57 ± 0.12	2700	2.24 ± 0.22	1.10 ± 0.13	900–1800
2b	Carbon (30 Mev)	4450 ± 540	1.72 ± 0.10	4030	2.24 ± 0.22	1.23 ± 0.17	1200 3000
3a	Carbon (67 Mev)	2600 ± 200	2.51 ± 0.12	2760	2.29 ± 0.22	1.04 ± 0.09	400 750
3b	Carbon (24 Mev)	5080 ± 460	1.29 ± 0.08	5370	2.29 ± 0.22	1.05 ± 0.12	1000–1500
4a	Alpha (Po^{210})	1250 ± 50	4.30 ± 0.23	1610	1.95 ± 0.20	0.862 ± 0.035	42,000
4b	X-ray (220 kv)	20 ± 5	2.16 ± 0.14	3200	1.95 ± 0.20	— —	800
5	Alpha (Po^{210})	1230 ± 50	3.86 ± 0.23	1800	2.12 ± 0.20	0.761 ± 0.037	5000

[a] Data of Sayeg *et al.* (1959) showing that the maximum killing effectiveness was obtained with Po^{210} α-particles.

fusion as regards the RBE of various radiations are discussed by Beam (1959). The present discussion will be concerned primarily with lethal genetic effects rather than nonlethal genetic effects since the latter are covered elsewhere in this treatise and earlier by Pomper and Atwood (1955).

As considered earlier, there is an interaction between the fungus and the radiation, as well as the environment and the radiation. We will discuss several of these interactions, namely, the effects of ploidy, mitotic stage, starvation, temperature, moisture, other radiations, and oxygen. It will be noted that a large proportion of the work cited deals with yeast. This is so for two reasons: first, it is the writer's own area of interest; and, second, in recent years the suitability of yeast as a closely controllable subject for radiation research has become better appreciated. It seems likely that, in the broad sense, what holds true for one fungus will hold true for others

and that translation of broad conclusions from one genus to another will be profitable.

1. Kinetics

Haploid yeast cells are inactivated exponentially by X-rays under properly designed test conditions; polyploid yeast cells are inactivated sigmoidally (see, for example, Latarjet and Ephrussi, 1949; Lucke and Sarachek, 1953; Pomper *et al.*, 1954). Various interpretations have been placed on this type of observation (Atwood and Norman, 1949; Lindegren *et al.*, 1959; Mortimer, 1958; Magni, 1959). The best synthesis at this time appears to be that the mechanism of inactivation is complex, involving primarily recessive and dominant lethal mutations, nongenetic effects playing a lesser role. Mortimer's (1958) evidence indicated that for haploid *S. cerevisiae* cells the primary cause of inactivation was recessive lethal mutation, whereas with higher ploidies dominant lethal mutations became more important. As a consequence, tetraploid cells were more radiosensitive than diploid cells. Mortimer (1958) did not find an integral relationship between ploidy and zero-dose extrapolate of the survival curves, as had been reported by Lucke and Sarachek (1953) (Fig. 1). Beam (1959) found that tetraploid *Hansenula anomala* was more resistant than diploid *H. anomala,* and this was interpreted as indicating a preponderance of recessive lethal mutations. It appears desirable that additional well-characterized clones be analyzed in this regard since unpublished experiments of the writer indicated that tetraploids were slightly more sensitive than diploids, but that triploids were more resistant than either. From this brief discussion, it should be clear why in an earlier discussion on inactivation of fungi by ionizing radiation (Pomper and Atwood, 1955) it was concluded that interpretation of survival kinetics could only be made on very well-defined systems and that the significance of much of the literature on irradiation of fungi could not be assessed.

Evidence that the kinetics of inactivation of haploid uninucleate fungal cells by ionizing radiation were more complicated than a simple logarithmic kill was seen in the data of Latarjet and Ephrussi (1949), and this problem has been studied extensively by Beam and his colleagues (1954; Beam, 1955, 1959) and by Elkind and Sutton (1959a,b). The basic observation was that the inactivation of haploid *S. cerevisiae* cells was logarithmic in part, but then "tailed" off as though a portion of the population were radioresistant (see Fig. 2) (Beam *et al.*, 1954). The resistant fraction was shown to consist primarily of cells in the process of budding rather than merely of clumps of cells. When rapidly growing cells were harvested and then stored in buffered sugar solution, the radioresistant fraction decreased

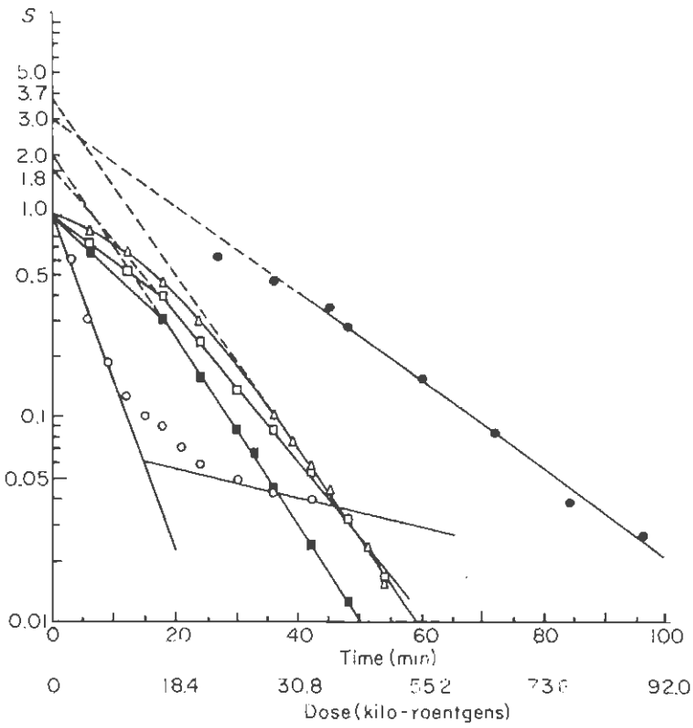

FIG. 1. Relationship between ploidy and survival curves. ○–○, Haploid (13,804), slope 0.10; ■–■, diploid (11,296), slope 0.11; □–□, diploid (11,294), slope 0.086; •–•, triploid (13,894 × 11,294), slope 0.050; △–△, tetraploid (11,294 × 11,296), slope 1.10. From Lucke and Sarachek (1953).

from about 10% of the population to 1% or less (Beam, 1959). As a further illustration of the complex interaction of fungi with ionizing radiations, we may note the interesting observation that the α-particle–X-ray RBE for the resting yeast cell can be changed from about 0.7 to about 2.0 either by starving the yeast or by putting it into a growth environment (Beam, 1959). The basic mechanism underlying these effects has yet to be elucidated.

2. *Interaction of Other Radiations*

There are unexpected interactions between ionizing and other radiations. The earlier literature is summarized by Pomper and Atwood (1955). Forssberg *et al.* (1960) working with *Phycomyces* found that X-irradiation of dark-adapted sporangiophores caused an immediate increase in

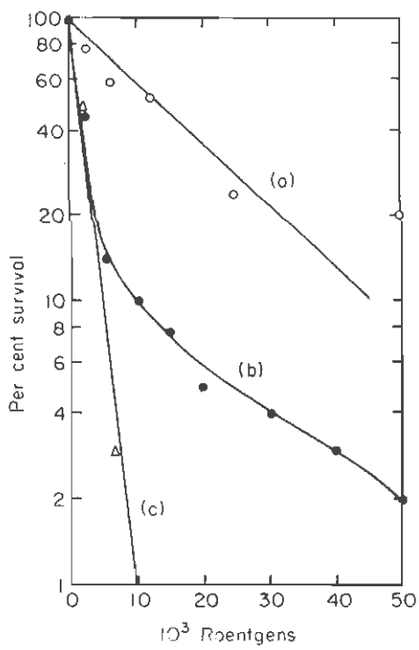

FIG. 2. X-ray survival curves by microcolony counting procedure: survival criterion, >40 cells. Age of culture prior to irradiation, 4 days. ●, Whole survival; △, single cells only; ○, budding cells only. From Beam *et al.* (1954).

lactic acid production, whereas X-irradiation of light-adapted sporangiophores caused an immediate decrease. Uretz (1955) and Elkind and Sutton (1959b) concluded that the lethal sites of X- and ultraviolet (UV) radiations overlap in both dividing and interdivisional yeast cells. The latter workers also observed (Elkind and Sutton, 1959a) that after a slight pretreatment exposure to ultraviolet, yeast cells were more resistant to X-rays. This was attributed to killing of the nondividing cells by the UV, leaving only the more radioresistant, budding fraction. They showed (1959b) that an extensive pretreatment with UV (such that all nondividing and 90% of the budding cells were killed) resulted in yeast cells which were more sensitive to X-rays than the usual budding cells. X-ray lethality was partially reversed by post-treatment with ultraviolet radiation. It seems reasonable to conclude from these experiments that the sites of action of both radiations were largely the same, and that the input of additional energy after the initial treatment might either reverse the initial damage or enhance it.

3. Other Modifying Factors

Very significant effects on the X-ray sensitivity of fungi are exerted by the level of oxygen present during irradiation, the temperature, and the moisture content. Anderson and Turkowitz (1941), working with *Torulopsis cremoris,* and Stapleton and Hollaender (1952), working with *Aspergillus terreus,* observed that the absence of oxygen reduced the effectiveness of killing by X-irradiation. A dose reduction factor (DRF), i.e., the extent of the protective effect, of about 2 was observed by Beam (1955) for haploid, diploid, and tetraploid cells of *S. cerevisiae* irradiated in the absence of oxygen. A similar DRF was obtained whether the cells were dividing or at rest. Wood (1960) found that acriflavine-induced petite colony mutants of haploid *S. cerevisiae* had similar X-ray sensitivity to the parental (respiring) strain and showed a similar response to anoxia, phase-state change, and respiratory inhibitors. Moustacchi (1958) had reported greater X-ray sensitivity for a petite haploid strain than for a respiring strain. Differences in the genetic characteristics of these yeasts may explain the differences in results.

Wood and Taylor (1957) reported an increased DRF for frozen yeast cells irradiated under anoxic conditions. Stapleton and Hollaender (1952) found that as the moisture level was reduced the efficiency of X-radiation treatment of *A. terreus* was reduced, and that there was an interaction between moisture content and anoxia. Thus, *A. terreus* spores at 25% moisture (desiccated) had an LD_{99} of 126 kr, at 42% moisture (normal) an LD_{99} of 74 kr, and at 80% moisture (in water suspension) an LD_{99} of 52 kr. At a dosage of 60 kr, anoxia permitted about threefold more survivors at 42% moisture, but about tenfold more survivors at 80%. Schwinghamer (1958) found the sensitivity of urediospores of *Melampspora lini* to be constant to about 52% relative humidity, with a twofold increase at 98% RH. It is interesting to note, in connection with moisture effects, that Hutchinson (1957) and Hutchinson *et al.* (1957) found the sensitivities of coenzyme A, invertase, and alcohol dehydrogenase in wet and dry yeast cells to be 100:1, 2:1, and 20:1, respectively. They calculated that the maximum distance over which chemical intermediates produced by radiation in the water phase of these cells would diffuse would be about 30 Å.

The effect of temperature as a modifying factor of radiation damage has been carefully analyzed for *S. cerevisiae* by Wood (1954, 1959). He found essentially no effect on survival of yeast at temperatures of exposure in the range 0–45°C; the temperature coefficient was 0.0034 per degree. At temperatures of 50 C and above, where heat inactivation of yeast becomes important, there was a marked increase in radiosensitivity. Either heat or

X-ray pretreatment sensitizes haploid yeast to the subsequent treatment. Yeast cells supercooled to −10° C showed little change in radiosensitivity whereas yeast cells frozen at −10 °C showed a twofold decrease in sensitivity. Wood (1959) interprets his observations as indicating that if free water is trapped within the cell by very rapid freezing, then radiosensitivity is not affected because the free radicals would be released within the cell on thawing. If cells are frozen slowly, some dehydration occurs and there is less free water trapped within the cell, with a dilution effect of the free radicals in the water outside of the cells.

In addition to the interactions noted above, there may be interactions between ionizing radiations and various chemicals. Stapleton (1960) recently summarized the literature on protective effects. It should be noted also that some chemicals may sensitize fungi to ionizing radiation. For example, vitamin K_5 (4-amino-2-methyl-1-naphthol hydrochloride) sensitized *Torulopsis rosea* and various bacteria to X-radiation (Shebata, 1961).

IV. EFFECTS OF ULTRAVIOLET RADIATION ON FUNGI

A. Mechanism of Action

The principal feature of ultraviolet radiation as applied to fungi (and other systems as well) is the general correlation between activity and the wavelengths corresponding to those of nucleic acid absorption. In general terms, we can conceive of the nucleic acid molecules of the chromosomes and genes specifically absorbing the light energy input, and undergoing chemical changes which in the extreme case could be fatal for the organism.

Some of the earlier literature on ultraviolet irradiation effects on fungi was reviewed by Pomper and Atwood (1955). It should be noted that all the fungi covered in this review showed a fairly similar action spectrum, i.e., maximum ultraviolet effectiveness at approximately 2600 Å, with a single exception. The fungi noted as giving "nucleic acid"-type action spectra were *Saccharomyces cerevisiae* (Oster, 1934c), *Ustilago zeae* [*U. maydis*] (Landen, 1939), *Aspergillus niger* (Zahl *et al.,* 1939), *Trichophyton mentagrophytes* (Hollaender and Emmons, 1941), *A. terreus* (Hollaender and Emmons, 1946), *Neurospora crassa* (Hollaender *et al.,* 1945b), and *Penicillium notatum* (Hollaender and Zimmer, 1945). The exception was *Chaetomium globosum,* which showed maximum production of mutations at about 2800 Å, i.e., a protein type excitation (McAulay and Ford, 1947). This work should probably be reinvestigated. In any event, it does not appear to change the general conclusion that ultraviolet radiation exerts its effect through nucleic acid—most likely, primarily through the nuclear deoxyribonucleic acid (DNA).

The current understanding of the action of ultraviolet light is based on molecular considerations (reviewed by Deering, 1962). Thymine and cytosine (the pyrimidine bases of DNA) were found to be more sensitive than adenine and guanine (the purine bases of DNA). About 1% of absorbed quanta altered the pyrimidines whereas only 0.01% were effective in altering the purines. The particular alteration that appears to occur most readily is formation of a thymine dimer in the DNA, such that a strong interchain chemical bond is formed. To round out the picture, a mechanism possibly involved in the light reversal of ultraviolet irradiation effects was found in the light activation of an enzyme which splits thymine dimers (Wulff and Rupert, 1962). It is reasonable to speculate that organisms naturally resistant to ultraviolet radiation may process a large amount of the thymine dimer-splitting enzyme, or may possess the ability to activate it in the absence of light energy.

B. "Nongenetic" Effects

As with ionizing radiation, the effect of ultraviolet irradiation may be recognized in terms of "nongenetic" or "genetic" manifestations. Early reports in the literature on growth-stimulating effects of ultraviolet radiation must be regarded with caution, since for the most part proper controls were not set up to take into account release of stimulatory material from injured cells. The fundamental work of Loofbourow (1948) clarified this aspect of ultraviolet action on microbial cells by establishing that various compounds, particularly nucleic acid fragments, were released after irradiation. Studies on amino acid leakage from ultraviolet irradiated yeast were recently made by Swenson and Dott (1961).

Delay in budding was observed in several species of *Saccharomyces* (Wyckoff and Luyet, 1931; Oster, 1934a,b,c). Analogous delays in spore germination have been reported for *Rhizopus suinus* (Dimond and Duggar, 1940a,b), *Ustilago maydis* (Landen, 1939), *Aspergillus niger* (Zahl *et al.*, 1939), and *R. nigricans* [*R. stolonifer*] (Tagüena, 1959). Recently, Svihla *et al.* (1960) described in some detail the sequence of morphological events that occurred in *Candida utilis* after ultraviolet (2650 Å) irradiation. They observed first a swelling of the vacuole accompanied by a gradual decrease in cell size and suddenly followed by collapse of the cell with apparent precipitation of the cytoplasm. Figure 3, from Svihla *et al.* (1960), illustrates this effect of ultraviolet irradiation. It is interesting to note that loss of viability occurred at much lower dosages than were required to liberate significant amounts of ultraviolet-absorbing materials; i.e., normal-looking cells after ultraviolet irradiation may be incapable of budding, but cells

with enlarged vacuoles and shrunken cell walls are definitely incapable of budding and have probably lost vital constituents to the medium. These authors report similar observations with *S. cerevisiae*. It seems likely that the exact details of the sequence of events described above may reflect in part at least particular experimental conditions, since the description provided for ultraviolet-treated commercial bakers' yeast by Townsend and Sarachek (1953) is quite different. The irradiated bakers' yeast was re-

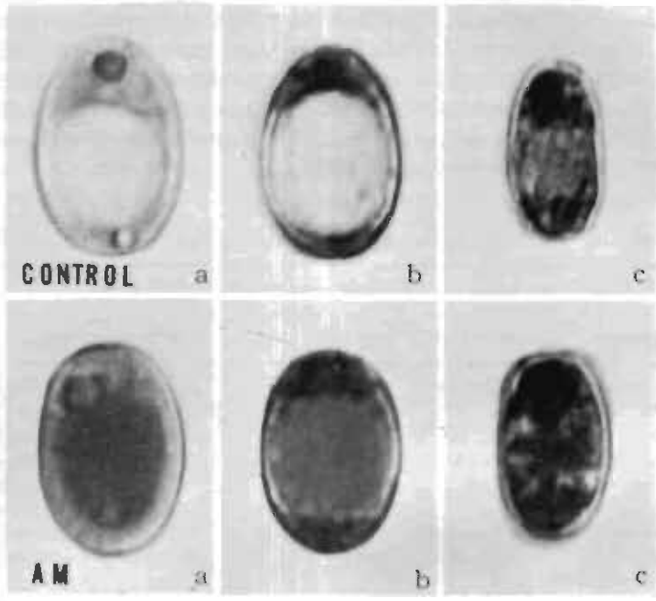

FIG. 3. *Candida utilis*. Ultraviolet (265 mμ) photomicrographs of control and *S*-adenosylmethionine-enriched cells showing the effects of ultraviolet (265 mμ) irradiation. Sequential pictures were obtained at 0, 2, and 4 minutes in each case. *a*, Normal cell; *b*, enlarged vacuole; *c*, collapsed cell. From Svihla *et al.* (1960).

ported to develop a granular cytoplasm, giant cells, and an unusual dumbbell-shaped appearance on being put into growth medium.

Ultraviolet radiation has been shown to prevent adaptation to galactose fermentation by yeast (Swenson, 1950). Interestingly enough, irradiating yeast that has already adapted to galactose has no effect on the rate of fermentation of the carbohydrate. The action spectrum for the prevention of adaptation resembles the absorption spectrum of nucleic acid, and we can speculate that the radiation might have been interfering with the transfer of

information from the gene via ribonucleic acid by alteration of the latter. X-radiation does not interfere with adaptation to galactose.

Two additional "nongenetic" effects of ultraviolet radiation on fungi should be noted. Leach (1962) observed that near-ultraviolet radiation [3100–4000 Å] initiated or stimulated sporulation in most fungi tested, e.g., in *Botrytis cinerea, Alternaria chrysanthemi, Fusarium roseum, Gliocladium* sp., *Helminthosporium avenae*. For most fungi tested, a 12-hour dark–12-hour ultraviolet schedule produced zones of sporulation. These zones were absent under conditions of continuous ultraviolet irradiation or continuous darkness. Berliner and Brand (1962) found that luminescence in dikaryotic cultures of the basidiomycete *Panus stipticus* was markedly inhibited by ultraviolet radiation at 2800 Å, suggesting absorption by the enzyme protein. A temporary stimulation of luminescence was obtained with radiation at 2450–2650 Å and at 3660 Å.

TABLE II

TYPES OF SURVIVAL CURVES OBTAINED AFTER ULTRAVIOLET IRRADIATION[a]

Organism	Type of curve	Morphological element irradiated	References
Rhizopus suinus	Sigmoidal	Conidiospores	Dimond and Duggar (1941)
Mucor dispersus	Sigmoidal	Conidiospores	Dimond and Duggar (1941)
Aspergillus melleus	Exponential	Conidiospores	Dimond and Duggar (1941)
Trichophyton mentagrophytes	Sigmoidal	Conidiospores	Hollaender and Emmons (1939, 1941, 1946); Emmons and Hollaender (1939)
Aspergillus terreus	Sigmoidal	Conidiospores	Hollaender, *et al.* (1945a)
Saccharomyces cerevisiae (haploid)	Exponential	Resting cells	DeLong and Lindegren (1951)
Saccharomyces cerevisiae (haploid)	Sigmoidal	Resting cells	Sarachek and Lucke (1953); Pomper (1951)
Saccharomyces cerevisiae (diploid)	Sigmoidal	Resting cells	Wyckoff and Luyet (1931); DeLong and Lindegren (1951); Pomper (1951)
Ustilago maydis (haploid and diploid)	Sigmoidal	Sporidia and chlamydospores	Landen (1939)
Streptomyces flaveolus	Exponential	Conidiospores	Kelner (1948)
Streptomyces griseus	Sigmoidal	Conidiospores	Savage (1949)
Aspergillus niger	Sigmoidal	Conidiospores	Zahl *et al.* (1939)
Rhizopus stoloniter	Sigmoidal	Conidiospores	Luyet (1932)
Neurospora crassa	Exponential	Uninucleate microconidia	Norman (1951)
Neurospora crassa	Sigmoidal	Multinucleate conidia	Norman (1951)

[a] After Pomper and Atwood (1955).

C. "Genetic" Effects

1. Kinetics

We have already noted that ultraviolet radiation, particularly in the wavelength range about 2600 Å, may produce mutations or lethality in fungi, i.e., "genetic" effects. Table II, from Pomper and Atwood (1955), summarizes information on the inactivation kinetics of a variety of fungi. The work of Norman (1951, 1954) showed that conidia of *Neurospora crassa* were killed by ultraviolet according to kinetics that fit well the theory that a sensitive target was inactivated by a single event in each nucleus. Uninucleate microconidia were inactivated according to an exponential curve, and multinucleate conidia according to sigmoidal curves whose extrapolates to zero dose corresponded to the average number of nuclei per conidium. These findings are illustrated in Fig. 4 (Norman, 1954). There is some confusion in the literature as to the shape of the survival curve for haploid

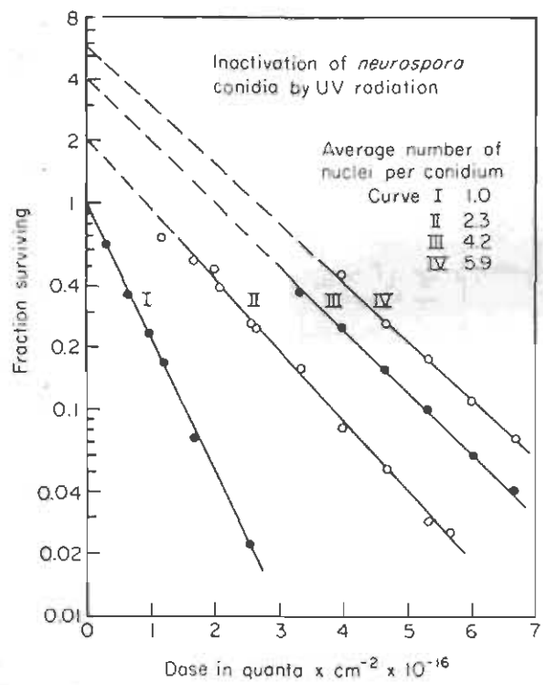

FIG. 4. The inactivation of *Neurospora* conidia by ultraviolet radiation. The wavelength is 254 mμ. From Norman (1954).

yeast; most reports indicate sigmoidal curves (e.g., Sarachek and Lucke, 1953), but some indicate exponential curves (DeLong and Lindegren, 1951). This may be a matter of differences in experimental design resulting in a different interaction between the microorganism and the radiation. In any event, most workers agree that haploid yeast cells are more sensitive than yeast of higher ploidy. Elkind and Sutton (1959a) demonstrated a resistant "tail" of survivors in a population of *S. cerevisiae* after ultraviolet irradiation, which they interpret as corresponding to the X-ray "tail," i.e., cells in the early phases of division. Swann (1962) observed the UV sensitivity of *Schizosaccharomyces pombe* cells early in division was at least tenfold greater than that of cells later in the cycle (Fig. 5).

The kinetics of the production of mutations by UV radiation appears to follow a complex curve. In most of the earlier work (cf. Hollaender and Emmons, 1941), morphological mutations were used as criteria for effective mutagenicity. This was relatively crude, but had the advantage of permitting the investigator to get high mutation rates with relatively mild irradiation doses. Later on, when specific biochemical mutants of various

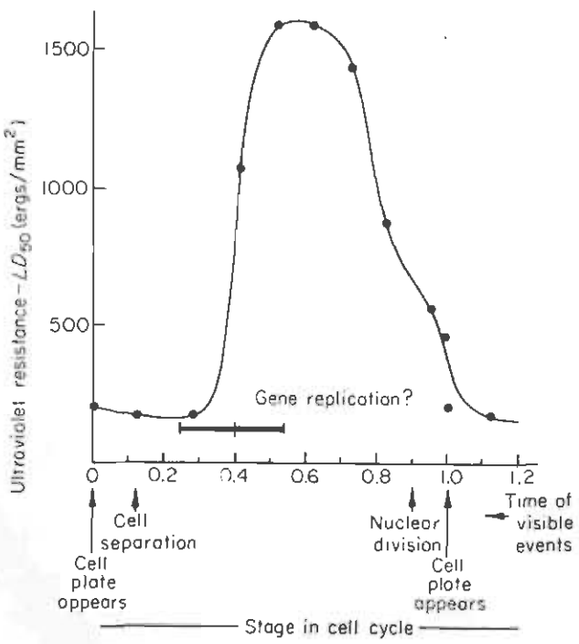

FIG. 5. Resistance to killing by ultraviolet rays, and the timing of various events in the cell cycle. From Swann (1962).

fungi were available it was possible to follow the "back-mutation" rate of specific genes (cf. Giles, 1951). However, genetic work, which we shall not discuss here, showed that more rigorous criteria were necessary than merely a return to nutritional independence ("prototrophy") for a particular biochemical factor. The possibility is that genes other than the particular one under study may be altered by the ultraviolet radiation and may give rise to prototrophy without an actual back mutation at the original mutant locus. Most of the work with morphological mutations indicated that there is a maximum ratio of mutants to survivors, and that beyond this dosage there is a decrease in mutant production. This was reported recently for *Aspergillus nidulans* by Kuzyurina (1959), and earlier for *A. terreus* (Hollaender *et al.*, 1945a), *Penicillium notatum* (Hollaender and Zimmer, 1945), *Trichophyton mentagrophytes* (Hollaender and Emmons, 1941), etc. This was not seen with reverse mutations in *Neurospora crassa* (Giles, 1951) or *Saccharomyces cerevisiae* (Pomper, 1951).

2. Photoreactivation

One of the most interesting aspects of the action of ultraviolet radiation on fungi is that a large portion of the effect, whether it be killing, mutation, suppression of adaptation, etc., is reversible by exposing the organism to visible light. This phenomenon, "photoreactivation," generally is defined as the reversal (in part, at least) of the effect of ultraviolet radiation of approximately 2600 Å by light of about 3300–4800 Å (Dulbecco, 1955). Photoreactivation was first recognized by Kelner (1949), working with *Streptomyces griseus*. It has been seen with a variety of fungi [for example, species of *Botrytis*, *Uromyces*, and *Erysiphe* (Buxton *et al.*, 1957)] and is probably a general phenomenon. Pittman and Pedigo (1959) and Pittman *et al.* (1959) reported that photoreactivation of yeast was not mediated through the cytochrome system and followed a multihit curve whether haploid or tetraploid yeasts were exposed. Production of respiratory mutations by ultraviolet radiation was also found to be photoreactivable. Norman (1954) concluded that photoreactivation of multinucleate *Neurospora crassa* conidia could be brought about by reactivation of a single nucleus per conidium. As discussed earlier, it now appears possible that at least a part of the photoreactivation effect is brought about by light-stimulation of an enzyme that splits thymine dimers.

The interaction of various physical and chemical factors with ultraviolet radiation was discussed by Pomper and Atwood (1955). They noted that for the most part this work had only limited value because of failure to recognize the effect of factors such as photoreactivation and genetic homogeneity. Slight protection against the lethal and mutagenic effects of UV was reported by Whittingham and Stauffer (1956) when spores of *Penicil-*

lium chrysogenum were treated with cyanide, azide, fluoride, or were exposed under nitrogen. The wavelength used in this work was 2750 Å, i.e., somewhat longer than the generally considered optimal for UV genetic effects. Wainwright and Nevill (1955) reported that the mutation rate and survival level were increased by holding irradiated *Streptomyces* spores in distilled water or in iodoacetate.

V. CONCLUSION

Fungi, because of their diversity and flexibility remain a valuable research tool for the radiation biologist. Radiations, including ionizing, ultraviolet, and visible, are a valuable research tool for the mycologist because the interactions observed reveal basic information about the organism itself. In nature, fungi are exposed continually to low levels of radiation. In the laboratory, these exposures are increased and the time span of events shortened, but the laboratory observations basically reflect what may happen to fungi in nature. Therefore, the student of fungi should be aware of the effects of various radiations on the organism of his interest if he wishes to understand it completely in nature.

REFERENCES

Alexander, P. (1957). *In* "Advances in Radiobiology" (G. C. de Hevesy, A. G. Forssberg, and J. D. Abbott, eds.), pp. 8–13. Oliver & Boyd, London.

Alper, T. (1956). The modification of damage caused by primary ionization of biological targets. *Radiation Res.* 5:573–586.

Anderson, R. S., and H. Turkowitz. (1941). The experimental modification of the sensitivity of yeast to roentgen rays. *Am. J. Roentgenol., Radium Therapy Nucl. Med.* 46:537–542.

Atwood, K. C., and A. Norman. (1949). On the interpretation of multi-hit survival curves. *Proc. Natl. Acad. Sci. U.S.* 35:696–709.

Bair, W. J., and F. P. Hungate. (1958). Synergistic action of ethylenediaminetetraacetate and radiation on yeast. *Science* 127:813.

Bair, W. J., and J. N. Stannard. (1955). Role of electrolytes and starvation in altering apparent radiosensitivity of bakers' yeast. *J. Gen. Physiol.* 38:493–504.

Barron, E. S. G. (1954). *In* "Radiation Biology" (A. Hollaender, ed.), Vol. I, pp. 283–350. McGraw-Hill, New York.

Beam, C. A. (1955). The influence of ploidy and division stage on the anoxic protection of *Saccharomyces cerevisiae* against X-ray inactivation. *Proc. Natl. Acad. Sci. U.S.* 41:857–861.

Beam, C. A. (1959). The influence of metabolism on some radiation effects. *Radiation Res.* Suppl. 1, pp. 372–390.

Beam, C. A., R. K. Mortimer, R. G. Wolfe, and C. A. Tobias. (1954). The relation of radioresistance to budding in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophy.* 49:110–122.

Berliner, M. D., and P. B. Brand. (1962). Effects of monochromatic ultraviolet light on luminescence in *Panus stipticus*. *Mycologia* **54**:415–421.

Brace, K. C. (1950). Effects of X-rays on size of yeast cells. *Proc. Soc. Exptl. Biol. Med.* **74**:751–755.

Burns, V. W. (1956). X-ray-induced division delay in individual yeast cells. *Radiation Res.* **4**:394–412.

Buxton, E. W., F. T. Last, and M. A. Nour. (1957). Some effects of ultraviolet radiation on the pathogenicity of *Botrytis fabae*, *Uromyces fabae* and *Erysiphe graminis*. *J. Gen. Microbiol.* **16**:764–773.

Dale, W. M. (1954). *In* "Radiation Biology" (A. Hollaender, ed.), Vol. I, pp. 255–281. McGraw-Hill, New York.

Deering, R. A. (1962). Ultraviolet radiation and nucleic acid. *Sci. Am.* **207**:135–144.

DeLong, R., and C. C. Lindegren. (1951). Ultraviolet resistance as a criterion for determining the degree of ploidy in yeasts. *Bacteriol. Proc.* p. 63.

de Serres, F. J. (1961). *Symp. Soc. Gen. Microbiol.* **11**:196–216.

Dimond, A. E., and B. M. Duggar. (1940a). Effects of monochromatic ultraviolet radiation on the growth of fungus spores surviving irradiation. *Am. J. Botany* **27**:906–914.

Dimond, A. E., and B. M. Duggar. (1940b). Effects of ultraviolet radiation on the germination and morphology of spores of *Rhizopus suinus*. *J. Cellular Comp. Physiol.* **16**:55–61.

Dimond, A. E., and B. M. Duggar. (1941). Some lethal effects of ultraviolet radiation on fungus spores. *Proc. Natl. Acad. Sci. U.S.* **27**:459–468.

Dulbecco, R. (1955). *In* "Radiation Biology" (A. Hollaender, ed.), Vol. II, pp. 455–486. McGraw-Hill, New York.

Elkind, M. M., and H. Sutton. (1959a). The relationship between division and X-ray sensitivity, ultraviolet sensitivity, and photoreactivation in yeast. *Radiation Res.* **10**:283–295.

Elkind, M. M., and H. Sutton. (1959b). Site of lethal irradiation: overlap in sites for X-ray, ultraviolet, photoreactivation, and ultraviolet protection and reactivation in dividing yeast cells. *Radiation Res.* **10**:296–312.

Emmons, C. W., and A. Hollaender. (1939). The action of ultraviolet radiation on dermatophytes. II. Mutations induced in cultures of dermatophytes by exposure of spores to monochromatic ultraviolet radiation. *Am. J. Botany* **26**:467–475.

Engelhard, H., K. E. Schneweis, and H. Bülow. (1959). Strahlenschädigung und mineralstoffwechsel. Versuche mit röntgenhestrahlung von *Saccharomyces cerevisiae*. *Z. Naturforsch.* **14b**: No. 3, 152–157. [Abstr. in *Biol. Abstr.* 33:30099 (1960).]

Forssberg, A. G., and R. Novak. (1960). Growth rate and biochemical changes in *Phycomyces* at low dose-levels of X-rays. *Intern. J. Radiation Biol. Suppl. Immediate Low Level Effects Ionizing Radiations, Proc. Symp., Venice, 1959* pp. 205–216. [Abstr. in *Chem. Abstr.* **55**:20040 (1961).]

Forssberg, A. G., R. Novak, G. Dreyfus, and A. Pehap. (1960). The radiation sensitivity of *Phycomyces*, interaction of visible light and ionizing radiation. *Radiation Res.* **13**:661–668.

Gal'tsova, R. D., and A. T. Novichkova. (1962). Effect of ionizing radiation on nitrogen metabolism of yeast. *Radiobiologiya* **2**:690–694. [Abstr. in *Chem. Abstr.* **58**:2 (1963).]

Giles, N. H., Jr. (1951). Studies on the mechanism of reversion in biochemical

mutants of *Neurospora crassa*. *Cold Spring Harbor Symp. Quant. Biol.* **16**:283–313.
Gray, L. H. (1959). Cellular radiobiology. *Radiation Res.* Suppl. 1, pp. 73–101.
Hollaender, A., and C. W. Emmons. (1939). The action of ultraviolet radiation on dermatophytes. I. The fungicidal effect of monochromatic ultraviolet radiation on the spores of *Trichophyton mentagrophytes*. *J. Cellular Comp. Physiol.* **13**:391–402.
Hollaender, A., and C. W. Emmons. (1941). Wavelength dependence of mutation production in the ultraviolet with special emphasis on fungi. *Cold Spring Harbor Symp. Quant. Biol.* **9**:179–186.
Hollaender, A., and C. W. Emmons. (1946). Induced mutations and speciation in fungi. *Cold Spring Harbor Symp. Quant. Biol.* **11**:78–84.
Hollaender, A., and E. M. Zimmer. (1945). The effect of ultraviolet radiation and X-rays on mutation production in *Penicillium notatum*. *Genetics.* **30**:8.
Hollaender, A., K. B. Raper, and R. D. Coghill. (1945a). The production and characterization of ultraviolet induced mutations in *Aspergillus terreus*. I. Production of the mutations. *Am. J. Botany* **32**:160–165.
Hollaender, A., E. R. Sansome, E. M. Zimmer, and M. Demerec. (1945b). Quantitative irradiation experiments with *Neurospora crassa*. II. Ultraviolet irradiation. *Am. J. Botany* **32**:226–235.
Holweck, F., and A. Lacassagne. (1930). Action sur les levures des rayons X mous (K du fer). *Compt. Rend. Soc. Biol.* **103**:60–62.
Howard-Flanders, P., and T. Alper (1957). The sensitivity of microorganisms to irradiation under controlled gas conditions. *Radiation Res.* **7**:518–540.
Hutchinson, F. (1957). The distance that a radical formed by ionizing radiation can diffuse in a yeast cell. *Radiation Res.* **7**:473–483.
Hutchinson, F., A. Preston, and B. Vogel. (1957). Radiation sensitivity of enzymes in wet and dry yeast cells. *Radiation Res.* **7**:465–472.
James, A. P., and I. Müller. (1961). Radiation-induced mitotic stimulation. *Radiation Res.* **14**:779–788.
Käfer, E. (1963). Radiation effects and mitotic recombination in diploids of *Aspergillus nidulans*. *Genetics* **48**:27–45.
Kelner, A. (1948). Mutations in *Streptomyces flaveolus* induced by X-rays and ultra-violet light. *J. Bacteriol.* **56**:457–465.
Kelner, A. (1949). Effect of visible light on the recovery of *Streptomyces griseus* conidia from ultra-violet irradiation injury. *Proc. Natl. Acad. Sci. U.S.* **35**:73–79.
Kuzyurina, L. A. (1959). Production of mutants in fungi by means of ultraviolet rays. II. Morphological characteristics of *Aspergillus nidulans* variants obtained through irradiation with different doses of ultraviolet rays. *Microbiology (USSR)* (*English transl.*) 28:625–631. [Abstr. in *Biol. Abstr.* **35**:57747 (1960).]
Landen, E. W. (1939). The spectral sensitivity of spores and sporidia of *Ustilago zeae* to monochromatic ultraviolet light. *J. Cellular Comp. Physiol.* **14**:217–226.
Latarjet, R., and B. Ephrussi. (1949). Courbes de survie de levures haploïdes et diploïdes soumises aux rayons X. *Compt. Rend.* **229**:306–308.
Leach, C. M. (1962). Sporulation of diverse species of fungi under near-ultraviolet radiation. *Can. J. Botany* **40**:151–161.
Lindegren, C. C., D. Pittman, and A. Yuasa. (1959). The centriole as the primary radiation target in the resting cell. *Trans. N.Y. Acad. Sci.* **21**:524–530.
Loofbourow, J. R. (1948). Effects of ultraviolet radiation on cells. *Growth* **12**, Suppl., 77–149.

Lucke, W. H., and A. Sarachek. (1953). X-ray inactivation of polyploid *Saccharomyces*. *Nature* **171**:1014–1015.

Luyet, B. J. (1932). The effects of ultra-violet, X-, and cathode rays on the spores of *Mucoraceae*. *Radiology* **18**:1019–1022.

McAulay, A. L., and J. M. Ford. (1947). Saltant production in the fungus *Chaetomium globosum* by ultra-violet light, and its relation to absorption processes. *Heredity* **1**:247–257.

Magni, G. E. (1959). Genetic effects of radiation on yeast cells and genetic control of radiosensitivity. *Radiation Res.* Suppl. 1, pp. 347–356.

Morgan, K. Z. (1963). Permissible exposure to ionizing radiation. *Science* **139**:565–571.

Mortimer, R. K. (1958). Radiobiological and genetic studies on a polyploid series [haploid to hexaploid] of *Saccharomyces cerevisiae*. *Radiation Res.* **9**:312–326.

Moustacchi, E. (1958). "L'effet oxygène" dans l'irradiation X de microorganismes déficients en enzymes de la respiration aérobe. *Ann. Inst. Pasteur* **94**:89–96.

Norman, A. (1951). Inactivation of *Neurospora* conidia by ultraviolet radiation. *Exptl. Cell Res.* **2**:454–473.

Norman, A. (1954). The nuclear role in the ultraviolet inactivation of *Neurospora* conidia. *J. Cellular Comp. Physiol.* **44**:1–10.

Oster, R. H. (1934a). Results of irradiating *Saccharomyces* with monochromatic ultra-violet light. I. Morphological and respiratory changes. *J. Gen. Physiol.* **18**:71–88.

Oster, R. H. (1934b). Results of irradiating *Saccharomyces* with monochromatic ultra-violet light. II. The influence of modifying factors. *J. Gen. Physiol.* **18**:243–250.

Oster, R. H. (1934c). Results of irradiating *Saccharomyces* with monochromatic ultra-violet light. III. The absorption of ultra-violet light energy by yeast. *J. Gen. Physiol.* **18**:251–254.

Pittman, D., and P. R. Pedigo. (1959). Photoreactivation studies on yeasts. I. Ultraviolet inactivation and photoreactivation of respiration-sufficient and respiration-deficient yeasts. *Exptl. Cell. Res.* **17**:359–367.

Pittman, D., B. Ranganathan, and F. Wilson. (1959). Photoreactivation studies on yeasts. II. Photoreactivation of the ultraviolet damage producing respiratory deficiency in haploid and tetraploid yeasts. *Exptl. Cell Res.* **17**:368–377.

Pomper, S. (1951). Unpublished data.

Pomper, S., and K. C. Atwood (1955). *In* "Radiation Biology" (A. Hollaender, ed.), Vol. II, pp. 431–453. McGraw-Hill, New York.

Pomper, S., K. M. Daniels, and D. W. McKee. (1954). Genetic analysis of polyploid yeast. *Genetics* **39**:343–355.

Rothstein, A. (1959). Biochemical and physiological changes in irradiated yeast. *Radiation Res.* Suppl. 1, pp. 357–371.

Sarachek, A., and W. H. Lucke. (1953). Ultraviolet inactivation of polyploid *Saccharomyces*. *Arch. Biochem. Biophys.* **44**:271–279.

Savage, G. M. (1949). Improvement in streptomycin-producing strains of *Streptomyces griseus* by ultraviolet and X-ray energy. *J. Bacteriol.* **57**:429–441.

Sayeg, J. A., A. C. Birge, C. A. Beam, and C. A. Tobias. (1959). The effects of accelerated carbon nuclei and other radiations on the survival of haploid yeast. II. Biological experiments. *Radiation Res.* **10**:449–461.

Schwinghamer, E. A. (1958). Relation of survival to radiation dose in rust fungi. *Radiation Res.* **8**:329–343.

Shebata, A. M. El-Tabey. (1961). Effect of combined action of ionizing radiation and chemical preservatives on microorganisms. I. Vitamin K₅ as a sensitizing agent. *Radiation Res.* **15**:78–85.

Sinclair, W. K., S. E. Gunter, and A. Cole. (1959). The relative biological effectiveness of 200-Kvp X-rays, cobalt-60 gamma-rays, and 22-Mevp X-rays, determined from the dose-survival curve of *Saccharomyces cerevisiae*. *Radiation Res.* **10**:418–432.

Spoerl, E., D. Looney, and J. E. Kazmierczak. (1959). Polyphosphate metabolism in X-irradiated division-inhibited yeast cells. *Radiation Res.* **11**:793–803.

Stapleton, G. E. (1960). *In* "Radiation Protection and Recovery" (A. Hollaender, ed.), pp. 87–116. Pergamon Press, New York.

Stapleton, G. E., and A. Hollaender. (1952). Mechanism of lethal and mutagenic action of ionizing radiation on *Aspergillus terreus*. II. Use of modifying agents and conditions. *J. Cellular Comp. Physiol.* **39**: Suppl. 1, 101–113.

Stapleton, G. E., A. Hollaender, and F. L. Martin. (1952). Mechanism of lethal and mutagenic action of ionizing radiation on *Aspergillus terreus*. I. Relationship of relative biological efficiency to ion density. *J. Cellular Comp. Physiol.* **39**: Suppl. 1, 87–100.

Stocken, L. A. (1959). Some observations on the biochemical effects of X-radiation. *Radiation Res.* Suppl. 1, pp. 53–72.

Svihla, G., F. Schlenk, and J. L. Dainko. (1960). Some effects of ultraviolet irradiation on yeast cells (*Candida utilis*). *Radiation Res.* **13**:879–891.

Swann, M. M. (1962). Gene replication, ultra-violet sensitivity and the cell cycle. *Nature* **193**:1222–1227.

Swenson, P. A. (1950). The action spectrum of the inhibition of galactozymase production by ultraviolet light. *Proc. Natl. Acad. Sci. U.S.* **36**:699–703.

Swenson, P. A., and D. H. Dott. (1961). Amino acid leakage and pool levels of ultraviolet-irradiated yeast cells. *J. Cellular Comp. Physiol.* **58**:217–231.

Tagüeña, M. (1959). Comparative action of various radiations and of ultrasonics on the spores of *Rhizopus nigricans*. *Ciencia (Mex.)* **19**:189–196. [Abstr. in *Chem. Abstr.* **54**:15523 (1960).]

Tai, W. H. (1962). Effect of X-rays on the accumulation of catalase in conidia of *Aspergillus niger*. *Bull. Inst. Chem. Acad. Sinica* No. 6, pp. 71–78.

Townsend, G. F., and A. Sarachek. (1953). Characteristic alterations of the budding process of *Saccharomyces* induced by ultraviolet treatment. *J. Bacteriol.* **65**:747–749.

Uretz, R. B. (1955). Additivity of X-rays and ultraviolet light in the inactivation of haploid and diploid yeast. *Radiation Res.* **2**:240–252.

Wainwright, S. D., and A. Nevill. (1955). Induction of provariants as a function of dose of ultraviolet radiation. *J. Gen. Microbiol.* **12**:13–24.

Whittingham, W. F., and J. F. Stauffer. (1956). The protection of spores of *Penicillium chrysogenum* against the action of ultraviolet radiation. *Am. J. Botany* **43**:54–60.

Wood, T. H. (1954). Influence of temperature and phase state on X-ray sensitivity of yeast. *Arch. Biochem. Biophys.* **52**:157–174.

Wood, T. H. (1959). Inhibition of cell division. *Radiation Res.* Suppl. 1, pp. 332–346.

Wood, T. H. (1960). Comparative X-ray sensitivities of related respiring and fermenting yeasts. *Radiation Res.* **13**:335–342.

Wood, T. H., and A. L. Taylor. (1957). Dependence of X-ray sensitivity of yeast on phase state and anoxia. *Radiation Res.* 6:611–625.

Wulff, D. L., and C. S. Rupert. (1962). Disappearance of thymine photodimer in ultraviolet irradiated deoxyribonucleic acid [DNA] upon treatment with a photoreactivating enzyme from bakers' yeast. *Biochem. Biophys. Res. Commun.* 7:237–240.

Wyckoff, R. W. G., and B. J. Luyet. (1931). The effects of X-rays, cathode, and ultraviolet rays on yeast. *Radiology* **17**:1171–1175.

Zahl, P. A., L. R. Koller, and C. P. Haskins. (1939). The effects of ultraviolet radiation on spores of the fungus *Aspergillus niger*. *J. Gen. Physiol.* **22**:689–698.

Zirkle, R. E. (1940). The radiobiological importance of the energy distribution along ionization tracks. *J. Cellular Comp. Physiol.* **16**:221–235.

Zirkle, R. E., and C. A. Tobias. (1953). Effects of ploidy and linear energy transfer on radiobiological survival curves. *Arch. Biochem. Biophy.* **47**:282–306.

GENERAL REFERENCES

Lea, D. E. (1955). "Action of Radiation on Living Cells," 2nd ed., 416 pp. Univ. Press, Cambridge. London and New York.

Spear, F. G., ed. (1947). Certain aspects of the action of radiation on living cells. *Brit. J. Radiol.* Suppl. 1, 146 pp.

Symposium on radiation genetics. (1950). *J. Cellular Comp. Physiol.* **35**: Suppl. 1, 210 pp.

Symposium on recovery of cells from injury. (1961). *J. Cellular Comp. Physiol.* **58**: Suppl. 1, 248 pp.

CHAPTER 25

Kinetics of Fungal Growth

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I. DEFINITIONS AND CRITERIA

Any discussion of the kinetics of fungal growth necessitates a brief consideration of what we mean by kinetics, of the nature of growth, and of the manner in which fungal growth occurs.

We are using the term *kinetics* in the chemical sense, rather than physical, to mean the study of rates. We are, therefore, concerned here with the quantitation of rates of fungal growth. Such kinetic analyses provide a quantitative evaluation of the impact of environmental or genetic (i.e., *external* or *internal*) factors on a fungus. Kinetic analysis of growth phenomenon can also be useful in studies attempting to elaborate mechanisms.

In the words of Thompson (1948), ". . . growth is a somewhat vague word for a very complex matter." Needham (1942) has analyzed in detail this complex phenomenon, particularly with reference to the relations between growth and differentiation. While Needham's comments are concerned primarily with the development of the animal embryo, they are pertinent to fungi and can help clarify our thinking about the problem. Thus, growth can involve an increase in the number of nuclei, in the number of cells, in the size of cells, or in the amount of "non-living structural matter."

Fungi are of very diverse morphological form varying from unicellular yeasts to large fleshy basidiomycetes. Furthermore, the morphology of many fungi is markedly dependent upon growth conditions. Some yeasts can be induced to become filamentous and, conversely, some filamentous fungi to grow in unicellular fashion (Cochrane, 1958). Fundamentally, however, fungi are filamentous organisms whose morphology is determined by behavior of the hyphae, i.e., branching, aggregation into a complex

structure, division into individual cells, or differentiation of a hyphal strand.

For purposes of analysis of the kinetics of fungal growth, we are concerned primarily with two situations—growth as a population of individual cells or growth by elongation of a hypha. Branching of hyphae can, and usually does, occur so that this phenomenon is also involved.

General treatments of growth, in addition to those mentioned above, are by Brody (1945), Buchanan (1953), Hinshelwood (1946), Lamanna and Mallette (1953), Monod (1942), Thimann (1955), and Van Niel (1949).

II. METHODS OF MEASUREMENTS

To measure rates of growth, we can measure any quantity known to be proportional to the criterion for growth being used. Obviously, the measurement should also be one which is convenient and which has a degree of precision consistent with experimental variables and with intended goals. Thus, growth can be measured in terms of changes in numbers of cells, in linear dimensions, in mass, in volume, in total activity of any metabolic process, or in quantity of some cellular constituent.

A. Linear

The simplest method of measuring fungal growth is by determination of increase in linear dimensions on a medium solidified with some nonnutrient material such as agar. This is done most commonly in petri dishes, and the increase in colony diameter or radius is recorded at suitable intervals. For fungi which grow very rapidly, or for some special cases, it may be more convenient or useful to employ "race" tubes (Ryan *et al.*, 1943), or some modification thereof. In these tubes, measurements are made of the distance from the point of inoculation to the advancing front of mycelium at appropriate intervals. Linear measurements can also be made on a microscopic scale by observing individual hyphae. This can be advantageous in reducing the time element from days to hours, although large variations in rate of elongation of an individual hyphae can occur and must be taken into consideration (Smith, 1924).

The advantages of linear measurements reside in their simplicity and nondestructive nature. Repeated measurements can be made on the same culture thereby reducing the number of replications required while still providing statistically significant values.

There is one fundamental and important limitation to the use of linear measurements for quantifying growth rates or for assessing the effect of an environmental variable: there is no necessary correlation between the

spread of a mycelial front on a solid surface and the total amount of fungus produced. Thus, linear extension on agar lacking an available carbon source can be as rapid or even more rapid than on a complete medium. On the other hand, with a given medium the rate of spread can be used very effectively to measure the effect of temperature, for example. It is mandatory, therefore, that selection of linear measurements as a criterion of growth be evaluated critically to ensure that the variable being investigated operates as a limiting factor.

The reasons for the nonequivalence of linear growth rate and amount of organism produced do not appear to have been analyzed experimentally. Examination of the problem, however, suggests that four factors may be involved including, first, the amount of branching of hyphae; second, growth rate of branches; third, the translocation of nutrients along the hyphae to the growing apex; and, fourth, a phenomenon of apical dominance, similar to that sometimes found in higher plants where the growing hyphal tip can suppress branching or growth of branches by competition for nutrients or hormonal control.

B. Dry Weight

Dry weight measurement is probably the most widely used and most generally applicable method for assaying growth in fungi. It is the most direct way of representing the amount of organism produced and is the most basic and meaningful. Any other method should be referred to dry weight at some time to interpret properly the physiological significance of the parameter being used.

Although dry weight is a basic quantity which can be easy to measure, there are limitations and disadvantages in its use. It is a destructive test and consequently requires large numbers of cultures to provide significant measurements of the course of growth. It is most widely and usefully used in liquid cultures which are shaken to provide a more uniform environment and better gas exchange. Obviously, this is not a "normal" environment for most organisms. The slimy, filamentous nature of fungal growth may sometimes create problems in separation from the growth medium by filtration, in which case centrifugation may be useful.

If solidified media are desired, or if a constituent of the medium is insoluble, the separation problem may present real difficulties. Agar can be removed by hot water (Day and Hervey, 1946). Insoluble substrates such as wool can be removed by boiling with alkali (Mandels *et al.*, 1948) or other appropriate solvents. The uncertainty of correction factors for the inevitable loss of cellular constituents resulting from these drastic treatments prevents any high degree of precision.

C. Cell Volume

Cell volume determination can be a convenient and useful criterion in certain specialized cases where growth occurs in a unicellular manner or in studies involving spore germination where swelling is involved (Mandels and Darby, 1953). Where filamentous growth occurs, the method has not been useful because of nonuniform packing.

The advantages of cell volume determination reside in the simplicity and speed of measurement and in the small quantities of material required. Techniques include centrifugation in hematocrit tubes or use of a device such as the Coulter Counter (Kubitschek, 1960). In the latter case, continuous measurements are possible which give changes in distribution of size of cells.

D. Cell Number

Techniques employing changes in number of cells are restricted to those instances where growth occurs as in unicellular organisms. In addition to yeasts, certain normally filamentous fungi may grow as unicellular organisms (Cochrane, 1958) and the techniques employed by bacteriologists are applicable. Of great potential application are changes in light absorption or scattering, or devices such as the Coulter Counter and the de Bonet-Maury Biospectrophotometer (Coultas and Hutchinson, 1962). These instruments, or those using similar principles, make possible continuous measurements of growth.

E. Metabolic Activity

In certain cases growth can be measured indirectly by following the metabolic activity of the organism or culture. Such measurements can include respiratory activity (CO_2, O_2), product formation (acid, pigments, etc.), disappearance of substrate, or enzymatic activity. The applicability of any such system must be proved by demonstrating a correlation between the function analyzed and some more direct quantity such as dry weight.

The advantages of following metabolic activity are that simple, nondestructive, continuous measurements can be made. They are likely to be of particular value when transitionary changes are occurring such as in germination or in adaptation to new growing conditions. Such methods are adaptable to use with continuous recorders coupled to an oxygen ana-

lyzer or infrared CO_2 analyzer, although such techniques have not been exploited in studies on fungi to the extent that they have been in other fields.

F. Compositional Changes

Sometimes it can be useful to follow growth by changes in the quantity of some cellular constituent or of total organic matter (Lu *et al.*, 1959). As with measurements of metabolic activity, a frame of reference must be established to some direct parameter of growth since composition can change during growth (e.g. Woodruff, 1961; Herbert, 1961).

III. KINETIC ANALYSIS

The manifestations of growth in fungi are so varied, depending upon the organism as well as environmental conditions, that no generalized analysis can be made. In certain cases, however, useful mathematical expressions have been shown to apply. We discuss these analyzable situations below.

A. The Idealized Growth Curve

Growth curves have been analyzed for innumerable organisms and populations ranging from bacteria to higher animals. The relative simplicity of growth by binary fission in bacteria, and the consequent conformance to a predictable curve, has made bacteria most amenable to quantitative study and analysis. It will, therefore, be convenient and instructive to discuss growth as exemplified by a population of bacteria in liquid culture. This will provide a frame of reference in our analysis of fungal growth and illustrates the terminology used to characterize various phases of the growth curve. This discussion will, of course, be applicable directly to those instances where fungi grow as unicellular organisms.

A significant general characteristic of growth is its autocatalytic nature, i.e., where the products of growth further catalyze the growth process. This is most evident in unicellular organisms and results in cell division occurring at regular intervals, each cell giving rise to two daughter cells. Autocatalytic growth can also occur in more complex organisms but is likely to be modified due to processes of differentiation or to the inevitable consequences of changing environment around each cell.

If environmental factors are constant, or nonlimiting, and no internal changes occur other than the normal processes involved in cell enlargement and division, then cell division occurs at a constant rate. Since growth

occurs at a constant rate and since the number of cells present at any time is determined by the number at some preceding time, we can express this as

$$\frac{dN}{dt} = kN \tag{1}$$

where N is the number of cells, t is time, and K is a proportionality constant. By integrating

$$\log N = kt + c \tag{2}$$

From this it follows that a plot of the log of cell number versus time should give a straight line whose slope, k, is a measure of the growth rate. The constant c represents the intercept on the log N axis and would be the cell number at zero time if logarithmic growth had started at this time. Equation (2) can also be expressed in exponential form

$$N = ce^{kt} \tag{3}$$

where e is the base of natural logarithms.

Exponential growth does not normally occur in fungi, although Plomley (1959) has shown that in *Chaetomium globosum* the total length of a

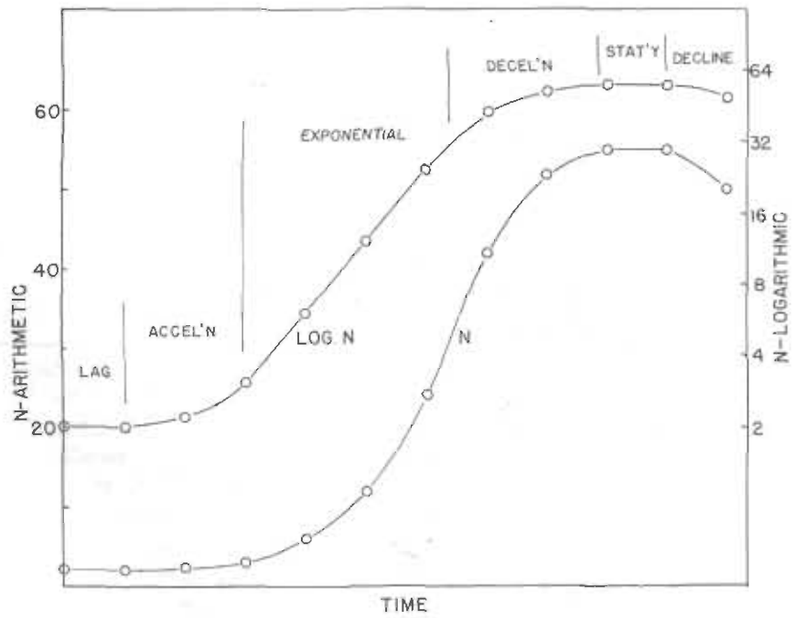

FIG. 1. Idealized representation of exponential growth and of the phases of the growth curve.

hypha and its branches increases in this manner. He has also obtained data indicating that the radius of a colony may increase exponentially in the very early stages of its establishment, i.e., until the radius is about 0.1 mm.

A diagrammatic growth curve showing the increase in numbers of a population of single-celled organisms is shown in Fig. 1. The data are also plotted on a semilog basis to demonstrate the exponential nature of the curve. It is evident that growth can be divided into several phases. Somewhat varied terminology has been applied to these phases (Lamanna and Mallette, 1953; Thimann, 1955). For our purposes, the following will suffice:

1. *Lag phase*—no cell division occurs but cell enlargement and increase in metabolic activity may occur.
2. *Acceleration phase*—increase in rate of cell division from zero to some maximum value characteristic of the next, or exponential, phase.
3. *Exponential phase* (also commonly called "logarithmic phase")—propagation at a uniform rate of cell division.
4. *Deceleration phase*—characterized by a decreasing rate of cell division.
5. *Stationary phase*—cessation of cell division. In experimental application this phase may be one in which cell division occurs but is balanced by death of cells. The interpretation depends upon the technique used to measure cell number, i.e., whether total cells or only viable cells are counted.
6. *Phase of decline*—decrease in number of viable cells.

Three basic phenomena determine this idealized growth curve. First, there is not immediate initiation of growth after inoculation of a culture. Secondly, after cell division starts, it soon reaches a steady rate. Lastly, due to depletion of food or for other reasons, growth stops. The growth curve can thus be characterized by only three phases, lag, exponential (or log phase), and stationary. It is frequently meaningful, however, to include the acceleration and deceleration phases which are periods of changing kinetics and are significant in stages of transition from dormancy to active growth, of adaptation to new conditions of growth, or of senescence.

The above analysis is usually applicable to fungi in only a qualitative way. The various phases of the growth curve are recognizable, but the quantitatively defined exponential phase becomes a phase of rapid growth since growth is not autocatalytic in filamentous fungi.

In any method for measuring growth rates, a single measurement after a given time is of questionable significance. The time interval between inoculation and initiation of a linear growth rate, for example, can be as

long as 30 days and may vary greatly depending upon such variables as type of inoculum used or the nature of the medium (Mandels, 1955; Page, 1961).

B. Linear Growth

Growth of fungi is essentially limited to the terminal portion of the hyphae. Changes must occur in the cell walls fairly soon after they are laid down which prevent elongation or expansion. This phenomenon of apical growth was studied most carefully by Smith (1924) in *Botrytis* and also in *Fusarium, Pyronema, Phytophthora, Aspergillus, Penicillium, Rhizoctonia,* and *Rhizopus* (Smith, 1923). By careful measurements of individual hyphae, Smith showed that growth was only apical and that the rate of elongation was independent of the length of the filament. Observations of *Rhizopus* growing in media containing carbon black particles to mark positions of hyphae show elongation only at the extreme tip (Stadler, 1952).

In *Neurospora* (Ryan *et al.,* 1943), only the terminal centimeter of a hypha contributes to the growth rate. A hyphal tip 1 mm long grew at a rate about 60% of that of a 10-mm hypha. The mechanisms involved have not been explored, although it has been shown that translocation of growth substances in which the fungus is deficient can occur over the terminal centimeter of hypha. In further studies with *Neurospora,* Zalokar (1959) has concluded that the rapid apical growth, whose maintenance is dependent upon the terminal centimeter of hyphae, is due to transport of protoplasm by streaming and that ". . . at least a 12 mm long portion of the hypha would have to reduplicate its cytoplasm in order to supply the tips." In cases of growth of aerial hyphae, translocation is obviously essential.

The aerially borne sporangiophores of *Phycomyces* have also been shown to increase in length at a uniform rate (Castle, 1937). In this case, there is a zone of elongation over a region extending approximately from 0.3 to 2 mm below the sporangium. Subsequent studies (Castle, 1940) show small rapid fluctuations in rate amounting to 2.5% of the average rate.

The linear extension of fungus hyphae in a growing culture is universally accepted as occurring at a constant rate. There are very few data, however, regarding the changing kinetics which must occur between initiation of growth after inoculation and establishment of a constant rate. A diagrammatic representation of these phenomenon is shown in Fig. 2. Smith's measurements (1924) of very young hyphae from germinated spores of *Botrytis cinerea* showed the rate of elongation to increase linearly with time for about 30 hours, at which point the hyphae were about 2 mm long. Subsequently, the hyphae maintained a constant rate of elongation.

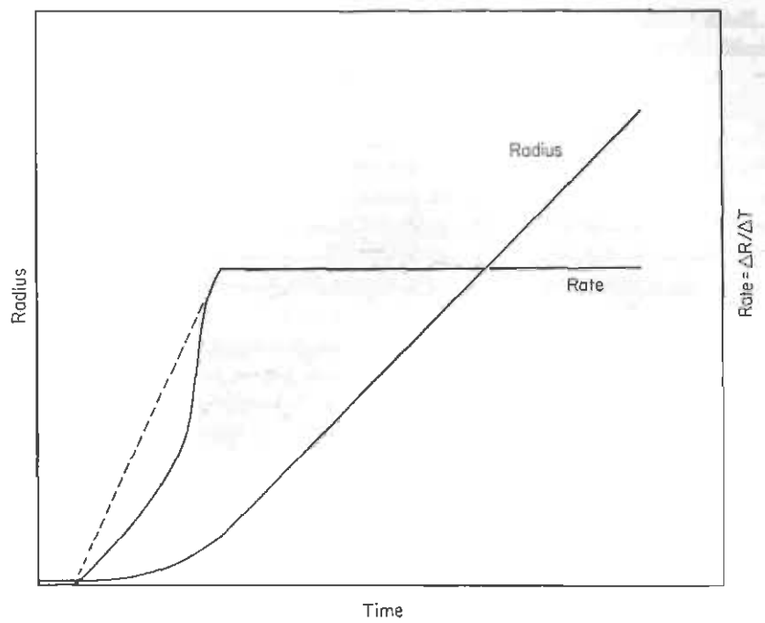

FIG. 2. Idealized representation of linear growth. The dashed line (— — —) indicates the linear increase in rate to a constant level as shown for *Botrytis* by Smith (1924).

This behavior is not universal, however, as shown by Plomley's studies of *Chaetomium globosum* (1959). In this case, hyphae increased in length at a constant rate after germination (2.5 hours) and continued over the period of measurement, i.e., for the next 3 hours. Measurements of the increase in radius of young colonies of *Chaetomium* show a gradual increase in rate. While Plomley notes that this is exponential over a short period, the significance of this is not clear since many curves of increasing rate may be exponential over a brief period. Evidence available is therefore too limited to permit any generalization respecting this period of establishment of a colony. It would appear that different species do not behave in the same manner and/or that experimental conditions influence the kinetics of early growth.

C. Growth in Three Dimensions

Changes in linear dimensions provide reasonably satisfactory criteria for evaluating growth rates on solidified media. In agitated liquid cultures,

however, there is no generalized, rational solution to kinetic analysis. This is due to the variable nature of fungus growth in such cultures.

In standing liquid cultures, fungi usually grow as floating mats, the mycelium being partly in the liquid phase and partly in air. In some cases growth can be completely submerged, consisting of amorphous masses of mycelium. When cultures are agitated by shaking or aeration, however, the germinated spores or mycelial fragments of the inoculum will most commonly aggregate into small spheres or pellets which grow by enlargement. The spherical or quasispherical shape implies uniform three-dimensional growth where the active proliferating hyphal tips are located. This has provided a basis for analysis of the growth curve.

S. Emerson (1950) has pointed out that the mycelial pellets occurring in liquid culture can be considered as spheres whose surface growth results in a constant rate of increase in radius. Consequently, the cube root of the volume or mass of these spheres should increase linearly with time (Fig. 3). These cubic relations were shown by Emerson to obtain for *Neurospora*

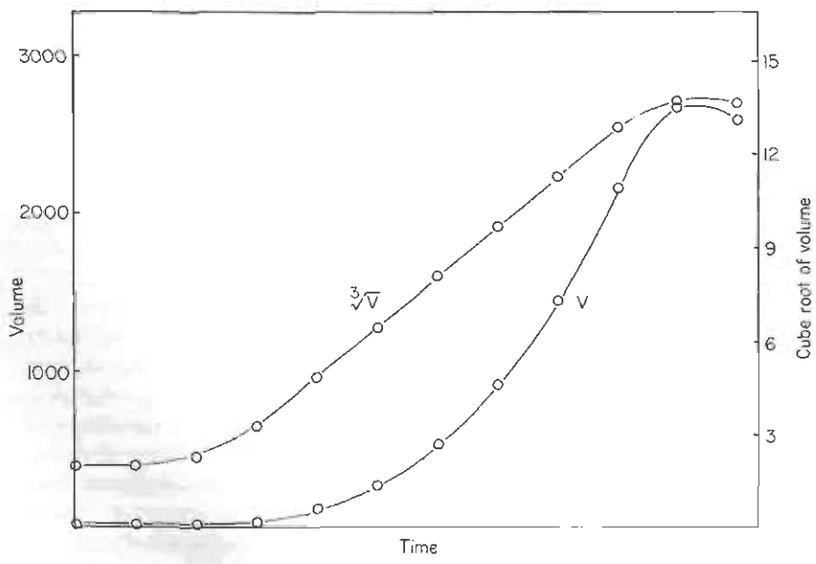

FIG. 3. Idealized representation of cubic growth relations for spherical mycelial pellets in liquid culture.

and Machlis (1957) has demonstrated this with *Allomyces*. Marshall and Alexander (1960) have shown similar relations for the actinomycete *No-*

cardia and for several fungi using oxygen uptake as a criterion for growth.

Unfortunately, this cube root relation is not of general applicability because of the variable nature of the pellets and the fact that they are not produced by some fungi. The variability of pellets formed by different fungi and under different conditions has been illustrated by Burkholder and Sinnott (1945) and Darby and Mandels (1954) and discussed by Foster (1949). Variables involved include such factors as density of inoculum, species, or even strain (Gilbert, 1960) of the organism, as well as composition of the medium. It should also be noted that no one appears to have proved that only apical growth occurs in liquid culture.

D. Transitions from Dormancy to Active Growth

We are interested here primarily in the changes occurring between the time of inoculation and the inception of strict vegetative growth. More generally speaking, we are concerned with the problems of analyzing the process of germination, which is, after all, a growth process.

Measurements of the changes in respiratory activity during germination of *Myrothecium verrucaria* spores show that the rate increases linearly with time during the swelling, germ tube protrusion, and early stages of vegetative growth (Mandels *et al.*, 1956). No break in the curve was observed which could coincide with protrusion of germ tubes.

Expressed mathematically,

$$R = \frac{dy}{dt} = Kt + b$$

where R = rate of respiration, y = oxygen uptake, t = time, K is the slope, and b the intercept. The constants b and K should have definite physiological significance. K, the acceleration in oxygen uptake, should be directly proportional to the quantity of spores and, when corrected for this, should be a measure of their physiological activity, or metabolic capacity. The intercept b is also proportional to spore quantity and should represent the rate of respiration at zero time under the prevailing experimental conditions.

The generality of this equation for application to other situations is not known, although published data of Goddard and Smith (1938) and M. R. Emerson (1954) with *Neurospora* ascospores also show linear increases in rate of respiration. Furthermore, Smith's (1924) measurements show that the rate of linear extension of *Botrytis* hyphae increases proportionally with time after germination until a constant growth rate is attained. Additional comments on the establishment of linear growth during this transitional stage have been presented in Section III, B.

IV. APPLICATIONS

Fungi are used extensively as biological reagents for the commercial production of various substances, such as enzymes, antibiotics, and steroids. They are also used as analytical tools for bioassay of growth factors, fertilizers, fungicides, and antibiotics. The rational use of fungi for these purposes is dependent upon a basic understanding of the phenomenon of growth and its kinetics. Consideration of growth in large fermentations or in microbiological assays is outside the scope of this chapter. The interested reader is referred to reviews by Deindoerfer (1960), Gaden (1955), Hutner *et al.* (1958), Kavanagh (1960), and Maxon (1955, 1960).

V. CONCLUSIONS

In summarizing what we know of the kinetics of fungal growth, it is evident that no generalized treatment is possible. Yeasts, and presumably other fungi growing in a unicellular manner, can grow exponentially. On the other hand, growth of the large, fleshy fungi or of fungal pathogens on plant or animal hosts has not been analyzed and is undoubtedly complex. Between these extremes are the situations in artificial culture where fungi grow in essentially undifferentiated form as filamentous organisms. In these cases we can generalize to the extent that growth is apical and that the rate of apical growth, i.e., linear extension, is constant under uniform conditions. The problem arises in attempting to analyze growth in three dimensions. In some fungi, a cubic relation has been shown to obtain. In others, no meaningful, mathematical relation is apparent. Possibly additional quantitative data with a greater variety of organisms and with the development of a better undertsanding of the variables involved in determining the extent of hyphal branching may reveal more definable growth patterns.

REFERENCES

Brody, S. (1945). "Bioenergetics and Growth, with Special Reference to the Efficiency Complex in Domestic Animals," 1023 pp. Reinhold, New York.

Buchanan, R. E. (1953). Some elementary mathematics of plant growth. *In* "Growth and Differentiation in Plants" (W. E. Loomis, ed.), pp. 101–111. Iowa State Univ. Press, Ames, Iowa.

Burkholder, P. R., and E. W. Sinnott. (1945). Morphogenesis of fungus colonies in submerged shaken cultures. *Am. J. Botany* 32:424–431.

Castle, E. S. (1937). The distribution of velocities of elongation and of twist in the growth zone of *Phycomyces* in relation to spiral growth. *J. Cellular Comp. Physiol.* 9:477–489.

Castle, E. S. (1940). Discontinuous growth of single plant cells measured at short

intervals, and the theory of intussusception. *J. Cellular Comp. Physiol.* **15**:285–298.

Cochrane, V. W. (1958). "Physiology of Fungi," 524 pp. Wiley, New York.

Coultas, M. K., and D. J. Hutchison. (1962). Use of a Biophotometer in growth-curve studies. *J. Bacteriol.* **84**:393–401.

Darby, R. T., and G. R. Mandels. (1954). Inorganic nutrition of *Myrothecium verrucaria*. *Mycologia* **46**:276–288.

Day, D., and A. H. Hervey. (1946). Phycomyces in the assay of thiamine in agar. *Plant Physiol.* **21**:233–236.

Deindoerfer, F. H. (1960). Fermentation kinetics and model processes. *Advan. Appl. Microbiol.* **2**:321–334.

Emerson, M. R. (1954). Some physiological characteristics of ascospore activation in *Neurospora crassa*. *Plant Physiol.* **29**:418–428.

Emerson, S. (1950). The growth phase in *Neurospora* corresponding to the logarithmic phase in unicellular organisms. *J. Bacteriol.* **60**:221–223.

Foster, J. W. (1949). "Chemical Activities of Fungi," 648 pp. Academic Press, New York.

Gaden, E. L. (1955). Fermentation kinetics and productivity. *Chem. & Ind. (London)* pp. 154–159.

Gilbert, F. A. (1960). The submerged culture of *Morchella*. *Mycologia* **52**:201–209.

Goddard, D. R., and P. E. Smith. (1938). Respiratory block in the dormant spores of *Neurospora tetrasperma*. *Plant Physiol.* **13**:241–264.

Herbert, D. (1961). The chemical composition of microorganisms as a function of their environment. *Symp. Soc. Gen. Microbiol.* **11**:391–416.

Hinshelwood, C. N. (1946). "The Chemical Kinetics of the Bacterial Cell," 284 pp. Oxford Univ. Press, London and New York.

Hutner, S. H., A. Cury, and H. Baker. (1958). Microbiological assays. *Anal. Chem.* **30**:849–867.

Kavanagh, F. (1960). A commentary on microbiological assaying. *Advan. Appl. Microbiol.* **2**:65–93.

Kubitschek, H. E. (1960). Electronic measurement of particle size. *Research (London)* **13**:128–135.

Lamanna, C., and M. F. Mallette. (1953). "Basic Bacteriology, its Biological and Chemical Background," 677 pp. Williams & Wilkins, Baltimore, Maryland.

Lu, B. C., J. E. Dawson, and M. Alexander. (1959). A microchemical method for detecting antifungal substances. *Arch. Mikrobiol.* **33**:182–185.

Machlis, L. (1957). Factors affecting the lag phase of growth of the filamentous fungus, *Allomyces macrogynus*. *Am. J. Botany* **44**:113–119.

Mandels, G. R. (1955). Biotin and interrupted growth of *Myrothecium verrucaria*. *Am. J. Botany* **42**:921–929.

Mandels, G. R., and R. T. Darby. (1953). A rapid cell volume assay for fungitoxicity using fungus spores. *J. Bacteriol.* **65**:16–26.

Mandels, G. R., W. H. Stahl, and H. S. Levinson. (1948). Structural changes in wood degraded by the ringworm fungus *Microsporum gypseum* and other microorganisms. *Textile Res. J.* **18**:224–231.

Mandels, G. R., H. S. Levinson, and M. T. Hyatt. (1956). Analysis of respiration during germination and enlargement of spores of *Bacillus megaterium* and of the fungus *Myrothecium verrucaria*. *J. Gen. Physiol.* **39**:301–309.

Marshall, K. C., and M. Alexander. (1960). Growth characteristics of fungi and actinomycetes. *J. Bacteriol.* **80**:412–416.

Maxon, W. D. (1955). Continuous fermentation. A discussion of its principles and applications. *Appl. Microbiol.* 3:110–122.

Maxon, W. D. (1960). Continuous fermentation. *Advan. Appl. Microbiol.* 2:335–349.

Monod, J. (1942). "Recherches sur la croissance des cultures bactériennes," 210 pp. Hermann, Paris.

Needham, J. (1942). "Biochemistry and Morphogenesis," 785 pp. Cambridge Univ. Press, London and New York.

Page, O. T. (1961). Induced variation in *Fusarium oxysporum*. *Can. J. Botany* 39: 1509–1519.

Plomley, N. J. B. (1959). Formation of the colony in the fungus *Chaetomium*. *Australian J. Biol. Sci.* **12**:53–64.

Ryan, F. J., G. W. Beadle, and E. L. Tatum. (1943). The tube method of measuring the growth rate of *Neurospora*. *Am. J. Botany* **30**:784–799.

Smith, J. H. (1923). On the apical growth of fungal hyphae. *Ann. Botany* (*London*) **37**:341–343.

Smith, J. H. (1924). On the early growth rate of the individual fungus hyphae. *New Phytologist* **23**:65–79.

Stadler, D. R. (1952). Chemotropism in *Rhizopus nigricans*: The staling reaction. *J. Cellular Comp. Physiol.* 39:449–474.

Thimann, K. V. (1955). "The Life of Bacteria, their Growth, Metabolism, and Relationships," 775 pp. Macmillan, New York.

Thompson, D'Arcy W. (1948). "On Growth and Form," 1116 pp. Macmillan, New York.

Van Niel, C. B. (1949). The kinetics of growth of microorganisms. *In* "The Chemistry and Physiology of Growth" (A. K. Parpart, ed.), pp. 91–105. Princeton Univ. Press, Princeton, New Jersey.

Woodruff, H. B. (1961). Antibiotic production as an expression of environment. *Symp. Soc. Gen. Microbiol.* **11**:317–342.

Zalokar, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* **46**:602–620.

CHAPTER 26

The Mechanism of Cellular Extension and Branching

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I. THE HYPHA

The fungal hypha is a special type of cell or aggregate of cells. It is superficially similar to the algal filament, but it is possibly of a separate evolutionary origin and it is certainly differently organized. In the algae—although the evidence is not complete—we can visualize the development of a chain of cells by the division of a single cell in one plane and the gradual development of a meristematic function by the apical cell. In the fungi, on the other hand, such evidence as exists from comparative morphology (e.g., the Blastocladiales) suggests that the fungal hypha arose by the elaboration of a single cell and its gradual increase in size and independence. It is also possible that the early hyphae were coenocytic and nonseptate and that septate hyphae are derivative.

In the nonseptate hypha the liquid contents and the included organelles move freely from base to growing apex. Where septa are present a similar movement takes place through the septal pores.

A. Evidence for Extension at the Apex

The tubular hypha extends apically. The evidence for this lies in the recognition that there is no increase in the interseptal distance (Butler, 1958) and that when measurements are made of growing apices, with reference to markers, extension takes place only at the very tip (Smith, 1923). That extension can take place over a very small area of the actual apex can be seen from the various figures showing penetration of surfaces by means of peglike growths (e.g., Boyce, 1948, Figs. 143 and 144) and

also by manipulations, such as those of Robertson (1958), which resu in regrowth by a narrow extension from the very tip of the hypha. It is tru that no one has demonstrated a localization of extension in the subapic region in vegetative hyphae although very clear subapical extension zon have been demonstrated in sporangium-bearing fungi such as *Phycomyc blakesleeanus* and *Thamnidium elegans* (Castle, 1942; Lythgoe, 1961 and Castle (1937) has shown that in vegetative hyphae of *Phycomyce* although the extreme tip is the main region of growth in area of the prima wall, extension takes place over a larger part of the apex, decreasing amount farther away from the tip.

B. Nature of the Wall at the Apex

Mycologists have always had difficulty in visualizing the way in whi fungal hyphae mold and shape themselves at the tip, where accretion the structural material is taking place. We clearly need electron microsco evidence for the wall structure at the apex, but there are certain aspe we can ascertain by a consideration of the evidence before us. If the hyp consists of a rigid tube with viscous contents that emerge from the end the tube, we should expect the viscous material to assume a spherical s face and the hyphal apex to be rounded.

In fact, while the actual hypha does approximate this shape (Fig. 1A reference to photographs and drawings (Middlebrook and Preston, 19 Robertson, 1958; Zalokar, 1959) shows a narrowing of the hyphal ap (Fig. 1B), which suggests that the hyphal apex cannot be considered a simple tube with viscous contents. This is made more plain when considers that the system shows turgor pressure equivalent to 6–7 atm pheres (atm), e.g., in *Neurospora*. When the apex is placed in water, turgor increases and the apex is seen to stretch and eventually to burst very rarely shows the spherical deformation that we might expect if it w entirely viscous. Instead we find that the expanded apex assumes a diam shape (see Robertson, 1958, for figures).

We must assume, therefore, that this apical region which contains a eous cytoplasm is bordered by a wall of such structure that an increas internal pressure causes little stretching or obvious deformation of extreme apex, but does cause substantial irreversible stretching in the apical shoulder. The simplest explanation of such behavior would found in a model of wall formation such as that postulated by Pre (1952) for the alga *Valonia*, where a tight spiral of wall material over apex, and presumably continually generated from within, may be i preted as being slowly uncoiled backward by the extension of the a and helices, as they become progressively further apart, are crosse

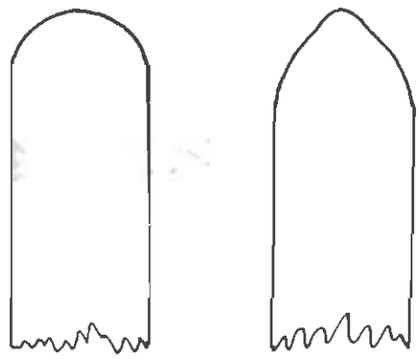

FIG. 1. Hyphal tips. For explanation see text.

meridians also spirally arranged. There is also evidence (Rizvi, 1964) that the older parts of the hypha in, for example, *Neurospora* are stretched by turgor pressure, but this is an elastic (reversible) stretching.

C. Loss of Plasticity of the Wall behind the Apex

There is evidence that the wall near the apex is initially sufficiently plastic to grow and be shaped, but that it becomes modified behind and is no longer plastic. This evidence is provided by Robertson (1958) using the apexes of *Fusarium oxysporum*. When colonies of *F. oxysporum* are flooded with water the hyphal apexes cease to elongate and begin to swell. About half the apexes grow on from an initially narrowed apex after ca. 40 seconds, but the others continue to swell and eventually grow on by subapical branches. When colonies are transferred to water for less than 40 seconds and then to a nontoxic solution of about 3 atm (a so-called balancing solution), the hyphal apices which would normally grow on do not do so and eventually branch subapically. This situation is illustrated in Fig. 2, and the data from which the hypothesis is derived are given in Table I. We are now able to see that a change in wall structure is taking place behind the hyphal apex such that a plastic cell wall structure is laid down which becomes changed and loses its plasticity as it moves away from the apex. An analogous change between plastic and nonplastic states has been postulated by Nickerson *et al.* (1956), who were commenting on the change from yeast-like to mycelium-like states in *Candida albicans* and other fungi. They were able to show experimentally that selenium added to the medium converted certain strains from the mycelial to the yeast form. They sug-

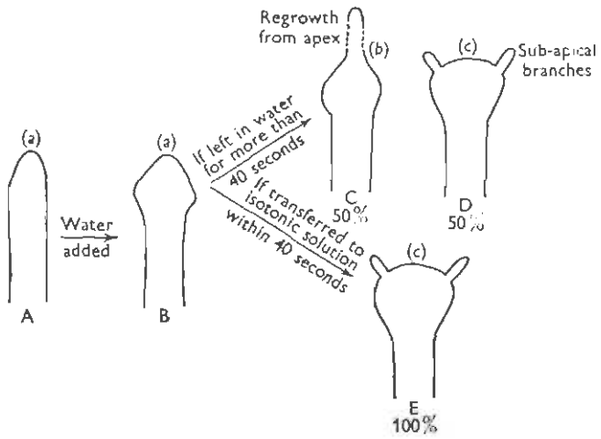

FIG. 2. Diagrammatic explanation of behavior of hyphal apexes of *Fusarium oxysporum* in water and isotonic solutions. (A) Normal hypha. (B) Hypha showing arrestment and beginning of swelling. (C) Hypha showing regrowth from the apex after immersion in water. (D) Hypha which has swelled and branched at the apex after arrestment in water. (E) Hypha which in water would have behaved as (C) above behaves as (D) above when transferred from water to an isotonic solution within 40 seconds of first immersion. It is suggested that the new equilibration following transfer maintains arrestment past the point at which the apex becomes incapable of regrowth. *a*, Undifferentiated apex; *b*, narrowing of hypha due to some wall differentiation; *c*, differentiated apex. Reproduced from Robertson (1959).

TABLE I

EFFECT OF IMMERSION OF HYPHAL APICES IN DISTILLED WATER, FOR VARYING PERIODS, FOLLOWED BY IMMERSION IN 0.076 M KCl[a]

Time in distilled water	Number of apices		
	Unbranched	With one branch	With two branches
5 sec	7.2	6.2	16.6
10 sec	5.4	3.2	22.4
20 sec	6.8	2.8	20.4
40 sec	10.4	1.2	18.4
60 sec	19.4	0.8	9.8
80 sec	19.6	1.2	9.2
20 min	18.8	0.6	10.6

[a] From Robertson (1958).

[b] Values are means of five observations, of samples of thirty hyphal apexes each, on separate plates.

gest that this change could be induced by the substitution of selenium for sulfur and the failure to form sulfhydryl bonds, which, they suggest, are responsible for the rigidity of the tubular wall. Such a theory has obvious implications for the assumption of rigidity at the hyphal subapex which is postulated above, but as yet there is no chemical or electron microscopic evidence to help in the elucidation of the problem.

Indeed, Zalokar (1953) has shown no morphological effect of sodium selenite on *Neurospora crassa*, so that either selenium is ineffective or the sulfhydryl links are not of universal importance. Bartnicki-Garcia and Nickerson (1962a,b) have shown that changes in other environmental factors can bring about the conversion of *Mucor rouxii* from mycelial to yeast-like morphology. These transformations are reviewed in greater detail in Volume II of this treatise.

D. Branch Initiation

The hyphal apex is not so organized that an increase in its size leads to a simple partition of materials and the development of dichotomy, and this is further evidence for the unique nature of the apex. In fact dichotomy can be produced experimentally by subapical branching, but usually it is not an exactly equal dichotomy and there is evidence to suggest that one branch is formed before the other. There is also evidence that the branches form by the resumption of plasticity in the already hardened wall, at the point where the new branch originates. In *Fusarium oxysporum* this resumption of plasticity and initiation of branching occupies about 7 minutes.

The hyphal apex is a unique structure which is, at least theoretically, potentially immortal. This apex is fed from behind—how far absorption takes place at the actual apex is difficult to say. This single tube has, however, the capacity to duplicate itself by branching and colony formation. The branching takes place near the apex in acropetal succession and is independent of the septation of the hyphae because branches are produced by nonseptate fungi and by the nonseptate apical portions of *Neurospora*, for example. The converse may also be true, however, for there are cases where the branching is marked by the proximity of a septum and a clamp connection (Butler, 1958, 1961). The simplest picture is of a main hypha with a series of branches borne alternately and in the form of a two-dimensional Christmas tree.

II. THE PATTERN OF HYPHAL GROWTH

Such a picture implies a marked apical dominance, and if it were maintained it would make impossible colony formation as we know it. In-

oculation at a point shows that leading hyphae quickly radiate from the point and in turn develop the acropetal series of branches we have already mentioned. However, as the leading hyphae diverge from one another the apical dominance appears to become weaker and a branch escapes and grows on to become a leading hypha to fill in the margin of the colony. So marked is this phenomenon that any shape of inoculum in which the main axes are approximately equal in size will develop a colony with a circular margin when grown in an unrestricted space. This is an artificial concept because in liquid culture the colony would theoretically become spherical. The problem of the filling-up behavior by fungi was first commented on by Ryan *et al.* (1943). They noted that when *Neurospora* was growing in growth tubes there was a regular array of more or less parallel hyphal apices. This array has a more or less sharply defined outer margin at right angles to the direction of growth of the colony, and Ryan *et al.* suggested that when a hypha grows ahead of the colony branching becomes possible, and on branching the apex slows down. So far there has been no experimental confirmation of these findings, and such detailed observations as have been made in other fungi (Butler, 1961) do not support this hypothesis.

There is a variety of hyphal patterns in colonies growing on agar, and these patterns are modified by the nutritional state of the agar on which the colonies are growing, but they are similar in that in each is a leading hypha from which a series of branches are given off. In certain fungi (e.g., *Neurospora*) it is possible to trace some hyphae from the point of inoculation right to the edge of the colony. Other hyphae of equal thickness arise from these to fill the circumference. In other fungi, such as *Pyronema omphalodes,* main hyphae seem to stop growth and a subterminal hypha grows from below the apex. Yet again, in *Fusarium oxysporum* and other species of *Fusarium,* the leading hyphae on occasion spontaneously divide dichotomously, in a way similar to the experimental branching demonstrated by Robertson (1958).

The main hypha and its series of branches form a system on agar, and observation of hyphal wefts in nature—on dung, on rotted wood surfaces and in the litter layer of pinewoods—suggests that a similar pattern of branching obtains to that found on agar. Butler (1957) made some of her observations on *Merulius lacrymans* [*Serpula lacrimans*] on glass slides in a humid atmosphere. It thus seems fairly clear that the system of branching often is independent of the substrate.

The hierarchical nature of the branching system can be generally observed, but it has been most thoroughly studied by Butler in a series of papers which contain a wealth of detailed observation. The system was established for *S. lacrimans,* which can be shown to have a very regular

branching system with a main hypha, primary branches, secondary branches, and tertiary branches. This system is to be found also in *Coprinus disseminatus,* and the growth measurements establish that the branches of each order grow at a slower rate than the order next above. No evidence was found for any changes in growth rate of main hypha or branches with increase in length, although the distances on which the measurements were made were small and branches accelerating to catch up with the main hypha were not observed. The evidence was clear that main hyphae and such side branches as were not accelerating maintained a more or less steady state and did not show a falling off in growth rate even where, as in this case the greater part of the food supply was being transferred from a food base through hyphae and independently of the nutrients in the agar base.

III. THE PROBLEM OF APICAL DOMINANCE

This is an intriguing situation. The vigor of the main hypha and the reduced vigor of the primary and secondary branches can be likened to the branch system of a coniferous tree where a hormone-controlled apical dominance is postulated. What is the system in the fungi? It is clear that the amount and pattern of branching may be affected by external nutrient supply (see below), but since the pattern of hyphal branching is well marked in fungal aerial hyphae and in hyphae remote from a nutrient substrate, the problem can best be considered experimentally in relation to those fungi in which the nutrients are transported to the growing apices through the hyphae. With that proviso there are four possibilities: (1) The dominance could be controlled by nutritional relationships between the main apex and the side branches. This could only hold if the passage of elaborated materials is general through the hyphae and some material is limiting. (2) The dominance could be controlled by internal hormones. (3) The dominance could be controlled by the secretion of a liquid toxin by the fungus. The only parts of the fungus which would not be growing in this toxin would be the main hyphal apices. (4) The dominance could be controlled by a gaseous toxin which would act similarly to the aqueous toxin.

1. The dominance could be controlled by nutritional relationships between the main apex and the side branches. The simplest situation for analysis would be where the nutrients were all transported through the hyphae to the growing apex from a food base some distance behind the advancing edge of the colony. This situation obtains in the experiment of Butler (1961) where *Coprinus disseminatus* was used. In such situations the competition for nutrients would depend upon the relative positions of the apex and the side branches, and it might relate to the cross-sectional

area of the hyphae. Butler has shown that the primary branch hyphae are narrower than the main hyphae in *C. disseminatus* and that there is a significant correlation between extension rate and hyphal diameter. This might at first sight appear to be the beginning of the answer to the problem of the hyphal hierarchy except that the extension rate for equally wide hyphae was significantly higher for main as compared with branch hyphae, suggesting that the difference between main and branch hyphae is not simply one of diameter. There is, of course, no evidence that diameter alone would directly control the flow of nutrients at diameters such as these we are dealing with. Butler gives figures that allow us to calculate the cross-sectional area and to compare the ratio of extension rate : cross-sectional area for main and primary branches, and the correspondence of the ratios is good. The nub of this problem lies in the mechanism for uptake and transport in these fungi, and without more details about this we have difficulty in understanding the nature of the competition which may exist. It is difficult to see how, if we are dealing with a branching system where all the hyphae are of equal diameter, competition would act at all or not act to the detriment of the main apex. Where diameter differences exist we can begin to see—allowing that there is a simple relationship between cross-sectional area and the movement of nutrients—how competition could control the dominance pattern. We are left with the question of how the diameters of the main and branch hyphae are themselves determined. It may be that the initial openings in the hyphal wall are nutritionally determined, but it is more likely that they are determined by the physical properties of the softened wall under hydrostatic pressure and the final diameter is the result of an adjustment by the growing apex. It can be seen clearly that where a branch hypha first emerges there is a constriction (*Fusarium:* personal observations, 1958, and Zalokar, 1959, p. 604, Figs. 1–5). It is possible that there is a physical relationship between the main hypha, the primary branch, and the secondary branch such that the branch initials each must be smaller than the category above. But this precludes the situation where the hyphae are known to adjust in diameter after their initiation. The initial opening from one hypha to another cannot really be important if we consider that the septal pore itself has apparently no limiting effect on fungal growth.

2. Dominance relationships could be controlled by an internal hormone, or hormones, produced apically and inhibiting the growth of side branches. There is no evidence for this whatsoever except the analogy with the auxin-gibberellin system in higher plants. It is clear that hormones are produced by fungi—auxins or auxin-like substances are isolated from fungal cultures—and the gibberellins were identified first in fungi, but so far none have been shown to be active inside the fungal hypha. It is doubtful, however, if the

appropriate experiments have ever been done, for the most searching examination of the action of the auxins or auxin-like substances on fungi has been made by Banbury, who has largely been concerned with the relation between external concentrations of the appropriate substances and the degree of curvature of the sporangiophore of *Phycomyces*. In fact none of the substances tried was effective. A discussion of other recent work on fungal growth substances is given in Chapter 19 of this volume.

3. Dominance relationships could be maintained on the agar plate by the secretion into the substrate of a soluble toxin or a substance which affects the growth rate. This material, if produced at equal rates by young growing hyphae, would occur in a concentration such that the later-formed branches grow more slowly than the leading hyphae. This possibility might be stretched to include hyphae that are growing on any moist substances, but it would not be applicable to the situation where fungal hyphae grow and ramify with apparent coordination in a humid atmosphere, as for example in the litter layer of a pinewood.

4. Dominance relationships could be obtained through the production of a gaseous substance which at high concentrations brought about a slowing down of growth. Again there is no evidence to relate a gaseous inhibition to the morphogenetic pattern of fungal hyphae, but it is known that spore germination of *Agaricus* and fruiting of *Rhizopus*, can be affected by gases produced by fungi (McTeague *et al.*, 1959; Hepden and Hawker, 1961).

IV. BRANCHING

A. Direction of Branching

Fungal hyphae appear to grow in straight lines, and, although some show a slight curvature (e.g., *Pyronema omphalodes* and *Ascobolus immersus*, mutant "vague"; Chevaugeon, 1959), they never grow back into the colony and the branches come out at a more or less acute angle to the main hypha and in a forward-pointing direction. In fact close observation shows that hyphal branches appear almost at right angles to the hypha and pointing slightly forward on initiation and that they never grow backward except under unusual experimental conditions. But there is one case, the clamp connection, where the branch on initiation grows backward, and we know that this is genetically controlled.

B. The Internal Factor in Branching

We know that the nutrition of the fungus profoundly affects the density of the colony, which is in turn a reflection of the branching pattern. But

we are lacking detailed analyses of the effect of nutrition on branching and on the spacing of branches. We have information from a thesis of Grover (1961) and from Larpent (1962) that in certain fungi, nutrition has a modifying effect on the pattern of apical dominance. How this is effected is not clear, but two pieces of evidence can be adduced to show that there is an internal control of branching which can work in some instances with extreme precision. The first evidence is that of Chevaugeon, who suggests that it is an internal factor passing from the older mycelium to the younger that determines the zones of little and of much branching in the mutant "vague" of *Ascobolus immersus*. The second is that when dichotomous branching has been induced in *Neurospora* by appropriate manipulation (Robertson, 1959), if a side branch is initiated on one of the dichotomous branches there is always a mirror image produced at precisely the same time and at an equal distance along the other dichotomous branch. With continued growth this synchrony is lost.

V. SCOPE FOR FUTURE WORK

Enough has been said to indicate the great scope for future work in this field. The nutritional aspects of the problem have not advanced far enough for worthwhile review and the genetical aspects, the study of which is increasing, are in need of some simple basic interpretations of the hyphal apex and branches for further advance. It is hoped that this chapter may make a beginning in this direction.

REFERENCES

Bartnicki-Garcia, S., and W. J. Nickerson. (1962a). Induction of yeast-like development in *Mucor* by carbon dioxide. *J. Bacteriol.* 84:829–840.

Bartnicki-Garcia, S., and W. J. Nickerson. (1962b). Nutrition, growth and morphogenesis of *Mucor rouxii*. *J. Bacteriol.* 84:841–858.

Boyce, J. S. (1948). "Forest Pathology," 2nd ed., 550 pp. McGraw-Hill, New York.

Butler, G. M. (1957). The development and behaviour of mycelial strands in *Merulius lacrymans* (Wulf.)Fr. I. Strand development during growth from a food base through a non-nutritive medium. *Ann. Botany (London)* [N.S.] 21:523–537.

Butler, G. M. (1958). The development and behaviour of mycelial strands in *Merulius lacrymans* (Wulf.) Fr. II. Hyphal behaviour during strand formation. *Ann. Botany (London)* [N.S.] 22:219–236.

Butler, G. M. (1961). Growth of hyphal branching systems in *Coprinus disseminatus*. *Ann. Botany (London)* [N.S.] 25:341–352.

Castle, E. S. (1937). The distribution of velocities of elongation and of twist in the growth zone of *Phycomyces* in relation to spiral growth. *J. Cellular Comp. Physiol.* 9:477–489.

Castle, E. S. (1942). Spiral growth and reversal of spiralling in *Phycomyces* and their bearing on primary wall structure. *Am. J. Botany* 29:664–672.

Chevaugeon, J. (1959). Sur le déterminisme interne du rythme de croissance chez un mutant "vague" de l'*Ascobolus immersus*. *Compt. Rend.* **248**:1841–1844.

Grover, S. (1961). An experimental study on the growth of fungal colonies in relation to hyphal behavior. Ph.D. Thesis, Univ. of London.

Hepden, P. M., and L. E. Hawker. (1961). A volatile substance controlling early stages of zygospore formation in *Rhizopus sexualis*. *J. Gen. Microbiol.* **24**:155–164.

Larpent, J. P. (1962). La notion de dominance apical chez *Rhizoctonia solani* Kuhn. *Compt. Rend.* **264**:1137–1139.

Lythgoe, J. N. (1961). Effect of light and temperature on growth and development in *Thamnidium elegans* Link. *Brit. Mycol. Soc. Trans.* **44**:199–213.

McTeague, D. M., S. A. Hutchinson, and R. I. Reed. (1959). Spore germination in *Agaricus campestris* L. ex. Fr. *Nature* **183**:1736, 1959.

Middlebrook, M. J., and R. D. Preston. (1952). Spiral growth and spiral structure III. *Biochim. Biophys. Acta* **9**:32–48.

Nickerson, W. J., W. A. Taber, and G. Falcone. (1956). Physiological basis of morphogenesis in fungi V. Effect of selenite and tellurite on cellular division of yeast-like fungi. *Can. J. Microbiol.* **2**:575.

Preston, R. D. (1952). "The Molecular Architecture of Plant Cell Walls," 211 pp. Chapman & Hall, London.

Rizvi, S. R. H. (1964). Morphological and physiological studies on the hyphae of *Neurospora crassa* and other fungi. Ph.D. Thesis. University of Hull.

Robertson, N. F. (1958). Observations of the effect of water on the hyphal apices of *Fusarium oxysporum*. *Ann. Botany (London)* [N.S] **22**:159–173.

Robertson, N. F. (1958). Personal observation.

Robertson, N. F. (1959). Experimental control of hyphal branching and branch form in hyphomycetous fungi. *J. Linnean Soc. London, Botany* **56**:207.

Ryan, F. J., G. W. Beadle, and E. L. Tatum. (1943). The tube method of measuring the growth of *Neurospora*. *Am. J. Botany* **30**:784–799.

Smith, J. H. (1923). On the apical growth of fungal hyphae. *Ann. Botany (London)* **37**:341–343.

Załokar, M. (1953). Reduction of selenite by *Neurospora*. *Arch. Biochem. Biophys.* **44**:330–337.

Zalokar, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Botany* **46**:602–610.

CHAPTER 27

Growth Rhythms

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I. INTRODUCTION

That rhythms, which affect a large variety of processes, are widely distributed in plants and animals is now accepted. But, according to Aschoff (1960), if certain of these rhythms manifest themselves only when external physical conditions fluctuate periodically (exogenous rhythms), many others persist under uniform environmental conditions, thereby demonstrating the functioning of a periodic internal mechanism (endogenous rhythms).

Although endogenous cycles of very different periods and character have been reported (Baillaud, 1957; Cloudsley-Thompson, 1961; Jerebzoff, 1961b), most of the work in progress bears on the endogenous rhythms in which the period has, or can acquire, a value equal to that of periodic changes in the natural environment, including daily, tidal, semilunar, lunar, and annual ones (F. A. Brown, 1960; Bünning, 1958; Fingerman, 1957, 1960; Harker, 1958; Hauenschild, 1960; Menzel, 1962). This is due largely to the possible role of "clocks" and "internal calendars" in the survival of species and to the contributions that their study may make to experimental ecology and to medicine.

Actually the function of these rhythms is poorly known. The very large duration of some periods, approaching even a year, and their relative independence of temperature cannot be explained by the classical concepts of biochemical reactions. Up to now no substances are known that are capable of inducing the manifestation of an internal rhythm as an approach to the study of causal mechanisms. Finally, the multiplicity of processes which can show the same rhythm even within the same organism (Bünsow, 1960; Glick *et al.*, 1961; Halberg, 1960; Pirson *et al.*, 1954; Richter and Pirson, 1957; Sweeney, 1960), leads to conjecture about the fundamental mecha-

nism which can only be hypothetical at present. What that is new in this field has been, or can now be, adduced from studies of fungi?

Among the fungi, different processes show rhythms, which often are endogenous, such as protoplasmic streaming in the plasmodium of *Physarum polycephalum* (Kamiya *et al.*, 1957), the discharge of ascospores in *Daldinia concentrica* (Ingold and Cox, 1955) or of sporangia in *Pilobolus* (Schmidle, 1951; Uebelmesser, 1954), and even the intensity of luminescence in certain basidiomycetes (Berliner, 1961). However, the most common periodicity involves the development on solid media of a series of regularly spaced concentric circles of growth, which are called zonations. These zonations can affect a single process as, for example, the thickness of the mycelium of *Ascochyta chrysanthemi* (Stevens and Hall, 1909), or the distribution of spores of *Penicillium* and *Aspergillus* (Gallemaerts, 1911; Hedgecock, 1906; Munk, 1912), and pycnidia of *Ascochyta rabiei* (Hafiz, 1951). But more often, several phenomena are involved, such as sporulation and pigmentation in *Trichoderma viride* (Milburn, 1904), sporulation and thickness of the mycelium in *Sclerotinia fructicola* (Hall, 1933; Sagromsky, 1959a), and pigmentation and formation of fertile and sterile aerial hyphae in *Alternaria tenuis* (Jerebzoff, 1961b). Thus, two types of hyphal growth can be observed, one characterized by monopodial ramification, and the other by the formation of a cyme, as in the cases of *Sordaria fimicola* (Hawker, 1950), *Ascobolus immersus* (Chevaugeon, 1959b), and other organisms.

We will examine successively the responses of organisms with rhythms that are exclusively exogenous, followed by those that have endogenous zonation rhythms, to the action of different physical conditions, and as a function of the composition of the nutrient medium. The genetics of zonations will then be considered.

II. EXOGENOUS RHYTHMS

No zonation rhythm has been detected among the fungi under uniform external physical conditions. Zonations are induced by fluctuations of light or temperature and are stopped when uniform conditions are reestablished.

A. Action of Light and Temperature

Numerous fungi show zonation rhythms under the stimulus of different photoperiods (Jerebzoff, 1961b). Daily illumination of 1000–3000 lux for several seconds suffices to induce zonations in *Fusarium discolor sulfureum* (Bisby, 1925), *Trichothecium roseum* (Sagromsky, 1959a), and *Verticillium lateritium* (Isaac and Abraham, 1959). In spite of this great sen-

sitivity, the maintenance of the above amount of illumination for 12 hours to a day is not sufficient to induce an endogenous rhythm after the cultures have been returned to darkness. The duration of the dark period that is needed to make a photoperiod effective varies greatly. For example, 9 hours is needed for *V. lateritium* and 12 hours for *F. discolor sulfureum,* whereas *Botrytis gladiolorum* forms a daily zonation if the dark period is only 1 hour (Bjornsson, 1959). In contrast to the three previous cases, continuous light inhibits sporulation and a dark period of 1.5 hours suffices to reestablish it whereas 7 hours of darkness induces optimum zoning in *Peronospora tabacina* (Cruickshank, 1963). By way of comparison, it is interesting to note that the endogenous daily rhythm of higher plants, like that of *Phaseolus,* is inhibited by continuous illumination, but reappears after a minimum dark period of 8–9 hours (Wasserman, 1959).

Furthermore, in *Penicillium,* alternations of strong and weak light, as well as of light and dark, are capable of evoking zonations (Sagromsky, 1952a). In addition, when light accelerates fruiting of *Fusarium fructigenum,* 10 minutes of light each day provokes the release of spores uniformly over the surface of the cultures, whereas 30 minutes are needed to localize them in a certain place (Hall, 1933).

Also, although the action of light in the formation of zonations may sometimes be due to the inhibition of spore formation, in general fluctuating light probably acts as an excitation having an effect upon vegetative growth which favors fructification by the excited hyphae (Jerebzoff, 1961b). Moreover, a dark period of at least 1–12 hours also may be necessary for the induction of a zonation.

Finally, a comparison of action spectra reveals that whereas blue light is always effective, green, yellow, and red may be either important (Gallemaerts, 1911; Munk, 1912), weekly active (Cruickshank, 1963; Isaac and Abraham, 1959), inactive (Hedgecock, 1906; Sagromsky, 1956), or of doubtful significance, as in the case of *Trichothecium roseum* (Hedgecock, 1906; Munk, 1912). It should be noted that Sagromsky (1956) has been able to sensitize a *Penicillium* to the action of light, and to render a race of *T. roseum* responsive to red light, by adding methylene blue to malt agar.

As far as the action of temperature is concerned, experimenters are limited, in general, to the use of thermocycles and thermoperiods of long duration. However, some fungi are very sensitive to temperature, such as *Penicillium* (Sect. Asymmetrica) which forms zonations in the dark if abrupt variations of 1°C in temperature are applied (Sagromsky, 1952b). Also, zonations appear in cultures of *Pleospora herbarum* which are transferred for 1 hour to a temperature differing by 3°C from the one at which they were growing (Ellis, 1931). Moreover, it appears quite often that the appearance

of zonations can be connected with transfer from high to low temperature in *Ascochyta rabiei* (Hafiz, 1951); *T. roseum* (Sagromsky, 1956); *Fusarium fructigenum* (Hall, 1933). In *P. herbarum* not only is transfer from high to low temperature effective, but the reciprocal also works. Thus, variations in temperature have similar effects upon zonation as variations in light but are a weaker form of physical excitation (Ellis, 1931; Hafiz, 1951; Sagromsky, 1952a).

Finally, it is worth noting that certain fungi appear to respond only to a single type of stimulation, such as light in *Penicillium luteum* (Knischewsky, 1909) and heat in *Pleospora herbarum* and *Ascochyta rabiei*.

B. Role of the Culture Medium

First, it appears that substrates ought to satisfy a certain number of conditions, independently of their composition: they ought to be fairly rich and not permit too much thickening of the mycelium, in order to avoid the coalescence of the zonations; all conditions that might disturb growth, and prevent the mycelium that is sensitive to excitation from being in an even physiological state, ought to be avoided; the pH ought to be acid (W. Brown, 1925; Hafiz, 1951; Hall, 1933; Sagromsky, 1956).

Most frequently, complex media are employed, including extracts of potato (Bjornsson, 1959; W. Brown, 1925; Hafiz, 1951), malt (Sagromsky, 1952a; 1959a), prune (Gallemaerts, 1911), or beef (Bisby, 1925; Hafiz, 1951). When defined media have been used, the content of phosphate has been shown to have a marked effect on the zonations, as in *Ascochyta rabiei* and in *Fusarium*.

III. ENDOGENOUS RHYTHMS

A. Action of Light and Temperature

All the fungi mentioned below show endogenous zonation rhythms under certain conditions, but their responses to physical excitations are very different. On this basis, three principal groups can be recognized:

Group 1. Certain fungi are unresponsive to light or heat; the zonations, which are due to an endogenous rhythm, are a function solely of the nutritional environment. Included in this group are *Ascochyta chrysanthemi* (Stevens and Hall, 1909), and certain mutants of *Podospora anserina, Ascobolus immersus,* and *Pestalotia annulata* (Chevaugeon, 1959b; Nguyen, 1962; Tavlitzki, 1954).

Group 2. In this group, physical excitations induce exogenous zonation rhythms. *Alternaria tenuis* (Gallemaerts, 1911) and *Trichoderma viride*

FIG. 1. Repeated induction of endogenous zonation rhythm in *Sclerotinia fructicola*. Cultures maintained in the dark for 2 days received a first photoperiod of 12 hours at 250 lux, and a second after 5 days. After each stimulus, four zonations of decreasing intensity were found, one every 24 hours.

respond thus to treatment with photocycles, and *Aspergillus ochraceus* and *A. niger* are sensitive also to the action of thermocycles (Munk, 1912).

Group 3. In this group, weak physical excitations induce an exogenous rhythm while strong excitations provoke an endogenous zonation rhythm which is continued for some time after the cessation of the stimulus. The endogenous rhythm induced by a single illumination in *Sclerotinia fructigena, S. laxa* (Hall, 1933), and *Leptosphaeria michotii* (Jerebzoff and Lacoste, 1962) has a period of approximately 24 hours. In the case of *S. fructicola* cultures illuminated once with intense enough white light, or with wavelengths < 500 mμ, and placed in the dark or in very weak light, show a 24-hour rhythm for 3 days (Fig. 1). This same rhythm also is released in cultures held in the dark by simply altering the temperature $\geqslant 2°$ C (Jerebzoff, 1961b). In mutant no. 21,863 of *Neurospora crassa,* it is the transfer from continuous light to darkness that will induce an endogenous rhythm (Brandt, 1953; Pittendrigh *et al.,* 1959).

B. Role of the Culture Medium

First of all, it should be noted that the general remarks concerning the nutritional environment which favors the appearance of zonations in organisms having exogenous rhythms apply to those having endogenous rhythms.

Induction of endogenous rhythms by physical excitations can take place in very diverse natural or defined media in *Sclerotinia* (Hall, 1933; Jerebzoff, 1961b), and *Leptosphaeria michotii* (Jerebzoff and Lacoste, 1962). As for those rhythms which can be manifested without any physical induction, little or nothing has been determined as yet about the nutritional conditions which favor such rhythms in *Trichoderma viride* (Milburn, 1904), *Ascochyta chrysanthemi* (Stevens and Hall, 1909), and *Pestalotia annulata* (Chevaugeon, 1959b).

Some other fungi, like *L. michotii* appear to have weak nutritional deficiencies. *Podospora anserina* is a somewhat special case for, on a simple synthetic medium, the zonations appear at a pH $\geqslant 6$ if manganese is absent and at a pH $\leqslant 6$ if it is present (Tavlitzki, 1954).

Finally, an endogenous rhythm has been observed in the dark and at 23°C in *S. fructicola* if Difco yeast extract is added to a complex, or entirely synthetic medium, and in *Alternaria tenuis* grown on malt extract agar (Jerebzoff, 1958, 1960, 1961b).

1. Factors in Yeast Extract

The fraction in yeast extract that is active on *S. fructicola* is that retained on Dowex 50, which is rich in amino acids (Fig. 2). It has been possible

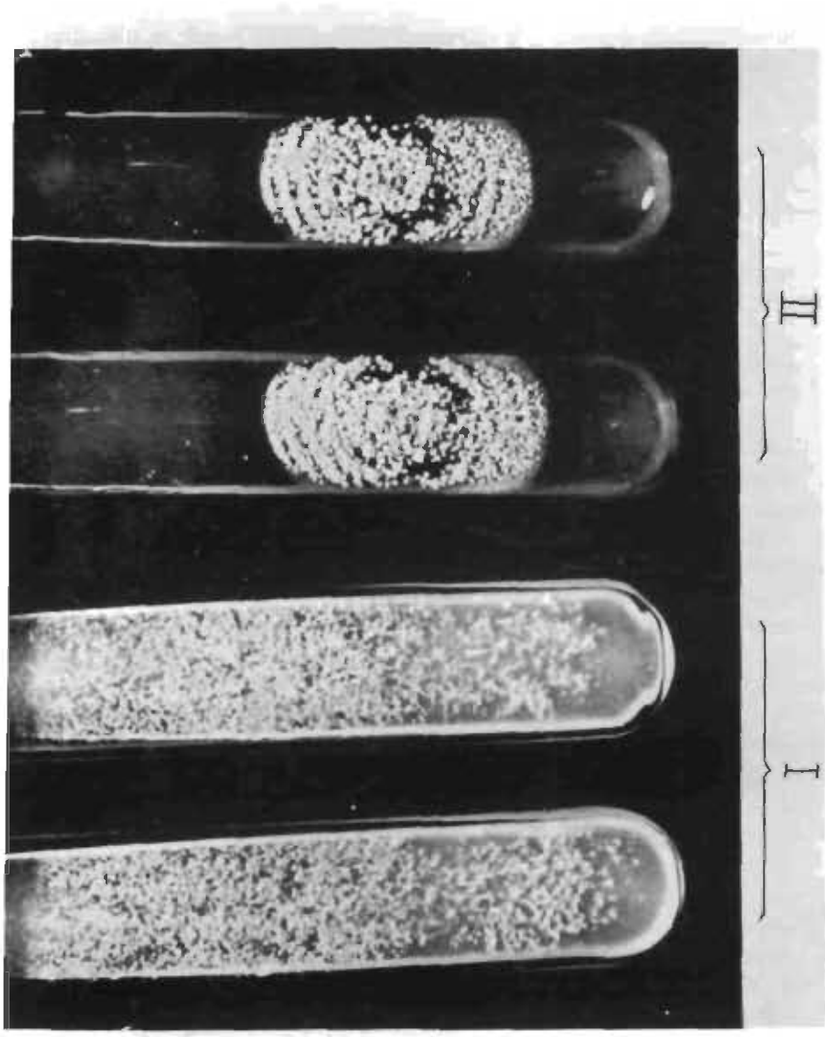

FIG. 2. Appearance of *Sclerotinia fructicola* under uniform conditions in the dark at 23°C. (I) Grown on a synthetic medium. (II) Grown on the same medium to which the "basic" fraction of yeast extract was added.

to substitute a combination of 14 amino acids, including aspartate, glutamate, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine, in the same proportions that they are contained in yeast extract. Nevertheless, the results of these experiments are somewhat variable.

This same fraction from the column, as well as the combination of 14 amino acids, when added to a synthetic medium, induces zonations in a repeatable way in another organism, *Aspergillus ochraceus* (Jerebzoff, 1961b).

When *A. niger* is cultivated in the presence of ammonium tartrate, ammonium nitrate, monopotassium phosphate, magnesium sulfate, and glucose, only the addition of isoleucine in concentrations between 0.1 and 2×10^{-3} *M* is needed to induce an endogenous zonation rhythm in the dark at 23°C. Leucine, norleucine, valine, norvaline, and threonine have proved to be incapable of duplicating this effect (Jerebzoff, 1963).

Thus, in three instances the addition of amino acids found in yeast extract suffices to induce an endogenous zonation rhythm under uniform external physical conditions. In the case of *A. niger*, a single factor is responsible, namely, isoleucine.

In addition, a mutant of *Neurospora crassa* forms zonations in the dark on potato-dextrose agar or, better still, on Gray's medium, containing Difco yeast extract (Brandt, 1953). These data suggest that the persistent rhythm described above could be due to the presence in the two media of factors that are analogous to those which are necessary for *S. fructicola* and the two aspergilli.

2. *Action of Malt Extract*

Alternaria tenuis grows uniformly on potato extract but shows zonation if an extract of crude malt, or a "nonionic" fraction therefrom, which is rich in glucose, mannose, sucrose, and especially maltose, is added. Although it is assimilated, lactose, an isomer of maltose, is almost inactive. At first sight it could be supposed that substances contained in potato extract can serve as cofactors that are necessary for the rhythm in *A. tenuis*. In fact, it appeared subsequently, that if, in the dark at 23°C, the fungus is grown upon an agar medium containing, for example, KNO_3, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and maltose, the manifestation of the rhythm is a function of the relative concentrations of nitrate, phosphate, and maltose. Changing the pH of the medium from 5.5 to 6.2 or 7.2–7.4, does not affect the reaction (Jerebzoff, 1962).

Therefore, the appearance of zonations in *A. tenuis* is not determined by the addition of special substances from potato extract, but is dependent upon the equilibrium between common nutritional factors, while being a function of the kind of sugar furnished (present in favorable concentrations in malt extract).

It is to be noted that an endogenous rhythm induced by nutritional factors under uniform physical conditions persists a long time. Thus, it continues for 3 weeks in *S. fructicola,* 1 month in *Aspergillus ochraceus* and *A. niger,* and 70 days in *Alternaria tenuis* without attenuating (Jerebzoff, 1961b, 1963).

C. Characteristics of Endogenous Zonation Rhythms

In describing research on the functioning of endogenous rhythms of a fairly long period, a zonation rhythm can be characterized by the: (1) conditions under which it can be manifested, such as we have just examined; (2) duration of its period, which is a function of the temperature, illumination, and the nutrient medium, under uniform conditions and, also may be affected by different photo- or thermocycles; (3) number, relative importance and sensitivity of the phases of the period under the influence of different physical or chemical treatments. This last question, although fully explored in a variety of endogenous plant and animal rhythms, to my knowledge, has not yet been approached in fungi showing zonation rhythms.

1. Duration of the Period under Uniform External Physical Conditions[1]

In *Neurospora crassa* the period is about 22 hours at 24° and 31°C [2] in the dark or in red light, but the rhythm disappears in continuous white light, or in very weak blue light (Brandt, 1953; Pittendrigh *et al.,* 1959).

[1] Also called the innate period, which is equivalent to the term "free-running period" proposed by Pittendrigh (1960).

[2] When the rate of a reaction is an exponential function of the temperature, the Q_{10}, or temperature coefficient, is the ratio of the rates (v and v') at which the phenomenon takes place at the two temperatures, θ and $\theta + 10$°C. According to the Vant'Hoff equation, where a is a constant, if $\log v = \theta \log a$, and $\log v' = (\theta + 10) \log a$, then $\log Q_{10} = [\theta + 10) - \theta] \log a = 10 \log a$. If rates ($v$, v') are known only at two temperatures which are not 10°C apart, the Q_{10} can be calculated according to the formula, $\log Q_{10} = (10/\Delta\theta)\log(v/v')$.

In the case of biological rhythms, the rate of a reaction is proportional to the frequency, which is the inverse of the period. The Q_{10} of the period is <1 if the period increases with the temperature, >1 if it decreases with the temperature, and equal to 1 if it is independent of this factor.

The rhythm in *Sclerotinia fructicola,* which can be induced by a physical excitation or by yeast extract, has a period of 24 hours in the dark between 17° and 27°C, but is 16–18 hours in weak continuous light and is independent of the amount of yeast extract used (Jerebzoff, 1961b).

The period of the rhythms induced by light approaches 24 hours in *S. fructigena, S. laxa,* and *Leptosphaeria michotii* (Hall, 1933; Jerebzoff and Lacoste, 1962). By contrast, when the rhythm is manifested in the absence of an inducing treatment, the period can be much longer. Thus, it is 2 days in *Sordaria fimicola* (Hawker, 1950), 3 days in *Ascochyta chrysanthemi* (Stevens and Hall, 1909), 4 days in *Aspergillus ochraceus,* 7½ days in *A. niger* (Jerebzoff, 1961b, 1963), and longer in *Trichoderma viride* (Milburn, 1904). However, these endogenous rhythms are still poorly understood.

In different races of *Podospora anserina* the period varies from 28 to 43 hours at 26°C (Chevaugeon, 1959a; Nguyen, 1962). At this temperature, the period in *Ascobolus immersus* and *Pestalotia annulata* is 35 and 57 hours, respectively, and at 16° C the period is doubled but remains insensitive to continued illumination (Chevaugeon, 1959a).

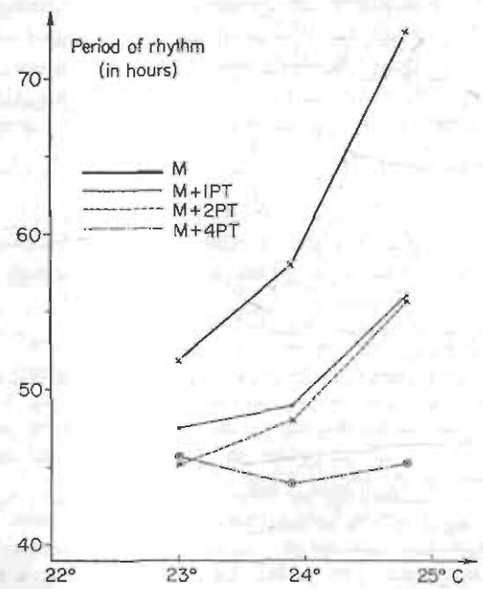

FIG. 3. Duration of the endogenous period of *Alternaria tenuis* in the dark, as a function of the temperature and composition of the medium. $M = 5$ gm of malt extract per liter. PT = water extract of 12.5 gm (*1*), 25 gm (*2*) and 50 gm (*4*) of peeled potatoes per liter.

Extreme dependence upon temperature is demonstrated by the rhythm of *Alternaria tenuis,* which is manifest in the dark on malt agar, but only between 22.5° and 25°C, and disappears in continuous light. Its period is 50 hours at 23°C and 71 hours at 24.8°C, so that its Q_{10} is about 0.14. The duration of the period is independent of the amount of malt extract furnished, but, on the other hand, the simultaneous addition of malt extract and a water-extract of potato has the following effects: (1) reduction in the period at a given temperature, such as, from 50 to 45, or even 35 hours, at 23°C. (2) modification of the dependence of the period upon temperature, making possible complete independence of this factor in certain concentrations of the extracts (Fig. 3).

Therefore, potato extract contains substances that affect the period of the rhythm of *A. tenuis* at a given temperature, as well as its dependence upon temperature (Jerebzoff, 1961b).

2. *Effect of Different Photocycles upon the Period*

Generally, it is considered that an organism having a classical endogenous diurnal rhythm undergoes "entrainment" [3] of its rhythm when its period coincides with that of a given outside factor, during the application of a stimulus like photo- or thermocycles. As soon as the organism is returned to uniform conditions it regains its innate period. Moreover, if the applied periodicity is too distant from the innate period the latter is manifested so that the rhythm then is independent of variations in the external environment.

Two zonation rhythms have been studied from this point of view. In *Sclerotinia fructicola,* homophasic photocycles of 8 and 12 hours induce zonation rhythms of 8 and 12 hours, whereas in photocycles of 4 and 6 hours a period of 12–14 hours is shown. The rhythm appears to be "entrained" in the first case, but independent of variations in light in the second case. Once the organism has been returned to darkness, a period of 24 hours is reestablished in all cases.

The second instance is that of *Alternaria tenuis* which, in 10 gm of malt extract in agar, shows as we have seen, a rhythm with a period of 50 hours at 23°C in the dark (Section III, C,1). If this organism is grown on the same medium but receives daily photoperiods of 12 hours in which the intensity is 250 lux, the zonation period is 24 hours (Fig. 4A). By contrast, if the intensity is lowered to 100 lux, two types of zonations appear, one of which shows a rhythm of 24 hours, and the other a rhythm of 50 hours (Fig. 4B). Consequently, the period of 24 hours is due to the applica-

[3] The term equivalent to "asservissement," as used by French workers.

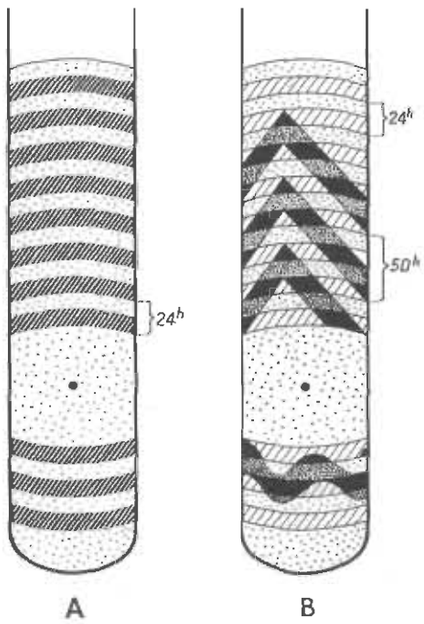

FIG. 4. *Simultaneous evocation of exogenous and endogenous zonations in Alternaria tenuis* grown on malt extract (10 gm/liter) and receiving daily 12-hour photoperiods. When the stimulus is 250 lux, only an exogenous 24-hour rhythm appears (A). If the stimulus is 100 lux, the preceding rhythm still is present, but an endogenous rhythm of 50 hours also appears (B).

tion of the photocycles and is of exogenous origin, whereas that of 50 hours is endogenous. It follows that the period of 24 hours which results from the application of physical stimuli to cultures of *A. tenuis* in malt extract cannot, in any of the cases studied, be considered to be a consequence of "entrainment" of the endogenous 50-hour rhythm. If the action of photocycles dominates that of nutritional factors, it may be due simply to the inhibition of the endogenous rhythm and a manifestation only of the exogenous zonation rhythm (Jerebzoff, 1961b).

To summarize, fungi can show very diverse endogenous rhythms. In *Neurospora crassa* and *Sclerotinia fructicola* the period is approximately, or equal to, 24 hours and independent of temperature. Blue light induces the rhythm in *S. fructicola*, as in other lower organisms, like *Gonyaulax polyedra* (Sweeney, 1960) and *Oedogonium cardiacum* (Bühnemann, 1955), but not in higher plants like *Phaseolus*, where only red light is effective in starting the endogenous diurnal rhythm (Bünning, 1960). In addition, the

circumstances for "entrainment" and for "free-running" period in *S. fructicola* are like those for the diurnal rhythms in the discharge of sporangia of *Pilobolus* (Uebelmesser, 1954), or of ascospores of *Daldinia* (Ingold and Cox, 1955), as well as like those for diurnal rhythms of higher plants like *Kalanchoe* (Bünsow, 1953). Therefore, *S. fructicola* and *Neurospora crassa* show classical endogenous diurnal rhythms.

The endogenous rhythms induced by light in *S. fructigena, S. laxa* (Hall, 1933), and *Leptosphaeria michotii* (Jerebzoff and Lacoste, 1962) also could be of the same type as the above. Moreover, *Leptosphaeria* can manifest a second endogenous rhythm, due to nutritional factors.

Finally, a series of endogenous rhythms that appear under very varied conditions have been listed. By reason of their innate period, which is longer than 24 hours, their Q_{10} and dependence upon continuous light and different photocycles, these rhythms are very odd. Such rhythms have only rarely been reported to exist in higher plants (Titz, 1942).

IV. GENETIC DETERMINATION OF ZONATION

The value of zonation as a taxonomic character has been known for some time, notably because of the work of W. Brown (1925) on *Fusarium*. However, precise genetic studies of the mechanism of zonation have scarcely begun.

Mutant no. 21,863 of *Neurospora crassa* is "prolineless," but the addition of proline to a minimal medium does not permit the origin of zonations in this mutant or in others which are deficient in the synthesis of compounds in the ornithine cycle (Brandt, 1953). Furthermore, the character "patch" (pat), which determines cyclic growth, and "prolineless," are controlled by genes that are not closely linked. Thus, the character, "appearance of zonations" does not appear to be linked to the metabolism of proline and, more generally, to the ornithine cycle in *Neurospora*. Besides, there is a good correlation between the ability to "escape" on media containing sorbose and "patch" segregants from a cross with race 74A so it is possible to observe the "patch" character under conditions where zonations do not occur. If the "patch" gene does not affect the functioning of a biological clock directly but, instead, affects growth in some way, it would be expected that pat+ strains would demonstrate clock activity (Stadler, 1959).

In addition, those races of *Podospora anserina, Ascobolus immersus,* and *Pestalotia annulata* which show zonations are all characterized by a chromosomal accident (Chevaugeon, 1959a; Nguyen, 1962; Tavlitzki, 1954). In particular, in *P. anserina,* it appears that the exogenous rhythm

induced by photocycles, and an endogenous rhythm that does not require light, are determined by hereditary differences based upon a single pair of genes (Nguyen, 1962).

V. DISCUSSION

One important new element, which the study of zonations in fungi has brought to the knowledge of endogenous rhythms of long duration, bears on the *manifestation* of these rhythms. In effect, concerning the relation between exogenous and endogenous rhythms affecting the same physiological process, it is known that weak physical excitations can induce an exogenous rhythm in certain organisms, whereas similar stimuli, but of greater intensity, induce an endogenous rhythm.

But, when cultivated on a simple nutrient medium, *Aspergillus ochraceus* and *A. niger* are incapable of showing an endogenous zonation rhythm under the stimulus of physical excitations. In order that the phenomenon can be demonstrated under uniform environmental conditions, they need substances found in yeast extract, such as isoleucine. On the other hand, very weak physical excitations induce exogenous rhythms in *Sclerotinia fructicola* grown on a basal medium, whereas stronger stimuli of the same kind, induce an endogenous daily rhythm. The latter effect can be duplicated entirely by substances in yeast extract.

The appearance in *S. fructicola* of the same endogenous diurnal rhythm after the application of either a physical or chemical stimulus suggests that the former permits the synthesis of the chemicals which are preformed in the latter case. Following such reasoning, it is suggested that, in *A. ochraceus* and *A. niger,* physical stimuli are insufficient to permit these organisms to accomplish the syntheses needed to induce an endogenous zonation rhythm.

In the case of *Alternaria tenuis,* it is possible that the organism is able to synthesize the hypothetical factors needed for the endogenous periodicity, if it has at its disposal certain common nutritional materials in the right proportions. Similar observations underscore the importance of the composition of the basal medium, even in the case of *Aspergillus,* or of *S. fructicola.* However, it remains to be explained why light is incapable of inducing an endogenous rhythm in *Alternaria tenuis* when it is grown on malt extract.

Be that as it may, a gradation can be perceived from exogenous, to induced-endogenous, to spontaneous endogenous rhythms, reflecting more and more complete capacity to synthesize products that are necessary somehow to the periodicities under consideration.

Moreover, is the appearance of an endogenous zonation rhythm, as is

generally thought to be so for other endogenous diurnal rhythms, the result of the synchronization of separate cellular endogenous rhythms that are out of phase with slightly different periods? According to such a hypothesis, the time required for the decay of an induced rhythm corresponds to the time needed for the complete desynchronization of the separate rhythms. Or, does its appearance constitute a true release of a rhythm?

As discussed above, when *S. fructicola* is grown in the dark on a basal medium, the application of strong light induces an endogenous rhythm which decays in 3 days. If the fungus develops under uniform external conditions, in the presence of yeast extract, the same endogenous rhythm continues without degenerating for at least 3 weeks. Likewise, the induced rhythms of *Aspergillus ochraceus, A. niger,* and *Alternaria tenuis,* under uniform external conditions, persist for 70 days without abatement.

According to the first hypothesis, the 3 days needed for the decay of the rhythm induced by a physical stimulus in *S. fructicola,* represent the time of desynchronization. But, in order to explain the long duration of the rhythm in the presence of the chemical inducer, the factors present in yeast extract would have to play a double role. They would have to synchronize the phases of the separate internal determinants of the periodicity. Then, these compounds would have to be able to induce a period of 24 hours in all the cells of a culture. Thus, the factors in yeast extract would have to participate in the fundamental mechanism of the periodicity in some way. One possibility is that the action of a factor in the medium could be to supersede, or modify, some elements of the periodic mechanism by imposing another value on the endogenous period. This appears much less probable, as it is proved that factors in yeast extract induce a period of several days in *A. ochraceus* and *A. niger*. Therefore, these factors are not specific as far as the duration of the period is concerned.

According to the hypothesis based upon a true release of the rhythm, the cells of a culture of *S. fructicola* would have the same endogenous period, but the action of light would be necessary to activate the rhythm which is initially at rest. The time taken for the rhythm to disappear would then be a function of the amount of substances produced by the organism as a consequence of the stimulus, and it would be practically indefinite if these substances were furnished preformed in the medium, at a constant rate. The similarity in the manifestation of endogenous rhythms in *Aspergillus ochraceus, A. niger,* and *Alternaria tenuis* also suggests that a true release is involved.

These examples can be added to those of *Gonyaulax polyedra* (Sweeney, 1960) and *Acetabularia major* (Sweeney and Haxo, 1960) where the appearance and disappearance of endogenous daily rhythms of photosynthetic capacity have been observed in single cells. *Consequently, at least in the*

fungi, the theory which explains the manifestation of an endogenous rhythm in a multicellular organism as being due to the synchronization of many separate rhythms, cannot have general application.

Let us now consider the function of an "internal clock," for example, one that shows a diurnal rhythm. Such a "clock" can only play a role in measuring time if the endogenous period can be "entrained," within certain limits, by exogenous photo- or thermocycles, and if it is independent of the temperature.

Taken by itself, *S. fructicola* could be considered to show a classical endogenous diurnal rhythm in response to periodically applied illumination. Depending upon the conditions used, "entrainment" and "independence" of its endogenous period are observed. But the reaction of *Alternaria tenuis* to analogous treatments is very different. Even though certain results simulate "entrainment" of the endogenous period under the stimulus of photocycles, these are due only to blocking of the endogenous rhythm and manifestation of a single exogenous zonation rhythm (Section III, C, 2).

It is evident that the periodicity of *A. tenuis* cannot be attributed to an "endogenous clock." However, if these results are compared to other extreme examples, it is possible to question again the notion of "entrainment" of endogenous rhythms in which the period is near, or equal to, that of natural periods. On the one hand, the diurnal endogenous rhythm of color change in *Uca pugnax* cannot be "entrained" but, contrary to *Alternaria,* the exogenous rhythm due to applied photocycles does not appear (F. A. Brown and Stephens, 1951). On the other hand, if *Hydrodictyon reticulatum* (Pirson *et al.*, 1954; Richter and Pirson, 1957) or *Platynereis dumerilii* (Hauenschild, 1960) are exposed to a series of very different photocycles and transferred to uniform conditions, they show an endogenous rhythm in which the period is equal to, or near, that of the applied periods.

So, it is possible to conceive of a gradation of endogenous rhythms characterized by more or less sensitivity of their period to cyclic variations in the external environment. One type of rhythm can be entirely independent of applied periods, or its appearance can be blocked if the physical stimulus is strong. Still another type can be brought under the control of periodic exogenous stimulation, but immediately reverts to its own period upon return to uniform conditions. Finally, we come to the case where the endogenous period has become so plastic that it can retain the applied period after the cessation of the stimulus.

As for the second essential character of "endogenous clocks," their relative independence of temperature, many workers stress the existence of rhythms whose Q_{10} is slightly higher or lower than 1, supposing that these values arise because different chemical reactions, possessing different tem-

perature coefficients, balance each other (Pittendrigh, 1960; Sweeney and Hastings, 1960). The behavior of *A. tenuis* when it receives malt and potato extracts simultaneously is an experimental proof in favor of this hypothesis. It represents, as do respiratory movements of young carps (Meuwis and Heuts, 1957), a new kind of endogenous rhythm which differs from a typical "endogenous clock," but whose period can be independent of temperature under certain conditions.

Finally, no substance controlling the duration of the period of endogenous diurnal zonation rhythms has been found. However, some factors that affect the period of *A. tenuis* have been revealed in aqueous extracts of potato. They differ from surface-active agents like alcohol, papaverine, and narcotine (Keller, 1960), or from mitotic poisons like colchicine and especially urethan (Bünning, 1958), which increase the period of the endogenous diurnal rhythm in the movement of leaves of *Phaseolus*.

The mechanisms which regulate the formation of zonations have not yet been localized. We know now that many unicellular organisms—*Euglena gracilis* (Bruce and Pittendrigh, 1956), *Gonyaulax polyedra* (Sweeney and Hastings, 1960), *Acetabularia major* (Sweeney, 1960), and others—can show typical diurnal endogenous rhythms. Still others, e.g., *Euglena limosa* (Bracher, 1937), *Strombidium oculatum, Chromulina psammobia,* and *Hantzchia amphyoxis* (Fauré-Fremiet, 1948, 1950, 1951) show endogenous "tidal rhythms." Moreover, diverse systems with diastatic capacity are capable of functioning rhythmically (Bünning, 1958; Glick *et al.*, 1961; Richter and Pirson, 1957). Nevertheless these systems do not constitute by themselves the fundamental mechanism. In fact, for example, three diverse enzymes manifest a rhythmic activity with the same period in *Hydrodictyon,* according to Richter and Pirson (1957). Another fact is very promising in this way. Sweeney and Haxo (1960) demonstrated that the rhythm of photosynthetic capacity continues for some days in enucleated *Acetabularia*.

In conclusion, the study of zonation rhythms in fungi already has provided experimental confirmation of certain hypotheses, while casting new light on certain problems concerning the functioning of endogenous rhythms with periods of long duration.

But above all, the fungi are a group in which, in spite of the few researches undertaken, there has been observed a wide diversity of types of endogenous rhythms. There is available now a group of nutritional factors, which are known or are in the process of being identified, that permit several approaches to the study of the internal mechanism of zonations including factors involved in the manifestation of a rhythm, the control of the duration of the period of a rhythm at a given temperature, and the dependence of the period on temperature, applied periodicities, and con-

tinuous light. Genetic studies of the mechanism of zonation periodicities ought right now to be undertaken, concomitantly with the physiological studies discussed above.

The results that are to be expected from such work will undoubtedly open new perspectives in the approach to the study of the mechanism of the more complex endogenous rhythms of longer duration in plants, as well as animals.

REFERENCES

Aschoff, J. (1960). Exogenous and endogenous components in circadian rhythms. *Cold Spring Harbor Symp. Quant. Biol.* **25**:11–28.

Baillaud, L. (1957). Recherches sur les mouvements spontanés des plantes grimpantes. Thèse Sciences Besançon No. 5.

Berliner, M. D. (1961). Diurnal periodicity of luminescence in three basidiomycetes. *Science* **131**:740.

Bisby, G. R. (1925). Zonation in cultures of *Fusarium discolor sulphureum*. *Mycologia* **17**:88–97.

Bjornsson, I. P. (1959). Responses of certain fungi, particularly *Trichoderma* sp. to light. *J. Wash. Acad. Sci.* **49**:317–323.

Bracher, R. (1937). The light relations of *Euglena limosa* Gard. Part. I. The influence of intensity and quality of light on phototaxy. *J. Linnean Soc. London, Botany* **51**:23–42.

Brandt, W. H. (1953). Zonation in a prolineless strain of *Neurospora*. *Mycologia* **45**:194–208.

Brown, F. A., Jr. (1960). Response to pervasive geophysical factors and the biological clock problem. *Cold Spring Harbor Symp. Quant. Biol.* **25**:57–71.

Brown, F. A., Jr., and G. C. Stephens. (1951). Studies of the daily rhythmicity of the fiddler crab *Uca*. Modification by photoperiod. *Biol. Bull.* **101**:71–83.

Brown, W. (1925). Studies in the genus *Fusarium*. II. An analysis of factors which determine the growth-forms of certain strains. *Ann. Botany (London)* **39**:375–408.

Bruce, V. G., and C. S. Pittendrigh. (1956). Temperature independence in a unicellular "clock." *Proc. Natl. Acad. Sci. U.S.* **42**:676–682.

Bühnemann, F. (1955). Das endodiurnale System der *Oedogonium*-Zelle. IV. Die Wirkung verschiedener Spektralbereiche auf die Sporulations-und Mitoserhythmik. *Planta* **46**:227–255.

Bünning, E. (1958). "Die Physiologische Uhr." Springer, Berlin.

Bünning, E. (1960). Opening address: Biological clocks. *Cold Spring Harbor Symp. Quant. Biol.* **25**:1–9.

Bünsow, R. C. (1953). Ueber tages-und jahresrhythmische Aenderungen der photoperiodischen Lichtempfindlichkeit bei *Kalanchoe blossfeldiana* und ihre Beziehungen zur endogenen Tagesrhythmik. *Z. Botan.* **41**:257–276.

Bünsow, R. C. (1960). The circadian rhythm of photoperiodic responsiveness in *Kalanchoe*. *Cold Spring Harbor Symp. Quant. Biol.* **25**:257–260.

Chevaugeon, J. (1959a). La zonation du thalle, phénomène périodique autonome chez l'*Ascobolus immersus*. *Compt. Rend.* **248**:1381–1384.

Chevaugeon, J. (1959b). Sur le déterminisme interne du rhythme de croissance chez un mutant "vague" de l'*Ascobolus immersus*. *Compt. Rend.* **248**:1841–1844.

Cloudsley-Thompson, J. L. (1961). "Rhythmic Activity in Animal Physiology and Behaviour," 236 pp. Academic Press, New York.

Cruickshank, I. A. M. (1963). Environment and sporulation in phytopathogenic fungi. IV. The effect of light on the formation of conidia of *Peronospora tabacina* Adam. *Australian J. Biol. Sci.* **16**:88–98.

Ellis, M. (1931). Some experimental studies on *Pleospora herbarum* (Pers.) Rabenh. *Brit. Mycol. Soc. Trans.* **16**:102–114.

Fauré-Fremiet, E. (1948). Le rythme de marée de *Strombidium oculatum* G. R. *Bull. Biol. France Belg.* **82**:1–20.

Fauré-Fremiet, E. (1950). Rythme de marée d'un *Chromulina psammophile*. *Bull. Biol. France Belg.* **84**:207–214.

Fauré-Fremiet, E. (1951). The tidal rhythm of the diatom *Hantzschia amphyoxis*. *Biol. Bull.* **100**:173.

Fingerman, M. (1957). Lunar rhythmicity in marine organisms. *Am. Naturalist* **91**:167–178.

Fingerman, M. (1960). Tidal rhythmicity in marine organisms. *Cold Spring Harbor Symp. Quant. Biol.* **25**:481–490.

Gallemaerts, V. (1911). De la zonation dans les cultures de champignons en boîtes de Petri. *Proc. Inst. Botan. Leo Errera* **8**:213–223.

Glick, D., R. B. Ferguson, L. J. Greenberg, and F. Halberg. (1961). Circadian studies on succinic-deshydrogenase, pantothenate and biotin of rodent adrenal. *Am. J. Physiol.* **200**:811–814.

Hafiz, A. (1951). Cultural studies on *Ascochyta rabiei* with special reference to zonation. *Brit. Mycol. Soc. Trans.* **34**:259–269.

Halberg, F. (1960). Temporal coordination of physiologic function. *Cold Spring Harbor Symp. Quant. Biol.* **25**:289–310.

Hall, M. P. (1933). An analysis of the factors controlling the growth form of certain fungi, with special reference to *Sclerotinia* (*Monilia*) *fructigena*. *Ann. Botany* (*London*) **47**:538–578.

Harker, E. (1958). Diurnal rhythms in the animal kingdom. *Biol. Rev.* **33**:1–52.

Hauenschild, C. (1960). Lunar periodicity. *Cold Spring Harbor Symp. Quant. Biol.* **25**:491–498.

Hawker, L. E. (1950). "Physiology of Fungi." Oxford Univ. Press (Athlone), London and New York.

Hedgecock, G. C. (1906). Zonation in artificial cultures of *Cephalothecium* and other fungi. *Rept. Missouri Botan. Garden* **17**:115–117.

Ingold, C. T., and V. J. Cox. (1955). Periodicity of spore discharge in *Daldinia*. *Ann. Botany* (*London*) [N.S.] **19**:201–205.

Isaac, I., and G. H. Abraham. (1959). Saltation and zonation formation in *Verticillium lateritium*. *Can. J. Botany* **37**:801–814.

Jerebzoff, S. (1958). L'extrait de levure contient un facteur qui permet la formation de zonations chez *Monilia fructicola* développé à l'obscurité et sous éclairement continu. *Compt. Rend.* **247**:1384–1387.

Jerebzoff, S. (1960). Remplacement de l'action d'une stimulation physique, dans le déclenchement du rythme endogène de *Monilia fructicola* par des composés de la fraction basique de l'extrait de levure. *Bull. Soc. Franc. Physiol. Vegetale* **6**:94–96.

Jerebzoff, S. (1961a). En relation avec la composition du milieu de culture *Alternaria tenuis* montre, soit un rythme exogène de zonations, soit un rythme endogène de 50 h. *Compt. Rend.* **252**:163–165.

Jerebzoff, S. (1961b). Etude de phénomènes périodiques provoqués par des facteurs physiques et chimiques chez quelques champignons. *Thèse Sciences Toulouse.*

Jerebzoff, S. (1962). Importance d'équilibres nutritionnels sur la manifestation du rhythme endogène de zonations chez *Alternaria tenuis* auct. *Compt. Rend.* **254**:2062–2063.

Jerebzoff, S. (1963). L'isoleucine, facteur de rhythmisme chez *Aspergillus niger* van Tieghem. *Compt. Rend.* **256**:759–761.

Jerebzoff, S., and L. Lacoste. (1962). Existence de deux rythmes endogènes de zonations chez *Leptosphaeria michotii* (West.) Sacc. *Compt. Rend.* **254**:1483–1485.

Kamiya, N., H. Nakajima, and S. Abe. (1957). Physiology of the motive force of protoplasmic streaming. *Protoplasma* **48**:94–112.

Keller, S. (1960). Uber die Wirkung chemischer Faktoren auf die tagesperiodischen Blattbewegungen von *Phaseolus multiflorus. Z. Botan.* **48**:32–57.

Knischewsky, O. (1909). Tagesringe bei *Penicillium luteum. Landwirtsch. Jahrb.* **38**:341–343.

Menzel, W. (1962). "Menschliche Tag-Nacht-Rhythmik und Schichtarbeit." Benno Schwabe, Basel/Stuttgart.

Meuwis, A. L., and M. J. Heuts. (1957). Temperature dependence of breathing rate in carp. *Biol. Bull.* **112**:97–107.

Milburn, T. (1904). Ueber Aenderungen der Farben bei Pilzen und Bakterien. *Zentr. Bakteriol., Parasitenk, Abt. II* **13**:129–138, 257–276.

Munk, M. (1912). Bedingungen der Hexenringbildung bei Schimmelpilzen. *Zentr. Bakteriol., Parasitenk., Abt. II* **32**:353–375.

Nguyen, V. H. (1962). Rôle des facteurs internes et externes dans la manifestation des rythmes de croissance chez l'Ascomycète *Podospora anserina. Compt. Rend.* **254**:2646–2648.

Pirson, A., W. J. Schön, and H. Döring. (1954). Wachstums und Stoffwechselperiodik bei *Hydrodyction. Z. Naturforsch.* **9b**:350–363.

Pittendrigh, C. S. (1960). Circadian rhythms and the circadian organization of living systems. *Cold Spring Harbor Symp. Quant. Biol.* **25**:159–184.

Pittendrigh, C. S., V. G. Bruce, N. S. Rosenweig, and M. L. Rubin. (1959). Growth patterns in *Neurospora. Nature* **184**:169–170.

Richter, G., and A. Pirson. (1957). Enzyme von Hydrodyction und ihre Beeinflussung durch Beleuchtungsperiodik. *Flora (Jena)* **144**:562–597.

Sagromsky, H. (1952a). Der Einfluss des Lichtes auf die rhythmische Konidienbildung von *Penicillium. Flora (Jena)* **139**:300–313.

Sagromsky, H. (1952b). Lichtinduzierte Ringbildung bei Pilzen II. *Flora (Jena)* **139**:560–564.

Sagromsky, H. (1956). Zur lichtinduzierten Ringbildung bei Pilzen. III. *Biol. Zentr.* **75**:385–397.

Sagromsky, H. (1959a). Lichtinduzierte Ringbildung. IV. Nachklingen der Rhythmik bei *Trichothecium roseum. Ber. Deut. Botan. Ges.* **72**:169–175.

Sagromsky, H. (1959b). Zur lichtinduzierten Ringbildung. V. Nachklingen der Rhythmik bei *Sclerotinia fructicola* (Wint.) Rehm. *Biol. Zentr.* **78**:589–597.

Schmidle, A. (1951). Die Tagesperiodizitat der asexuellen Reproduktion von *Pilobolus sphaerosporus. Arch. Mikrobiol.* **16**:80–100.

Stadler, D. R. (1959). Growth patterns in *Neurospora.* Genetic control of a cyclic growth pattern in *Neurospora. Nature* **184**:170–171.

Stevens, F. L., and J. Hall. (1909). The variation of fungi to environment. *Botan. Gaz.* **48**:1–30.

Sweeney, B. M. (1960). The photosynthetic rhythm in single cells of *Gonyaulax polyedra*. *Cold Spring Harbor Symp. Quant. Biol.* **25**:145–148.

Sweeney, B. M., and J. W. Hastings. (1960). Effects of temperature upon diurnal rhythms. *Cold Spring Harbor Symp. Quant. Biol.* **25**:87–104.

Sweeney, B. M., and F. T. Haxo. (1960). Persistence of a photosynthetic rhythm in enucleated *Acetabularia*. *Science* **134**:1361–1363.

Tavlitzki, J. (1954). Sur la croissance de *Podospora anserina* en milieu synthétique. *Compt. Rend.* **238**:2341–2343.

Titz, M. (1942). Makroskopische und mikroskopische Wachstumschwankungen am Blattstielen. *Botan. Arch.* **43**:215–251.

Uebelmesser, E. R. (1954). Über den endonomen Tagesrhythmus der Sporangienträgerbildung von *Pilobolus*. *Arch. Mikrobiol.* **20**:1–33.

Wassermann, L. (1959). Die Auslösung endogentagesperiodischer Vorgänge bei Pflanzen durch einmalige Reize. *Planta* **53**:647–669.

CHAPTER 28

Special Growth Techniques (Synchrony, Chemostasis)

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I. SYNCHRONY

The term synchrony denotes a situation in which a population of potentially independent individuals undergo the same sequence of events at the same times. Synchronization refers to any treatment to which an investigator subjects a population in order to cause or to improve synchrony. If the sequence of events is periodic, as in the case of cell division, synchronization consists of putting a collection of clocks in phase with each other. Synchronization of nonperiodic growth processes, e.g., spore germination and development, consists of applying some treatment that initiates a developmental sequence simultaneously in all individuals.

The synchronized individuals may be actually independent or they may interact with each other. Much of the published work on division synchrony has been done at high cell densities and it is sometimes difficult to be certain whether the clock studied is the individual cell or the entire culture. In spontaneous mitotic waves, such as observed in the coenocytic plasmodia of slime molds and occasionally in tissues of higher organisms, synchrony is almost certainly determined by chemical factors pervading a common cytoplasm.

The primary purpose of synchronization is to allow the study of physiological and biochemical changes which cannot conveniently be done directly on the individuals themselves. Some very ingenious and important studies have been made on single growing cells. However, most biochemical measurements require more material than is found in a single cell. One

therefore needs a population of cells at identical stages of growth or development.

One problem in the interpretation of such measurements is to distinguish those biochemical changes which characterize growth and cell division, on the one hand, from those which occur in response to the conditions imposed to induce synchrony on the other. One can afford to ignore this distinction in cases where the principal interest lies in establishing a well-defined growth system which then can be manipulated experimentally, e.g., in synchronous germination of spores. On the other hand, most biochemical measurements of synchronously dividing cells have been undertaken with the idea of elucidating the normal cycle of changes which occur from one division to the next.

Two criteria are useful in evaluating the data reported on synchronous division. First, the growth of cells during a normal doubling cycle must be balanced—i.e., twice as many cells of the same size, shape, and composition as at the start, must be present at the end. Second, biochemical measurements must be made during more than one cycle after the synchronizing treatment, to verify that any changes observed are indeed periodic ones. Because of the rather severe methods generally used to induce synchrony, growth during the first cycle has seldom been perfectly balanced, and the interpretation of the results is correspondingly difficult. This does not imply that the measurements are irrelevant to the normal division cycle, but only that a satisfactory proof is still lacking. The study of more than one cycle is complicated by the gradual loss of synchrony that occurs as time proceeds.

The above discussion refers to those methods of synchronization which we have called prior treatments, in which a cell population is first treated to synchronize it and then is placed in a constant environment and observed. Other workers have grown and observed cells maintained in periodic environments for an indefinite number of cycles. In this case, the two criteria given above are automatically fulfilled, and there is no way to be sure that the normal division cycle is really being studied.

A. Synchronous Division

Division synchrony in microorganisms have been induced by several methods. Single or multiple shifts in temperature, starvation for particular nutrients, changes in illumination, and shifts in pH have all been reported to have some synchronizing effect. Mechanical fractionation of a population into more homogeneous size classes by filtration or centrifugation have also been employed. The latter have the advantage that no known stress has been imposed on the cells. These methods and the results obtained

with them are discussed in recent reviews (Campbell, 1957a; Scherbaum, 1960; Williamson and Scopes, 1961b) and a book (Zeuthen, 1963). We shall consider here in some detail synchronization of two organisms of mycological interest—yeasts of the genus *Saccharomyces* and the slime mold *Physarum polycephalum.*

1. Saccharomyces

Several facts about *Saccharomyces cerevisiae* are pertinent to synchronization studies. One might ask whether synchronization is possible in an organism which divides asymmetrically by budding, as the division times of mother and daughter in the next generation might differ. Burns (1956) showed that, in diploid, triploid, or tetraploid strains grown at 30°C, mother and daughter remain joined until the bud is equal in size to the mother, and the two cells then begin to bud simultaneously for the next division. On the other hand, in a haploid strain grown at 30°C, and in all strains at 38 C, division of the mother cell preceded that of the former bud. There are therefore conditions under which synchrony is possible in principle.

Something is known about the normal division cycle from direct observation of single cells by various workers, especially Mitchison (1957, 1958). In the fission yeast, *Schizosaccharomyces pombe,* cell length and cell volume increase linearly during interphase and plateau for the 25% of the division cycle preceding division. Ribonucleic acid (RNA) seems to increase continually during interphase (Mitchison and Walker, 1959). Cell mass increases linearly throughout interphase. When the cells divide, the mass of each daughter cell begins to increase linearly, so that the total rate of increase is double that before division. In *Saccharomyces,* the results for cell volume and cell mass are similar except that the rate of mass increase doubles not at the time of bud formation, but about 20 minutes thereafter (total doubling time, 2 hours). Similar growth curves have been reported for other cell types, including algae and protozoa. The implication is presumably that the total number of synthetic centers for protein and RNA remain constant throughout one cycle and then double at some moment; this, for organisms which divide symmetrically, occurs at the time of cell division.

All the successful methods for synchronization of yeast have involved some sort of starvation procedure. When yeast is grown with aeration on a complex medium such as malt extract broth, old stationary phase cultures consist almost exclusively of single, nonbudding cells. In growing cultures, on the other hand, cells are rarely separate from their mother cell of the previous division until they themselves have budded or are about to bud. Apparently any cell which has begun to bud at the time the

stationary phase is reached continues to develop into a cell pair and then cleaves to two separate cells, whereas a cell which has not yet budded fails to do so. Some investigators, instead of starving the cells in the laboratory, have used commercial yeast cake directly as a source of old cells. When the cells of a stationary culture are introduced into a fresh medium, they may bud in unison. Various refinements, largely empirical, have been introduced over the years to improve the uniformity of the division cycles following replenishment after starvation. By far the best synchrony has been obtained by the method of Williamson and Scopes (1962).

A 10-day-old aerated culture is first subjected to differential centrifugation, and the smaller cells (about 40% of the population) are discarded. The larger cells are then subjected on three successive days to a 40-minute growth period in malt extract medium followed by 6 hours of starvation, with aeration, in a salt solution. Upon inoculation of the culture into fresh medium, division is synchronous for several cycles. This method has been perfected only recently, and the available biochemical data come from systems where the synchrony was good only for one or two cycles.

It is not certain which component of malt extract broth is growth limiting under the conditions employed, but probably the important factor is the energy supply. Aeration in the absence of substrate is known to deplete endogenous reserves, and the presence of other nutrients is not likely to have too much effect when the energy source has been exhausted. Synchronization by energy starvation is well documented in another case (Campbell, 1957b), but unfortunately the observed synchronization was quite poor. Starvation for nitrogen (Beam *et al.*, 1954), and shifts from sublethal to normal pH (Campbell, 1957b), can also induce an apparent synchrony. At least, a larger than normal fraction of the population is budding in unison in these cases, but how much synchrony is induced in subsequent divisions is not clear. A pseudosynchrony, in which simultaneous budding is not followed by subsequent synchrony, has been induced by subjecting yeast to a series of temperature shocks at 49°C (Louderback *et al.*, 1961). Real synchrony for at least one division cycle was reported following a combination of differential centrifugation and a 1-hour temperature-shock at 40°C (Nosoh and Takamiya, 1962).

In synchronized cultures, deoxyribonucleic acid (DNA) increases in a fairly stepwise fashion shortly after the time of budding. RNA seems to increase linearly throughout most of the cycle and to plateau before and during budding (Williamson and Scopes, 1960). Protein increases more or less linearly throughout the cycle, the rate of synthesis doubling at a time after budding has occurred (Williamson and Scopes, 1961a). These observations are sufficiently in accord with the single-cell findings to support

the contention that the normal doubling cycle is indeed being studied here. On the other hand, total cellular nitrogen showed a stepwise increase, the magnitude of which varied from one cycle to the next—indicating that the cultures used were indeed not in perfect balance.

Cyclic changes in the level of extractable peptidase and catheptic activities have also been reported (Sylvén *et al.*, 1959). The activity is highest immediately before budding and falls during budding sufficiently to cause a drop not only in specific activity, but in total activity as well.

2. Physarum polycephalum

Synchrony of nuclear division in this organism, and apparently in the plasmodia of other slime molds as well, is remarkable in that it occurs spontaneously. The synchrony is maintained by the chemical stimuli transmitted through the common cytoplasmic environment, well mixed by rapid protoplasmic streaming. This interpretation is verified by the fact that, when a plasmodium is fragmented, division in the different pieces eventually becomes out of phase, but when two pieces coalesce, their nuclei quickly come into phase with each other (Güttes *et al.*, 1961).

Chemical studies have been made by feeding C^{14}-orotic acid, which serves as a precursor of the pyrimidine bases of both DNA and RNA. DNA synthesis occurs during the hour following division (total doubling time, 12–14 hours), whereas RNA synthesis is fairly uniform throughout the division cycle (Nygaard *et al.*, 1960).

B. Synchronous Development

When a spore of the aquatic phycomycete *Blastocladiella emersonii* is inoculated into a nutrient medium, its volume increases exponentially by a factor of about ten thousand in 36 hours, after which there is no further increase. About this time, cleavage into two cells, one of which will become a sporangium, is observed. The development of a population of such spores is observed to be quite uniform, at least so far as linear dimensions at various times is concerned (Lovett and Cantino, 1960). The initiating event here is simply the act of placing the spores, previously suspended in water, into a nutrient medium. Some systematic attempt has been made to improve the uniformity in time of germination (Turian and Cantino, 1959). Treatment with indoleacetic acid did not improve the synchrony, but heat shocks at 37°C increased the fraction of individuals which germinated rapidly. The many interesting biochemical results obtained with this system are dealt with in Volume II.

For his studies on nucleic acid and protein content at different stages

of sexual reproduction in *Allomyces*, Turian (1963) has succeeded in synchronizing gametangial formation and development to a sufficient degree to make meaningful studies possible.

II. CHEMOSTASIS

When a culture of microorganisms grows in a closed system, the environment to which the individual is exposed inevitably changes with time, because the organisms are taking up certain molecules from the environment and producing others. The nutritional state of the organisms therefore changes, and this is ultimately manifested in a cessation of net growth. For many purposes, it is desirable to study populations growing in a constant environment. This necessitates an open system, to which fresh medium is continually being supplied to the cells, and old medium withdrawn.

These techniques have mainly been applied to organisms that grow in suspension, such as bacteria and yeasts, and therefore the withdrawal of medium implies the withdrawal of cells as well. If the contents of the vessel are well mixed, and if we let

V = volume of apparatus
$v(t)$ = total volume of medium which has flowed through apparatus at time t
$\alpha(t)$ = growth rate of cells
$n(t)$ = concentration of cells in apparatus

$$\beta(t) = \text{flow rate} = \frac{1}{V}\frac{dv}{dt}$$

then it is straightforward to show that

$$\frac{1}{n}\frac{dn}{dt} = \alpha - \beta \tag{1}$$

At the steady state, therefore, $\alpha = \beta$. If the apparatus is so constructed that the flow rate β is a constant, then α must ultimately equal it. This implies that the cell density must reach such a level that the alteration of the input medium by the cells is sufficient to reduce the growth rate from its maximum value to the steady-state value. If β is set at a higher rate than this maximum value, the culture cannot maintain itself and is gradually washed out.

The most desirable procedure is to use a synthetic medium where one nutrient is supplied in limiting amount. The cells will then reach such a density that the working concentration of this nutrient is reduced to a level corresponding to the flow rate set. If we consider a nutrient of such nature that the number of cells produced is proportional to the amount of nutrient consumed, it can then be shown that

$$\frac{dc}{dt} = (a - c)\beta - kn\alpha \tag{2}$$

where c = concentration of nutrient in apparatus; a = concentration of nutrient in input medium; and k = amount of nutrient required to make one cell.

At the steady state, $(dc)/(dt) = 0$ and $\alpha = \beta$, so that

$$c = a - kn \tag{3}$$

If the nutrient in question is the one which limits growth, then the steady-state value of c depends on the flow rate set, and this in turn determines the growth rate. The population density, on the other hand, is determined by the input concentration a as well. Thus, it is possible to study very dense populations growing at a very low concentration of the limiting nutrient.

It is also possible to construct an apparatus such that the value of β is not constant but variable. For example, density-regulated systems have been used such that new medium is introduced as needed to maintain a constant density measured turbidometrically (Fox and Szilard, 1955). The above equations apply equally to such a system, but there is no necessity in this case that any nutrient be limiting, and the working concentrations of all nutrients may be quite close to the input concentrations. From Eq. (3), it can be seen that in this case the value of n at which growth is regulated is small compared to the quantity a/k.

The constant flow-rate, nutrient-limited apparatus is called a chemostat, and the turbidity-controlled apparatus has sometimes been called a turbidostat. This nomenclatural distinction is slightly misleading. In either case, both the chemical environment and the turbidity are unchanging at the steady state.

The above equations do not tell us anything about the manner of approach of the population to the steady-state condition, and additional assumptions are necessary to draw such conclusions. Spicer (1955) has shown that, under the simplest assumptions, when growth limitation is due to exhaustion of nutrient, the steady state will be approached asymptotically, whereas limitation by accumulation of toxic products can result in oscillations around the steady state. Oscillations of nutrient-limited cultures are also possible under circumstances where the metabolic imbalance caused by nutrient exhaustion is itself effectively toxic. An example of this type seems to have been found in yeast using the energy source as limiting nutrient (Welch, 1957). Oscillations about the steady-state level were also observed by Finn and Wilson (1954), but in their case, the effect may be purely due to the toxic effect of low pH.

REFERENCES

Beam, C. A., R. K. Mortimer, R. G. Wolfe, and C. A. Tobias. (1954). The relationship of radioresistance to budding in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **49**:110–122.

Burns, V. W. (1956). Temporal studies of cell division. I. The influence of ploidy and temperature on cell division in *S. cerevisiae*. *J. Cellular Comp. Physiol.* **47**:357–375.

Campbell, A. (1957a). Synchronization of cell division. *Bacteriol. Rev.* **21**:263–272.

Campbell, A. (1957b). Division synchronization in a respiratory deficient yeast. *J. Bacteriol.* **74**:559–564.

Finn, R. K., and R. E. Wilson. (1954). Population dynamics of a continuous propagator for microorganisms. *J. Agr. Food Chem.* **2**:66–69.

Fox, M., and L. Szilard. (1955). A device for growing bacterial populations under steady state conditions. *J. Gen. Physiol.* **39**:261–266.

Güttes, E., S. Güttes, and H. P. Rusch. (1961). Morphological observations on growth and differentiation of *Physarum polycephalum* grown in pure culture. *Develop. Biol.* **3**:588–614.

Louderback, A. L., O. H. Scherbaum, and T. L. Jahn. (1961). The effect of temperature shifts on the budding cycle of *Saccharomyces cerevisiae*. *Exptl. Cell Res.* **25**:437–453.

Lovett, J. S., and E. C. Cantino. (1960). The relation between biochemical and morphological differentiation in *Blastocladiella emersonii*. II. Nitrogen metabolism in synchronous cultures. *Am. J. Botany* **47**:550–560.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exptl. Cell Res.* **13**:244–262.

Mitchison, J. M. (1958). The growth of single cells. II. *Saccharomyces cerevisiae*. *Exptl. Cell Res.* **15**:214–221.

Mitchison, J. M., and P. M. B. Walker. (1959). RNA synthesis during the cell life cycle of a fission yeast *Schizosaccharomyces pombe*. *Exptl. Cell Res.* **16**:49–58.

Nosoh, Y., and A. Takamiya. (1962). Synchronization of budding cycle in yeast cells, and effect of carbon monoxide and nitrogen-deficiency on the synchrony. *Plant Cell Physiol. (Tokyo)* **3**:53–66.

Nygaard, O. F., S. Güttes, and H. P. Rusch. (1960). Nucleic acid metabolism in a slime mold with synchronous mitosis. *Biochim. Biophys. Acta* **38**:298–306.

Scherbaum, O. H. (1960). Synchronous division of microorganisms. *Ann. Rev. Microbiol.* **14**:283–310.

Spicer, C. C. (1955). The theory of bacterial constant growth apparatus. *Biometrics* **11**:225–230.

Sylvén, B., C. A. Tobias, H. Malmgren, R. Ottoson, and B. Thorell. (1959). Cyclic variations in the peptidase and catheptic activities of yeast cultures synchronized with respect to cell multiplication. *Exptl. Cell Res.* **16**:75–87.

Turian, G. (1963). Synthèse differentielle d'acide ribonucleïque et differenciation sexuelle chez l'Allomyces. *Develop. Biol.* **6**:71–72.

Turian, G., and E. C. Cantino. (1959). The stimulatory effect of light on nucleic acid synthesis in the mould *Blastocladiella emersonii*. *J. Gen. Microbiol.* **21**:721–735.

Welch, G. P. (1957). Effects of chronic exposure to X-rays on a steady-state population of *Saccharomyces cerevisiae*. Univ. of California, Radiation Lab. Rept. No. 3763.

Williamson, D. H., and A. W. Scopes. (1960). The behaviour of nucleic acids in synchronously dividing cultures of *Saccharomyces cerevisiae*. *Exptl. Cell Res.* **20**:338–349.

Williamson, D. H., and A. W. Scopes (1961a). Protein synthesis and nitrogen uptake in synchronously dividing cultures of *Saccharomyces cerevisiae*, *J. Inst. Brewing* **67**:39–42.

Williamson, D. H., and A. W. Scopes. (1961b). Synchronization of division in cultures of *Saccharomyces cerevisiae* by control of the environment. *In* "Microbial Reaction to Environment" (G. G. Meynell and H. Gooden, eds.), pp. 217–242. Cambridge Univ. Press, London and New York.

Williamson, D. H., and A. W. Scopes. (1962). A rapid method for synchronizing division in the yeast *Saccharomyces cerevisiae*. *Nature* **193**:256–257.

Zeuthen, E. (1963). "Synchrony in Cell Division and Growth." Wiley (Interscience), New York.

Gene Action

CHAPTER 29

Gene Action

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I. INTRODUCTION

Genes have two distinct functions. One is to reproduce exactly once in each cycle prior to division of the nucleus. The other, with which this chapter is concerned, is to control some activity in a cell and so, collectively, in an organism as a whole. A gene is a segment of deoxyribonucleic acid which not only reproduces but generally also specifies a protein, or one constituent of a complex protein, which usually has enzymic properties, so catalyzing a specific metabolic reaction. The pathway of specification is operated by means of ribonucleic acids, involving the transcription by their agency of a code from deoxyribonucleic acids to polypeptides.

Many phases of these relationships cannot yet be demonstrated by reference solely to fungi, hence reference to work with other organisms will be made wherever necessary. Any other course, restricting the study to fungi alone, would distort the picture we now have of a process fundamental to all organisms. Further discussions and references will be found in Catcheside (1951) and Fincham and Day (1963).

Some genes may act other than by specifying proteins, but there is no certain evidence yet of this. Certainly there are species of ribonucleic acid, which are metabolically active and could be direct products of genes. However, no mutants of such genes are known, perhaps because they would be lethal. Secondly, metabolic reactions are subject to regulation which has a genetic basis. It is conceivable that some of the regulatory genes act otherwise than by the production of specific proteins, but no such mechanisms have been demonstrated.

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II. THE GENE

The definition of a gene has given rise to much difficulty and confusion, which has not been relieved by the introduction of new names (Benzer, 1956). The various criteria which have been proposed are (1) that the gene has a unitary function, any change in the gene being capable potentially of causing some loss of this function; (2) that the gene has an indivisibility by recombination; and (3) that all mutations affecting a given gene show effects on the same function. It has gradually become clear that definitions based on function and capacity to recombine, respectively, did not identify the same entity and, indeed, the unit of function is capable of very considerable subdivision by recombination. It is very probable that recombination is possible between the smallest separable elements of the deoxyribonucleic acid, namely between adjacent pairs of nucleotides. The best evidence is that presented by Henning and Yanofsky (1962). They showed that, in *Escherichia coli,* recombination is possible between genetic differences which specify different amino acid substitutions at a particular site in the A protein of tryptophan synthetase. It would appear therefore that rigorous application of the criterion of recombination would define nothing larger or more integrated than the ultimate molecular elements whose arrangement codes genetic information. On the other hand, application of a criterion of function would appear capable of defining integrated entities of higher significance. The chief problem lies in making such a criterion rigorous. It appears possible to do so at least in the case of auxotrophic mutants in fungi.

If a set of auxotrophic mutants of *Neurospora crassa,* all of which require tryptophan for growth, are examined by a suitable physiological test, they are divisible unambiguously into four groups (Ahmad and Catcheside, 1960). Pairs of the mutants will form heterokaryons[2] quite readily, provided they are of the same mating type and do not differ in respect of incompatibility factors (Garnjobst, 1953; Holloway, 1955). These heterokaryons can be tested to determine whether or not they will grow on a medium which does not supply tryptophan. If they do grow under these conditions they are said to complement one another. The four groups are defined by the fact that each member of one group complements all members of all other groups. Within a group one of two situations is found to exist, either (a) all pairs show no complementation (*try*-2 and *try*-4) or (b) most pairs show no complementation, but a minority show complementation to a

[2] Although the spelling "heterokaryon" is adopted in this book, the original usage, preferred by the author, was "heterocaryon" [Hansen and Smith, *Phytopathology* **22**: 955 (1932)].

greater or lesser degree (*try*-1 and *try*-3). In the latter case, it is usual for a substantial proportion, often a majority, of the mutants to show no complementation with one another and with each of the rest, some pairs of which may show complementation. The members of a group, defined in these ways, possess allelic genes. The entity in the normal strain, or wild type, that is capable of generating a series of mutants belonging to one group, is one gene.

This complementation test can be applied also where heterozygous diploids can be constructed, as in yeasts (Roman, 1956). It may also be done in those cases, e.g., *Aspergillus nidulans* (Roper, 1952), where diploidy may be induced to occur abnormally. The test has been applied particularly to mutants of the *ad*-8 series (Pritchard, 1955).

Strictly, of course, the absence of complementation in these tests is not complete evidence of functional allelism. It assumes that the mutational differences are individually recessive, as they can be shown to be, and also that they do not interact by some cumulative effect. To exclude the latter would, strictly, require the synthesis of the coupling (or *cis*) phase heterozygote $\left(\frac{m^1\ m^2}{+\ +}\right)$ or heterokaryon $[(m^1\ m^2) + (+\ +)]$ as well as the usual repulsion (or *trans*) phase heterozygote $\left(\frac{m^1\ +}{+\ m^2}\right)$ or heterokaryon $[(m^1\ +) + (+\ m^2)]$. If the former is wild and the latter mutant in phenotype, the case for noncomplementation (allelism) is complete, but the coupling phase combinations are extremely difficult to prepare. In general, therefore, the evidence of the mutant phenotype of the repulsion phase is all that is available. It is reasonable in general to accept it as defining the limits of groups of allelic genes.

The occurrence of recombination between the genetic differences of alleles, defined physiologically, allows the preparation of fine-structure maps showing the order and spacing of the sites at which each allele differs from a standard, the wild type. These maps will be of value to correlate with other differences between alleles.

III. GENE ACTION AND INTERACTION

Each group of mutants differentiated by the physiological test appears usually to be concerned in one physiological function in the organism. Where a number of functions appear to be affected, it is possible in some cases to demonstrate a single underlying cause; it is reasonable to assume such singularity unless the contrary is clearly shown. In many cases, the individual functions are those under the direction of enzymes, so that many genes are concerned with the specification of the structure of specific

enzymes, in general with one gene corresponding to one enzyme (Catcheside, 1960a). There are other genes that in some way limit or permit the occurrence of enzymes whose structures are specified by other genes. How these act is generally obscure.

Gene action may be regarded as comprising the nature of the path of command from gene to the ultimate function by which it is recognized. In fact, of course, the relationship is nearly always dealt with in terms of the alterations in function consequent upon a change in a normal gene or upon the total removal of it.

In the metabolism of an organism, the diverse chemical activities, leading to the degradation of some compounds, to the elaboration of other new ones and to the construction of large organized structures, occur in two contrasting ways. In both, a single-step reaction is the basic process.

In one, that characteristic of intermediary metabolism, a series of single steps, each catalyzed by a distinct specific enzyme, are linked together by a common product or reactant, thus

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C$$

Systems of enzymes working in concert in this way are called "multi-enzyme systems" (Dixon, 1949). Each step results in a rather minor modification of the reacting molecules. Collectively, these small changes make up the metabolism of the organism, within which all the multi-enzyme systems work together in a coordinated fashion, normally harmonious and mutually regulatory. Indeed any failure of regulation is liable to be disastrous to the organisms. The types of interaction are various (pp. 672–690).

The other constructional process is that concerned with the formation of highly specific macromolecules, the proteins and nucleic acids. Manufacture of these involves the assembly of small units, drawn from the general pool in each cell of the organism and arranged according to precise patterns provided by templates. These include the genes, segments of DNA, from which all the templates appear to be derived by transcription, the messenger RNAs and the proteins. It is true that the formation of these various macromolecules involves numerous small individual steps, each involving a characteristic enzyme. The difference lies not merely in the elaborate and precise assembly of small molecules to make a macromolecular pattern, rather than the substitution, addition or subtraction of atoms or radicals to a small molecule. Rather it lies in the intervention of a template, copied as such or in a different form.

These two metabolic mechanisms, both showing phenomena of gene action and interaction, need separate treatment from this point of view since it appears that they exhibit distinctive modes of action and interaction.

The pathway of specification between a gene and its various functions has been investigated chiefly in systems derived from bacteria and mammalian tissues. The gene is generally considered to be a segment of DNA, but the evidence for this is dependent chiefly upon transformation in bacteria, bacteriophage infection, and general circumstances.

The segment of deoxyribonucleic acid (DNA), with its individual sequence of nucleotide pairs, determines a messenger ribonucleic acid (mRNA) with an homologous sequence of nucleotide pairs. The mRNA passes into the cytoplasm and there is supported on a group of ribosomes, each consisting of an association of protein and ribosomal ribonucleic acid (rRNA). In this position the mRNA determines the synthesis of a specific polypeptide, a given sequence of nucleotides specifying a particular sequence of amino acids. A code of relationship is implied. The ordering is dependent on the mediation of a third class of ribonucleic acids, the transfer RNAs (tRNA), each kind of which will become attached to an individual kind of amino acid in the presence of a specific activating enzyme. The individual tRNA molecules, each carrying its particular amino acid, become associated with specific short regions of the mRNA, a sequence of tRNA nucleotides recognizing, as it were, an homologous sequence of nucleotides in the mRNA. Following association of the amino acids, the polypeptide formed would have a specific sequence of them. The resulting polypeptide would fold into a characteristic shape, determined by the sequence of the amino acids and by the specific environment in which folding occurs. Individual protein units, so formed, or associations of them, would form a specific protein.

IV. AUXOTROPHIC MUTANTS

The work on *Neurospora crassa,* initiated by Beadle and Tatum (1945), led almost at once to an important generalization. The great majority of mutants unable to grow on minimal medium (no more than sufficient to support the normal wild type), but able to grow on a supplemented medium, each require only a single substance more than does the normal wild type. On this basis it seemed very probable that most mutations affected only a single metabolic pathway and further evidence tended to show that in general only a single step in the pathway was blocked. The clear indication of a hypothesis of one gene-one enzyme was in fact the idea that motivated Beadle and Tatum to initiate the experiments which collectively support this theory, though with refinements and qualifications.

At one time considerable effort was devoted to argument and experiment for and against the generality of the one gene-one enzyme hypothesis. There were numerous cases of mutants which had multiple growth re-

quirements. Most of these have been satisfactorily explained as complex end effects of single enzyme defects, of kinds which are described below. A more serious case against the general character of the one gene-one enzyme hypothesis may lie in the suggestion that the auxotrophic mutants, reparable by an outside supply of a growth factor, may be unrepresentative of mutations in general. Mutants with multiple effects of primary origin would be difficult to detect, since they might be impossible to grow on any available medium. It is known that many mutations do have apparently irreparable effects (Atwood and Mukai, 1953) and can be maintained only in balanced heterokaryons, where they are supported by normal nuclei. Such irreparable effects could be due to single metabolic blocks in the syntheses of compounds which are unable to penetrate the cells from the outside. The argument is inconclusive, since total lack of knowledge of the nature of the irreparable mutants leaves us with no evidence that they represent a different type of mutation from the auxotroph. In any case, it is clear that the latter is one very significant kind of mutation.

Information from auxotrophs may be used to explore biosynthetic pathways, knowledge of which is useful to understanding of gene action and interaction. Discovery of pathways is aided by study of the ways in which related groups of mutants, all satisfied by one end growth factor, may also be satisfied by various other nutriments which might be precursors and, secondly, by examination of the compounds, presumed to be intermediates or their derivatives, which may be accumulated by mutants when grown on limiting amounts of their principal group factor. Anthranilic acid in the tryptophan pathway (Tatum *et al.*, 1944) and cystathionine in the methionine pathway (Horowitz, 1947) were first discovered by a combination of these two methods, i.e. that one kind of mutant in a pathway may accumulate a compound which a second mutant blocked at an earlier stage can use for its own growth. The success of this type of experiment is dependent upon the capacity of the organism to take up the precursors from the external medium. In some cases, notably in the histidine pathway, exogenous precursors are not accessible to use by the organism.

Whether precursors of a growth factor can be taken up by an organism, or not, they may be employed in a special way for deciding the relative order of the steps commanded by different genes. The principle is that if two mutants, *a* and *b*, have blocks in a given pathway and one of them, say *a*, accumulates a compound not normally found in the organism, then study of the properties, in this respect, of the double mutant could be used to decide their order of action. If *b* had a block before *a* in the pathway, then the double mutant would not accumulate the distinctive compound; if *b* had a block after *a*, then the double would accumulate

the compound just as *a* does alone. In histidine biosynthesis, several of the mutants accumulate imidazoles of various kinds and other mutants accumulate compounds which react with the Bratton-Marshall reagent. These compounds are generally accumulated within the mycelium and apparently are released only by death of the mycelium. None of the phosphorylated imidazoles, which are accumulated by some of the mutants, can be taken into the cells of *Neurospora*. Histidinol is also ineffective in the normal strains, but can be used by some special mutants, having an altered permease. Application of the principle that a double mutant will accumulate the same precursors as does the mutant blocked at the earlier stage in biosynthesis has been applied to all possible pairwise combinations of the seven genes which determine histidine biosynthesis (Haas *et al.*, 1952; Catcheside, 1960b; Webber and Case, 1960). The results are as illustrated in Fig. 1.

FIG. 1. Pathway of biosynthesis of histidine, showing positions of action of the seven genes in *Neurospora crassa*. Abbreviations: *BMC*, Bratton Marshall compound; *IGP*, imidazole glycerol phosphate; *IAP*, imidazole acetol phosphate; *HLP*, histidinol phosphate; *HL*, histidinol; *H*, histidine.

However, difficulties of various kinds are encountered in elucidating pathways and in assessing the function of particular genes. There are some genes which appear to cause a block in a particular pathway, in that their defective growth can be overcome by the terminal compound in the pathway and sometimes by some of its precursors. Thus there is a mutant, *nt* (*nicotinic tryptophan*), which can grow if supplied with nicotinamide or its precursor tryptophan or even by some precursors of tryptophan. However, there are genetic modifiers which render *nt* unable to use some or all of these precursors and so the modifiers appear to shift the block in biosynthesis (Haskins and Mitchell, 1952; Newmeyer and Tatum, 1953). Hence arguments based on nutritional requirements alone may be unsafe and insufficient. Indeed, in the case of *nt* the precise nature of the metabolic lesion is still unknown.

V. GENETIC CHANGES OF ENZYMES

Correlation of gene with enzyme may be achieved by a combination of evidence including, for a given mutant, its nutritional properties and capacities for accumulating precursors, the absence or reduction of a specific enzymic activity and that a specific enzyme is altered or reduced in respect

of part of its activity or has modified physicochemical properties. Numerous cases of the absence of specific enzymes in mutants have been reported; summaries are given in Catcheside (1960a) and Fincham (1959b, 1960). Changes in some of the properties of the enzyme, without total loss of activity, provide more distinctive correlations.

Horowitz and Fling (1953, 1956) showed that different strains of *Neurospora crassa* carry allelic genes which determine tyrosinases with distinctive properties. T^S determines a normal tyrosinase, relatively stable at high temperatures, whereas T^L determines a thermolabile tyrosinase. This difference is due to differences in the enzyme itself and cannot be due to the presence of an activator in the one or an inhibitor in the other. A mixture of the two behaves in the way expected if the enzymes are inherently different. Further, their half-lives at a given temperature are independent of their concentration. The tyrosinases determined by other alleles, T^{PR-15} and T^{Sing-2}, differ from T^S and T^L in having lower electrophoretic mobilities, as well as differing in their thermostabilities.

Yura (1959) showed that the *proline*-1 mutant (21863) of *Neurospora crassa* produced a pyrroline-5-carboxylate reductase with about 0.2–0.4% of the activity typical of the normal wild type. The mutant enzyme has an abnormally low thermostability and a decreased catalytic efficiency. The latter is shown by the increased activation energy of the enzyme-substrate complex. Whereas the activation energy is 8100 cal per mole in the wild type, it rises to 26,000 cal per mole in the mutant. Experiments with mixtures showed that each type of enzyme retains its characters in the presence of the other.

Many mutant varieties of glutamic dehydrogenase, determined by alleles of *am* (*amination*), have been described by Fincham and his co-workers (Fincham, 1957, 1959c, 1962; Fincham and Bond, 1960; Pateman and Fincham, 1958). These mutants all require an α-amino acid, any one of a number, but preferably alanine, to support their growth. They also tend to accumulate ammonia when supplied with nitrate. They all lack normal nicotinamide adenine dinucleotide (NADP)-linked glutamic dehydrogenase activity. This enzyme has been isolated from wild-type *Neurospora crassa* in practically pure form (Fincham, 1962; Barratt and Strickland, 1963). Similar procedures applied to several of the *am* mutants have resulted in the isolation of proteins which are very similar to the wild-type enzyme. The criteria include characteristics of fractionation, electrophoretic mobility, and the number and kinds of peptides yielded by tryptic digestion and separable by electrophoresis and chromatography. In am^1, this protein has no demonstrable enzymic activity, but in am^2 and am^3, neither of which can grow any better than am^1 on minimal medium, the homologous proteins have some glutamic dehydrogenase activity, though

of an anomalous kind. In each, the homologous protein is able to catalyze only the oxidation of glutamate, in the presence of NADP, and it requires very high concentrations of glutamate for maximum activity. Neither can catalyze the synthesis of glutamate, no activity occurring in the mixture of α-ketoglutarate, $NADPH_2$ and ammonia. However, the am^3 protein can catalyze glutamate synthesis, provided that the products of the reaction, glutamate and NADP, are present; moreover the concentration of glutamate must be fairly high. Each of the mutants, am^2 and am^3, appears to produce a protein capable of activation by glutamate and NADP, perhaps by a change in the pattern of its folding. In the case of am^2, this activation is prevented by the presence of α-ketoglutarate, $NADPH_2$ and ammonia.

Various other alleles of *am*, obtained by partial reversion of primary *am* mutants (Pateman, 1957), also have peculiar properties. The enzmye produced by am^{2t} (Fincham, 1957) is activated by heating. The mutant itself grows on minimal medium at 25°C, but requires an exogenous supply of α-amino nitrogen if grown at 20°C or lower. The purified enzyme can be activated by brief warming to 35°C, maximum activation being induced by as short a period as 3 minutes. The activity is lost slowly at 20°C, decaying with a half life of 30 minutes; the enzyme may be reactivated by reheating. This mutant enzyme is also activated by incubation with fairly high concentrations of the substrates, α-ketoglutarate and $NADPH_2$. Another partial revertant, am^{3a}, produces an enzyme with abnormally high Michaelis constants for all substrates, especially ammonium ions, the constant for which is about thirty times that of the wild type (Fincham and Bond, 1960).

Mutants affecting tryptophan synthetase (formerly called tryptophan desmolase, hence the designation of many mutants by the symbol *td*) are of considerable interest. They are all alleles at the *try*-3 (*tryptophan*-3) locus and the nutritional and enzymatic properties of many of them have been described (Yanofsky, 1960; Ahmad and Catcheside, 1960; Bonner *et al.*, 1960; De Moss and Bonner, 1959; Suyama *et al.*, 1964; Kaplan *et al.*, 1963; Rachmeler and Yanofsky, 1961; Suskind, 1957; Suskind *et al.*, 1955, 1962; Suyama, 1960; Lacy and Bonner, 1961). Tryptophan synthetase activity is lost completely or partially in these mutants. In many of them the presence of protein homologous to the enzyme may be detected by its capacity to combine with antibodies specific for the enzyme and so neutralize the antibodies (Suskind *et al.*, 1955). Those which possess this cross-reacting material are commonly described as CRM^+, while those in which none can be detected are CRM^-. Many of the mutants also show survival of part of the catalytic function, since this is complicated enough, in the reaction catalyzed by tryptophan synthetase, to be "divisible," as follows:

indole glycerol phosphate + serine → tryptophan + triose phosphate
indole glycerol phosphate → indole + triose phosphate
indole + serine → tryptophan

Each of these reactions can be demonstrated, by suitable means, to be present in the normal, wild-type, organism. However, it is clear that the normal reaction is the first one and that in this reaction indole is not a free intermediate, but a transient one bound to the enzyme. The second and third reactions can be demonstrated only under special conditions and so may be regarded as not acting under normal physiological states. In some of the mutants which possess a part of the normal reaction, i.e., either the second or the third reaction, this activity sometimes requires conditions *in vitro* which are unnecessary for the corresponding reactions for extracts derived from the normal. In these ways mutants with the following range of properties may be demonstrated:

(1) Mutants which produce no CRM and have no catalytic activities, e.g., *td*-1 and *td*-6.
(2) Mutants which produce CRM but have no catalytic activities, e.g., *td*-3 and *td*-7.
(3) Mutants which produce CRM and can also grow on indole since they can carry out the third reaction, e.g., A78 and *td*-141.
(4) Mutants which produce CRM and can also produce indole, since they can carry out the second reaction. This group of mutants is divisible into three classes according to the dependence of this reaction upon co-factors:
   (a) Some have no requirement for any cofactor, e.g., *td*-96 and *td*-98.
   (b) Some require serine, e.g., *td*-71 and *td*-97, although serine is not required for this reaction as it occurs in extracts of the normal fungus.
   (c) Some require pyridoxal phosphate, e.g., *td*-2 and *td*-99, although in extracts derived from the normal fungus this cofactor is necessary only for the first and third reactions.
(5) There are also mutants which produce CRM and whose extracts can carry out the normal catalytic reactions under some conditions. Thus the mutant *td*-24 is able to grow without tryptophan at temperatures of 30°C and above. In fact it was found to produce an active tryptophan synthetase (Suskind and Kurek, 1959). However, this enzyme is completely inactive in crude extracts owing to inhibition by a normal component of the mycelium, thought to be a metal. Active enzyme was obtained by fractionation of the crude extract and was found to differ from the enzyme obtained from wild type in its abnormally high sensitivity to inhibition by zinc ions. The property of causing the formation of a tryptophan synthetase abnormally sensitive to zinc is shared also by *td*-3.

VI. EXCEPTIONS TO THE ONE GENE-ONE ENZYME RELATIONSHIP

In earlier work having the object of testing the hypothesis that each enzyme is controlled by a distinct gene, exceptions in each direction were sought. It is now believed that there are some genuine ones. Care has to be exercised in the definition of gene and enzyme. The former is taken as the entity in the wild type defined by one physiological function and delimited by the complementation test as described previously. The enzyme may be defined as being one protein, normally catalyzing one reaction. However, difficulty is encountered in delimiting what is a single reaction and in many cases, as with tryptophan synthetase, an enzyme may commonly be described as bifunctional. Often, however, such dual functions are not separate from one another under physiological conditions.

Multiple effects of simple metabolic lesions, i.e., inactivity of single enzymes, may arise in various ways, and some of these were at first taken as exceptions. Two examples will illustrate the possibilities.

In the biosynthesis of aromatic compounds, it appears that all arise from a common source from which they diverge. One mutant, *arom*-1, was discovered by its requirement for four compounds simultaneously, phenylalanine, tyrosine, tryptophan, and *p*-aminobenzoic acid. Later (Gross and Fein, 1960) it was found possible to replace these four with one compound, shikimic acid. The multiple requirement was the simple consequence of the absence of a common precursor at, or prior to, a branch in a system of biosynthetic pathways.

It was also found that the *arom*-1 mutant accumulates dehydroshikimic acid and also some protocatechuic acid. The former of these is to be expected, as being the precursor precedent to shikimic acid. The mutant also differs from the wild type in possessing the enzymes dehydroshikimic acid dehydrase and protocatechuic acid oxidase. These are not present in detectable amounts in the wild type grown under similar conditions. However, both of these enzymes are inducible. When the normal conversion of dehydroshikimic acid is blocked by the absence of the reductase, the acid accumulates and in so doing induces the formation of detectable levels of the "new" enzymes, both involved in the degradation of dehydroshikimic acid. The dehydrase degrades shikimic acid to protocatechuic acid and as this accumulates the oxidase is induced to form.

Secondly, a single enzyme may catalyze similar reactions in two pathways, in which the intermediates and the terminal products are analogues. The best instance concerns the biosynthesis of isoleucine and valine, in which a series of four enzymes, each specified by a different gene, is com-

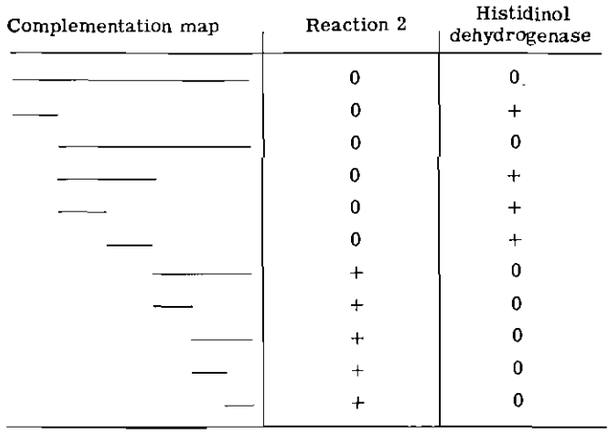
mon to the two pathways (see, for instance, Myers and Adelberg, 1954; Wagner *et al.*, 1960; Bernstein and Miller, 1961).

A similar behavior is found where one enzyme, controlled by one gene, catalyzes two distinct reactions in the same pathway. In adenine biosynthesis, all mutants at the *ad*-4 locus are alike in lacking two enzyme activities; (1) the splitting of 5-amino-4-imidazole-(*N*-succinylocarboxamide) ribotide (SAICAR) to the desuccinylated derivative AICAR) and (2) the splitting of adenylosuccinate to adenosine monophosphate (Giles *et al.*, 1957; Giles, 1959). Fractionation of extracts of wild type has not resulted in separating these two activities, and they generally remain in constant ratio in mutants in which adenylosuccinase activity is reduced. Consequently, it is highly probable that the two activities are functions of a single enzyme and that the same active site is involved in both activities. In one instance, it appears that a mutant which had regained adenylosuccinase activity by reversion had done so in such a way as to result in different relative activities toward the two alternative substrates. This secondary mutant has a disproportionally low activity in the splitting of SAICAR.

This situation may be compared with the unique case in *Salmonella* where one gene specifies two enzymes, one (histidinol dephosphorylase) being provided by the monomer of the protein specified by the gene and the other (imidazole glycerol phosphate dehydrase) being provided by the tetramer (Loper, 1961). In *Neurospora*, these two enzymes are controlled by distinct unlinked genes, perhaps one being evolved from the other. Whether any similar diversity of composition attends the two enzymic functions determined by *ad*-4 remains to be discovered.

Definite exceptions appear to be presented by the following cases. One gene in *Neurospora*, *his*-3, appears to specify at least two enzymic activities, perhaps borne by the same protein. One enzyme is histidinol dehydrogenase, and the other is the enzyme that catalyzes the second step in histidine biosynthesis. All mutants which affect one or other or both of these enzymic activities are allelic by the physiological test of complementation in heterokaryons (Catcheside, 1960b). There is a large group of noncomplementing mutants, which lack both enzymic activities. It is the existence of this large group of mutants, many at least due to point changes, as may be demonstrated genetically, which shows that a single gene is involved. The correlations between these properties are shown in Fig. 2 in which, it should be pointed out, the complementation map is but one of sixteen possibilities.

The early steps of biosynthesis of histidine are not yet very well understood, and there is a possibility that two or even three early enzymic steps, the second and third (and perhaps fourth), in biosynthesis of histidine are controlled by *his*-3^{+}. Moreover, these enzymes have yet to be



Complementation map	Reaction 2	Histidinol dehydrogenase
	0	0
	0	+
	0	0
	0	+
	0	+
	0	+
	+	0
	+	0
	+	0
	+	0
	+	0

FIG. 2. Complementation map of *his*-3 in *Neurospora crassa* showing the enzymes of histidine biosynthesis possessed by the groups of mutants (Catcheside, 1960b and unpublished).

isolated and examined to see whether they are proteins distinct from histidinol dehydrogenase. Alternative interpretations of the system as an operon have been put forward by Giles (1963).

There are also cases of two genes being concerned in the specification of the structure of one enzyme. For example, there is an enzyme (an isomerase), concerned in the biosynthesis of leucine (Gross, 1962), which converts β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxycaproate. There is evidence to support the view that this enzyme is constituted of at least two proteins, each specified by a distinct gene. Mutation at either of the loci *leu*-2 and *leu*-3 leads to loss of the isomerase. Many of the *leu*-2 mutants complement one another. Double mutants, involving one of the complementing *leu*-2 mutants (R86) with *leu*-1, *leu*-3, and *leu*-4, respectively, have been prepared and examined for their ability to complement 26 different *leu*-2 mutants with which R86 can normally complement. It was found that most of the combinations with *leu*-2 (R86) *leu*-3 showed a marked reduction in efficiency of complementation. No such dramatic reduction would be expected if *leu*-2 and *leu*-3 determined the structure of two different enzymes or if complementation involved some kind of recombination of protein fragments or protein forming systems to form completely normal protein.

A more complex situation is presented by a case in which two genes cooperate to produce one protein which appears to have two enzymic functions. These are malate dehydrogenase and aspartate aminotransferase

(Munkres and Richards, 1964). In *Neurospora* this bifunctional enzyme is composed of two distinct polypeptides, each determined by a distinct gene, *ma*-1 and *ma*-2, respectively. Both enzymic activities remain together in constant proportions during purification of the protein. The protein has been shown to consist of three units of the kind determined by one gene, combined with one unit of the kind determined by the other gene.

In tryptophan biosynthesis, it is probable that anthranilic acid synthetase, which catalyzes the conversion of chorismic acid to anthranilic acid (Gibson and Gibson, 1964), is composed of two heterologous polypeptides, determined respectively by *try*-1$^+$ and *try*-2$^+$. Moreover, *try*-1$^+$ also determines the structure of the enzyme, indole glycerol phosphate synthetase, which catalyzes the conversion of phosphoriboanthranilic acid to indole glycerol phosphate. It has been found (De Moss and Wegman, 1964) that some *try*-1 mutants lack both of these enzymes, while others lack only anthranilic acid synthetase. The latter are those found (Ahmad and Catcheside, 1960) to be able to grow on anthranilic acid. It is also possible that some other mutants have only an impaired indole glycerol phosphate synthetase.

These various instances, which refine the theory of one gene being responsible for one enzyme, provide valuable evidence about metabolic economy and also about possible routes of enzyme and gene evolution. It is perhaps too early to speculate much about possible connections, but on the kind of evolution of biosynthetic sequences by the reverse process proposed by Horowitz (1945), one would expect relationships between successive enzymes in a biosynthetic sequence since these have substrates in common and so might be expected to be similar in structure. Evolution, it now appears, may also involve production of a new enzymic activity by (1) a different degree of aggregation of the same basic protein unit, (2) the aggregation of two different protein units, or (3) the fusion of two units so that they are then determined primarily as one polypeptide. It may be predicted that the higher aggregates of the same protein units and the hybrid aggregates will be found to be more characteristic of the earlier steps in intermediary biosynthesis, than of later steps, if the Horowitz principle of reverse evolution applies.

VII. GENE INTERACTION

Genes control steps in biosynthetic reactions, by determining the specific enzymes. Each discrete step makes chemical sense. Organization is imposed on the system, even in homogeneous solution, by the specificity of the enzymes. Even in solution a highly ordered series of reactions can occur because of this specificity. Not only are the individual reactions

linked together by common substrates or products, but the different multi-enzyme systems may be linked together by their use of common substrates, by their use of common enzymes, by mutual inhibition, or by other mechanisms. Examples of several of these have already been described above. Indeed the whole of the metabolism of an organism is an interrelated system even though it is often convenient to consider the parts separately.

Some special properties of metabolism may be revealed by the ways in which metabolites act upon mutants. Inhibition of growth is an instance. Arginine mutants are inhibited by lysine and lysine mutants by arginine. Histidine mutants are inhibited by certain combinations of amino acids, including a basic amino acid such as either arginine or lysine together with one of a number of others, including tryptophan, methionine, leucine and phenylalanine. In this case, there is inhibition of uptake of histidine Mathieson and Catcheside, 1956), presumably by preventing histidine having access to a permease which provides an essential pathway into the cell. The same property is shown by the wild type, which is not dependent upon histidine for its growth. Competitive reactions are discussed by Emerson (1950). There are also negative feedback mechanisms in which the end product of a series of reactions acts to inhibit one or more of the earlier steps in the chain, thereby controlling the rate of its own formation. The inhibition may be either of enzyme formation (repression) or of enzyme action through allostery or inhibition. Very little is known about the genetics of these relations in fungi; the leads given by work on *Escherichia coli* need following. Inhibition of enzyme activity by genetically determined inhibitors has been found for tryptophan synthetase by Hogness and Mitchell (1954), but the mechanism is not understood.

If genes control the step reactions of metabolism which are themselves organized into multi-enzyme systems and if these systems interact, then the genes which control the step reactions will also appear to interact. Gene interaction in these cases is not really an interaction of the genes themselves, but is instead an interaction of the gene-controlled step reactions in nongenic parts of the organism.

It will be the purpose of this section to indicate two levels at which interaction may occur in nongenic parts of the organism. One operates at the level of the reactions catalyzed by the enzymes, the other at the level of the construction and shaping of the enzymes themselves.

A. Interaction of Reactions: Suppressors

It is a fairly common experience to find that a requirement for a growth factor caused by mutation of one gene may be relieved by mutation of

another gene. The latter is a suppressor mutation, and often this is its only obvious property. Closer study of various systems has disclosed a variety of mechanisms.

Suppressor genes do not duplicate the activity of the normal gene. Although suppressed mutants have enzymatic activity not present in the nonsuppressed mutant, the specificity of the system still resides with the mutant gene responsible primarily for the absence or abnormality of the enzyme. Among *tryptophan*-3 mutants, many have been found able to yield revertants through the action of suppressors. These are highly specific to individual *try*-3 alleles. The nutritional properties and growth characteristics of the four possible combinations of *try*-3 and the suppressor, *su*, are as follows: *try*-3 + requires tryptophan for growth; *try*-3 *su* grows without tryptophan, but often less vigorously than normal; + *su* nearly or quite like wild type in growth, in absence of tryptophan; + + wild type growing well without tryptophan.

There is a suppressor which acts on the temperature-sensitive allele *td*-24 (Suskind and Kurek, 1959); the enzyme produced is sensitive to zinc in a way similar to the enzyme formed when *td*-24, lacking the suppressor, is grown at a temperature above 30°C (see p. 668). In this case it appears that the enzyme may be shielded from the inhibitory action of zinc by a reaction which occurs above 30° or, at a lower temperature, in the presence of the suppressor gene. It is the inhibitor concentration that is altered, rather than the nature of the enzyme.

A fairly general method of suppressor action is the introduction of a second genetic block into the metabolic system, the second lesion in some way compensating for the effect of the first.

Strauss and Pierog (1954) described mutants of *Neurospora* which require acetate for their growth. They are inhibited by hexose sugars such as glucose, but will grow with pentoses if acetate is added to the medium. Two different nonallelic suppressors, or modifiers, relieve the inhibition by glucose and also permit some growth in the absence of acetate. Both suppressors lower the activity of pyruvic carboxylase. This is apparently merely a quantitative effect, without any qualitative change in the pyruvic carboxylase, which is indistinguishable from that of the normal. Lowering the pyruvic carboxylase activity reduces the amount of acetaldehyde formed from glucose by the mutants, and it is likely that the acetaldehyde or some derivative of it is the cause of the glucose inhibition. The small amounts of acetaldehyde still formed in the suppressed acetate mutants are oxidized directly to acetate, thus providing a bypass of the blocked reaction and permitting some growth in the absence of exogenous acetate. The suppressor genes restore the organism towards normal by introducing

a second block which lowers the production of an inhibitor, which tends to accumulate because of the primary lesion.

A famous and intricate case is presented by the suppressor of *pyrimidine*-3 discovered by Houlahan and Mitchell (1947). This was found to be specific in its action to some, but not all, *pyr*-3 mutants. The combination *pyr*-3^{a} *su-pyr*-3 was able to grow without uridine, but this relief of the requirement of the metabolite was reversed by addition of arginine to the medium. Later, further complexities were disclosed, including cases in which certain *pyr*-3 mutants, but not any suppressible by *su-pyr*-3, acted as suppressors or modifiers of the properties of mutants in the arginine pathway (Houlahan and Mitchell, 1948; Mitchell and Mitchell, 1952). This complex situation long lacked any apparent unifying principle, until it was suggested that certain *pyr*-3 mutants were abnormally sensitive to arginine and that the *su-pyr*-3 gene led to a relative deficiency in the synthesis of arginine (Davis, 1961).

It was also found that *pyr*-3 mutants fell into three major classes in respect of their biochemical properties (Davis, 1960; Davis and Woodward, 1962; V. W. Woodward, 1962). All members of one of these classes (I), the largest group, are unable to complement one another or, indeed, any members of the other two classes. Members of the two smaller classes (II and III) are able to complement one another, II with III, but not pairwise within either class. The members of classes I and III lack the enzyme aspartic acid transcarbamylase, by which the synthesis of ureidosuccinic acid from aspartic acid and carbamyl phosphate is catalyzed (Davis, 1960). All of the members of class II possess aspartic acid transcarbamylase, and it is these that are suppressible by *su-pyr*-3. It has become clear that these are impaired in the production of carbamyl phosphate, required specifically for the synthesis of pyrimidine. Thus the *pyr*-3^{+} gene controls two enzymic functions, or an enzyme with two functions, namely the synthesis of carbamyl phosphate and its utilization in the aspartic acid transcarbamylase reaction resulting in the formation of ureidosuccinic acid.

Carbamyl phosphate is also used in the biosynthesis of arginine, specifically in the synthesis of citrulline from ornithine catalyzed by ornithine transcarbamylase (OTC). The action of *su-pyr*-3 and the relation of the arginine and pyrimidine syntheses is accounted for by the two separate sources of carbamyl phosphate. One is specific for arginine synthesis and its production is catalyzed by carbamyl phosphate kinase, determined by *arg*-3^{+} (Davis, 1963; Davis and Thwaites, 1963). The other source is used for pyrimidine synthesis, the specific enzyme being determined by *pyr*-3^{+} (Davis and Woodward, 1962). The *su-pyr*-3 mutant has an un-

usually low ornithine transcarbamylase activity (Davis, 1962a,b), only about 3% of that characteristic of the normal organism. This means that normally much of the potential production of carbamyl phosphate in the pathway of arginine biosynthesis is not utilized for arginine synthesis in the *su-pyr*-3 mutant. Those *pyr*-3 mutants that are suppressible therefore have this unusual source of carbamyl phosphate made available to them and so are enabled to grow. This source of carbamyl phosphate is, however, extinguished in the presence of arginine, because the latter represses carbamyl phosphate kinase.

The relations of the known genes to the relevant enzymes and reactions are shown in Fig. 3.

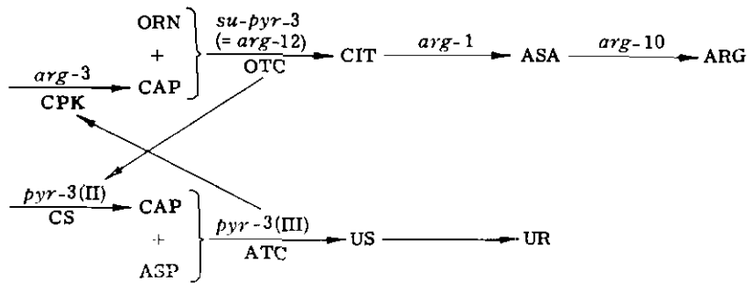


FIG. 3. Interaction of arginine and uridine biosyntheses. The arrows indicate the mutant genes which are suppressed and the mutant genes which suppress them. Abbreviations: Substrates: *ORN*, ornithine; *CAP*, carbamyl phosphate; *CIT*, citrulline; *ASA*, arginino succinic acid; *ARG*, arginine; *ASP*, aspartic acid; *US*, ureidosuccinic acid; *UR*, uridine. Enzymes: *CPK*, carbamyl phosphate kinase; *OTC*, ornithin transcarbamylase; *CS*, carbamyl synthetase; *ATC*, aspartic acid transcarbamylase.

It has been noted that suppressors are generally specific for each allele a given suppressor acting on some alleles, but not all. If the nature of the defect in the enzyme were specific for each allele, the specificity in the kind of suppressor mutant which could ameliorate its effects would be expected. This idea is particularly attractive partly because it avoids the necessity of supposing that two or more or many genes could directly affect the structure of the same enzyme. It also avoids the supposition that there are several equivalent potential pathways of biosynthesis and that a trivial change in a gene may result in opening up a pathway previously inoperative.

In the instances of suppressors considered so far, no change has occurred in the mutant enzyme, the nutritional effects of which have been relieved by an adjustment elsewhere in the genotype. In other cases significant amounts of normal enzyme are produced as well as large

amounts of homologous inactive protein, detectable by its cross reaction with antiserum. This is true for the suppressed combinations *td*-2 *su*-2 (Yanofsky, 1952) and *td*-201* *su*-201 (Yourno and Suskind, 1963). Several of these suppressors, when alone in wild type, actually depress growth to a small degree. Their precise mode of action has not been established, but possible modes of action include correction of the primary structure of the protein, by replacement of the wrong amino acid at the relevant position in the polypeptide, and correction of the tertiary structure without correction of the primary fault. The latter possibility has usually been dismissed on the assumption that a given primary structure could give rise to no more than one stable tertiary configuration. However, there are now several instances of mutants with no apparent change in the primary structure, and mutation of a regulatory gene affecting the tertiary structure assumed by a polypeptide may be suggested. There are other considerations which suggest that the tertiary structure assumed by a given polypeptide may change with other conditions. These include temperature, pH, and salt concentration. It is therefore not unlikely that genetic factors could influence shape and therefore activity, by modifying the cellular environment.

Some suppressors may act on more than one reaction. For example, in the biosynthesis of methionine (Giles, 1951; Fischer, 1957) the same suppressor restores activity to two nonallelic mutants (H98 at the *me*-2 locus and 4894 at the *me*-7 locus) concerned with the different stages in the pathway. The loci are in separate linkage groups, *me*-2 in IV and *me*-7 in VII; the normal alleles control distinct cystathionases, as shown in Fig. 4. The mechanism in this case is unknown.

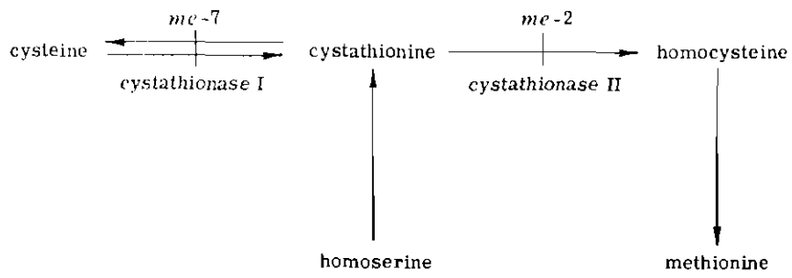


FIG. 4. Stages in methionine biosynthesis in *Neurospora crassa* affected by the same suppressor gene.

In *Escherichia coli*, Yanofsky *et al.* (1962) have been able to show that similar suppressors act by normalizing enzyme structure. While some

* *td*-201 is a new designation of A78: the latter should be used.

of these might act by correcting tertiary folding, the only direct evidence points to there being an alteration of the amino acid sequence of some of the polypeptide chains of the enzyme, but in an unspecific and accidental manner. It is suggested that such suppressors have a primary effect on one of the enzymes catalyzing the transfer of amino acids to transfer RNA, or else on a transfer RNA itself, since the latter is presumably also under the control of a gene. Such alterations would have the effect of resulting in one amino acid being incorporated in place of another. However, since such alterations would have general effects upon all proteins formed in the cell, some restrictions would be required to permit the cell to be viable. One suggestion is that the error is made only in a portion of cases, namely that only a small proportion of a given tRNA receives the wrong amino acid B instead of its correct one A. Now, if the primary alteration in a given suppressible mutant were missense, instructing the substitution of the amino acid B in the normal by the amino acid A in the mutant, it would be possible to make a small amount of normal protein if the suppressor mutant resulted in the occasional insertion of the amino acid B. That is, although the transfer RNA would be obeying the instruction of the messenger RNA at the site of the mutation, it would sometimes insert the wrong amino acid because it had been wrongly charged. For some mutants the mistake would result in the production of a small amount of normal enzyme, enough to promote growth in the absence of the growth factor. Naturally, all proteins would be affected and the organism could tolerate this general effect only if the probability of error were relatively small and perhaps only if the error affected the less common amino acids.

In yeast, Hawthorne and Mortimer (1963) have described supersuppressors, which act simultaneously upon several nonallelic mutants. There are several supersuppressors each with a nearly identical action spectrum; that is, all act similarly on the same series of mutants. They are specific in their action in that they act upon only a fraction of the different alleles at a given locus, but they do so at several different loci. The super-suppressors act upon about a quarter of all the gene mutants examined.

Although the mechanism postulated above for missense mutations might act on a number of allelic and nonallelic mutants, it is believed that the class of mutants affected by the supersuppressors is different and that these suppressible mutants are nonsense mutants. In nonsense mutants, the mutational alteration is supposedly to one which results in an interruption in the polypeptide chain when it is being formed on the messenger RNA. Thus only a fragment of the protein is produced. The action of the suppressor would be to alter a transfer RNA so that it would detect the non-

sense site as one at which to attach and so provide an amino acid for the developing protein. The effect would be to form a complete protein, *sometimes like the normal and sometimes of a missense type sufficiently* like the normal to possess enzymic activity.

B. Resistance to Inhibitors

Growth of wild-type *Neurospora crassa,* like that of many other organisms, is subject to inhibition by a variety of agents, including analogs of amino acids. The most extensive studies concern reaction to sulfanilamide (Emerson, 1947), which presumably often acts by interfering with metabolic reactions dependent upon *p*-aminobenzoic acid. Resistance to the analog usually appears to develop through an enhanced production of the normal metabolite, but occasionally bizarre relationships are found. Thus there is one mutant, *sfo,* which requires sulfanilamide for its growth, though its nutritional requirements are also met even more satisfactorily by the provision of threonine.

Mutants exhibiting resistance to analogs of amino acids are of particular interest since they may involve mutation of one or other of the elements of the system concerned in the building of amino acids into proteins. In the normal organism, which is sensitive to various of these analogs, it is found that the analog is incorporated into proteins replacing a certain amount of the amino acid which it resembles. The protein containing the analog is defective in character and unable to perform the function that it would have in the absence of the contaminating analog. Hence the organism is unable to grow satisfactorily.

Stadler (1963) has reported mutants which are resistant to 4-methyltryptophan and in which resistance is due to inability of the analog to enter the cells. The mutation affects a gene which determines the presence of the permease which is necessary to bring tryptophan and the analog into the cell. In this case resistance arises by exclusion of the analog from the cell.

Canavanine is an analog of arginine and several resistant mutants are known. Resistance is apparently due to the negligible replacement of arginine in the proteins of the resistant strain, whereas such replacement occurs significantly in the sensitive strain (Bauerle and Garner, 1963).

In a *Coprinus* mutant which is resistant to ethionine, there is good evidence that mutation has occurred of the structural gene for the enzyme that activates methionine (Lewis, 1963). In this mutant, there is almost no activation of ethionine, in contrast to the normal in which ethionine can replace methionine.

C. Interaction between Alleles

In this section there will be considered interactions which may occ between similar products of allelic genes, probably as a rule betwe proteins, but perhaps also between the nucleic acid systems responsil for protein synthesis. Broadly, two kinds of such interaction have be detected, but only one of them, allelic complementation, is known to oc in fungi, in which it was discovered.

The proportions of primary products, in the form of enzyme or p tein, may not always be equivalent to the proportional representation different alleles in the nuclei and cells. This has been observed with c tainty only in relation to the formation of hemoglobins in man. Thus, allelic genes for A and S hemoglobins, respectively, the normal and sic types are in the ratio of 1:1 in heterozygotes, but there is 6(of A and 40% of S in the hemoglobin. The disproportion is not alw in favor of the normal, e.g., the A I heterozygote shows only 30% o compared with 70% of I hemoglobin. The mechanism of this interac is obscure.

Secondly, in many cases, pairs of alleles, each producing inactive zyme, may together produce an active enzyme (Catcheside, 1962). T is the complementation between alleles noted at the beginning of this ch ter, and for which complementation maps, representing some kind functional relationship, may be drawn.

The principles may be illustrated by reference to a particular exam namely *tryptophan*-1 (Ahmad and Catcheside, 1960; Catcheside, unpul Some 120 *Neurospora crassa* mutants at this locus have been classifie

	A	B	C	D	E	F	G	H	I	No. in group
A	.	0	0	0	0	0	0	0	0	43
B	0	.	+	+	+	+	+	+	+	14
C	0	+	.	0	0	0	+	+	+	2
D	0	+	0	.	0	0	0	+	+	1
E	0	+	0	0	.	0	0	0	+	14
F	0	+	0	0	0	.	0	0	0	38
G	0	+	+	0	0	0	.	0	0	1
H	0	+	+	+	0	0	0	.	0	1
I	0	+	+	+	+	0	0	0	.	6
										120

FIG. 5. Matrix and complementation map of *Neurospora crassa try*-1 mu Catcheside (unpubl.).

complementation tests. These tests divide the mutants into nine groups (Fig. 5) distinguished by the patterns of complementation which are shown by each of them. In the matrix, the members of one large group, 43 in number, show no complementation with one another nor with any member of any of the other eight groups. As previously noted, it is this substantial group of noncomplementing mutants which provides the common factor holding the whole group together as alleles, representing various different alterations of one normal integrated function, presumably the determination of a protein with enzymic functions. The matrix of interaction of the group of alleles provides information about the organization of the function. The complementation map is a diagram which represents the information in the matrix in a more readily grasped pictorial form. The diagram assumes that the normal function can be represented by a geometrical figure, in which various parts are represented as defective in each of the distinguishable kinds of mutants. It has been found that in the majority of cases the normal may be represented by a line in which each kind of mutant may be shown as a single continuous segment of defect, either short or long (see Fig. 5). It is a very general finding that the map of function can be represented in one dimension, with one defective region for each mutant, even though the number of distinguishable segments may become relatively high.

Instances in *Neurospora* in which linear complementation maps have been found include *arg*-1 (Catcheside and Overton, 1958), *arg*-10 and *arg*-6 (Catcheside, 1960a); *his*-1, *his*-2, *his*-3 and *his*-5 (Catcheside, 1960b; unpubl.); *try*-1; *try*-3 (Ahmad and Catcheside, 1960; Lacy and Bonner, 1961); *lys*-5 (Ahmad and Catcheside, unpubl.); *pan*-2 (Giles, 1959; Case and Giles, 1960); *ad*-3B (de Serres, 1963, 1964; Brockman and de Serres, 1963). In a few cases, matrices which require two dimensions for their representation as complementation maps have been found. However, the one first reported for *his*-1 (Catcheside, 1960b) is incorrect. A circular complementation map has been reported for *leucine*-2 (Gross, 1962). The matrix for *ad*-8, which controls adenylosuccinate synthetase, is consistent with a circular complementation map (Ishikawa, 1962; Kapuler and Bernstein, 1963). The data reported by Bernstein and Miller 1961) for *iv*-3 are also compatible with a circular or polygonal map, subject to what some of the interactions not reported may be.

Similar behavior is also known to be characteristic of several genes in yeast, e.g., *try*-5 (Manney, 1964) and *ad*-5,7 (Roman, 1956); there are reports also of nonlinear maps.

Complementation between alleles does not restore the heterokaryon completely to the wild-type characteristics. Indeed, a wide range of vigor is shown. This may be measured in terms of the time taken for a mixture

of conidia to form a heterokaryon on minimal medium, the growth ra or the amount of growth in a given time on minimal medium, and tl level of enzyme activity, in those cases in which this can be assaye Where enzyme activities have been measured, they have never exceed 25% of that in a comparable specimen of the wild type (Fincham, 1959 Giles, 1959). Commonly the activities have been much smaller. The low activities are in marked contrast to the 50% levels found in heter karyons made between nonallelic mutants. Moreover, the enzymes in heter karyons formed by alleles are usually unlike the normal enzyme in respe to various physicochemical properties.

In some cases, allelic complementation may occur by a process anal gous to the pooled metabolism shown by pairs of nonallelic mutants heterokaryons. One mutant, partially blocked at one stage of a biosy thesis, is able to produce a compound which can be utilized by anoth mutant also partially blocked, but in a different manner, at the same sta of the biosynthesis. Thus, there are *try*-3 mutants which can produ indole upon which other *try*-3 mutants can grow (Ahmad and Catchesid 1960; Suyama *et al.*, 1964). A similar situation is shown by *try*-1 mutar with respect to the accumulation and utilization of anthranilic acid (Ahm and Catcheside, 1960). However, many *try*-3 mutants neither accumula nor grow upon indole, but yet pairs of them complement. Similarly, ma of the *try*-1 mutants that accumulate anthranilic acid complement o another in heterokaryons (Fig. 5). Hence not all complementary inte actions can occur at the level of intermediary metabolism.

It may be assumed that the site of interaction of the alleles is in tl cytoplasm, barring the possible movement of gene products betwe nuclei leading to interactions. More direct interaction between nuclei, lea ing to effective recombinants, is excluded by the failure to find recor binant nuclei in the heterokaryons. In every case where the compone nuclei have been recovered from the heterokaryons they have been fou to be unaltered by the association. Hence the interaction must be betwe gene products.

Several kinds of such interaction may be considered (Catcheside a Overton, 1958). The two principal levels of interaction are (1) the form tion and action of the templates upon which the polypeptides, out which enzymes are fashioned, are built; (2) the final shaping of enzym including the folding of polypeptides into their tertiary shapes and aggregation of these units into a polymer. In either case, complemen tion must involve the cooperation or aggregation of two or more homo gous, though somewhat different, gene products (ribonucleic acids) derivatives (proteins).

Of the two kinds of cooperation possible, actual or effective recombi

tion between differently defective ribonucleic acids or polypeptides seems unlikely, for it seems impossible to construct any consistent system whereby recombination could occur in some cases but not in others. *A priori,* it would seem that a recombinational mechanism ought to operate between all pairs of mutants which are defective at different genetic sites. This is not found especially between many mutants whose sites of mutation are far apart. Indeed, there are reports of complementation between mutants altered at the same site, presumably in different ways. Also, there are reports of different mutants, altered at the same site, only one of which will complement some other individual mutant. The effect therefore seems to depend upon qualitative rather than positional properties.

These considerations therefore rule out all hypotheses which entail actual recombination between ribonucleic acid templates or polypeptides, or any mechanism which is effectively similar in its results. An example of the latter would be the partial formation of a polypeptide on one imperfect template and its completion on a complementarily defective template. The most probable hypothesis is that the interactions are between polypeptides or more particularly the protein units formed when the polypeptides have folded to form their tertiary structure. This theory of interaction at the protein level (Catcheside and Overton, 1958; Brenner, 1959; Fincham, 1960; Crick and Orgel, 1964) supposes that complementation is possible only when the normal enzymes are aggregates of two or more homologous units, associated together to make the quaternary structure of the protein.

An alteration at a site in the deoxyribonucleic acid of a gene would be expected to cause a corresponding alteration in a ribonucleic acid and this, in turn, to cause a change in the sequence of amino acids specified in a polypeptide. In any particular case this may be merely the substitution of one amino acid for another at a particular site; more drastic effects resulting in the formation of a deficient polypeptide are less likely to allow allelic complementation. In a standard cellular environment, the tertiary shape assumed in a protein unit will depend upon the sequence of amino acids in the polypeptide. A change at any point in the sequence could mean a change in shape, a possible lack of correct mutual fitting in homogeneous aggregates and so a possible lack of enzyme activity. The precise change of shape, its extent and its effect on aggregation and function would depend upon the precise position and nature of the substitution in the polypeptide. Some changes would be fairly slight and others more drastic. In the heterokaryons, there would be the opportunity for formation of mixed polymers, any particular one of which might contain equal or unequal numbers of the two kinds of defective protein unit.

Several sorts of interaction might occur in such polymers. Defects in

two units might correct one another mutually. For example a defect in one monomer might, after aggregation, be situated beside a complementary defect in the other type of monomer, so that the two compensate mutually and active enzyme is formed. Such mutual local correction of the effects of two mutations is possibly responsible for some cases but, if it were at all common, circular and more complex complementation maps would be very frequent. In fact maps other than linear are the exception.

It is more likely that complementation is usually due to the correction, at least partially, of the misfolding of one monomer by an unaltered part of the other monomer. It is perhaps easier to conceive how such correction could occur if the misfolded region were in near contact in the regions where the monomers adjoin one another in the polymer. But the possibility of a folding correction being transmitted through the monomer to a more distant region of it, not in the contact zone but on the outer surface, should be kept in view. It is plausible that it is the precise shape of the active site on the surface of the polymer which is important.

Side-chain interactions between stretches of polypeptide chain adjacent to each other appear essential for protein shape and stability. In a polymer these interactions would be partly between different stretches of the same polypeptide and partly between stretches of different polypeptides. It is the latter which permit or determine correction of local misfolding and which may lead to correction of distortions of the shape which have occurred in more remote parts of the monomer. In general it would appear likely that the only mutants which should complement are those which distort the shape in those regions that are contact surfaces between monomers. Complementation might occur in pairs of mutants, one or both of which have a distorted shape in this region that may be corrected by a normal stretch of the other's protein.

Construction of a detailed hypothesis of complementation is handicapped by the general lack of knowledge of the architecture of proteins which show the effect. Our only model is the hemoglobin molecule and if its type of symmetry is general, the usual elements of symmetry would be axes of rotation. Crick and Orgel (1964) argue that to achieve complementation the misfolded region must lie so that it can only be corrected by part of the homologous region, correctly folded, in one of the other monomers of the polymer. This is most likely to occur in the regions adjacent to the axes of rotation of the polymer.

Mutants which complement would be expected to produce a protein homologous with the enzyme. Among *tryptophan*-3 mutants, Lacy and Bonner (1961) have found that complementation occurs only between mutants which produce a protein immunologically similar to the enzyme. Of course, only some pairs of such mutants do complement.

If the enzymes produced by heterokaryons were hybrid proteins of the kind suggested, they would be expected to differ from the normal by various physicochemical properties. Fincham, who has contributed most to knowledge of these matters, has pointed out that at least one of the components of a complementing pair produces an enzyme which, though virtually inactive, is capable of activation by appropriate treatments. These features have been well demonstrated (Fincham, 1957, 1962) for various *amination* (*am*) mutants, which are defective for the glutamic dehydrogenase which is dependent on $NADPH_2$ (reduced nicotinamide adenine dinucleotide). The heterokaryon formed of am^1 and am^2 produces an enzyme with 10% or less of the activity of the wild type at 20°C and has a very low stability at 60°C. This enzyme differs from the normal in its capacity for thermal activation when the temperature is raised to 35°. Quite brief heating at 35° fully activates it, but the increased activity is lost gradually following cooling to 20°, to be regained by further heating. The heterokaryon of am^1 with am^3 produces an enzyme with about 20–25% of the activity of the wild type and is more thermolabile at 60° than is the wild type, though less so than the enzyme of $am^1 + am^2$. It also exhibits a lower affinity for glutamate.

It may be predicted that conditions can be found in which complementation may be secured *in vitro* between preparations made from mutants grown separately. Probably the necessary conditions would be different for each enzyme and possibly show variations for different pairs of alleles. Nevertheless considerable success has attended the experiments of D. O. Woodward (1959) with *adenine*-4 mutants and of Fincham and Coddington (1963) with *amination* mutants. For adenylosuccinase (*ad*-4) Woodward found that reducing conditions were particularly favorable. For glutamic dehydrogenase (*am*), Fincham and Coddington found that acid conditions favored hybridization *in vitro;* indeed, interaction *in vitro* occurs at any pH removed from neutrality.

The reaction system catalyzed by glutamic dehydrogenase may be represented by the equation

$$\alpha\text{-ketoglutarate} + NADPH_2 + NH_3 \rightleftharpoons \text{glutamate} + NADP$$

in which the equilibrium position is normally well to the right. The protein isolated from am^1 shows no activity for the reaction in either direction, but am^3 may be activated by NADP and high concentrations of glutamate to catalyze the back reaction from glutamate to α-ketoglutarate. When eluted through a chromatographic column of DEAE-cellulose by means of a linear gradient of phosphate buffer, the am^3 protein appears somewhat sooner than does the am^1 protein. A mixture of the two may therefore be separated to a significant extent, though their distributions overlap some-

what. The hybrid protein comes through the column with intermediate speed. Thus the separation of the three, while imperfect, is sufficient for recognition. When purified *am*^{1} and *am*^{3} proteins are mixed at pH 7.4 and passed through the column, only these two are observed. However, if the mixture is first acidified to pH 5.8 and then returned to pH 7.4 before chromatographic separation, the hybrid protein, with activity in the forward direction from α-ketoglutarate to glutamate, is formed in substantial yield. Experiments in which the proportions of the two mutant proteins were varied, showed a well-defined optimum ratio of about 5 parts of *am*^{1} protein to 2 parts of *am*^{3} protein, if the concentration of protein was held constant. However, the optimum ratio could be further raised to 5 parts of *am*^{1} protein to 1 part of *am*^{3} protein, if the concentration of the latter was held constant. It appears as though the *am*^{3} enzyme were being replaced progressively by complementation enzyme, the latter being very greatly more effective in the forward reaction and slightly more effective in the back reaction.

The mechanism whereby activation of one defective protein by another occurs is obscure. Although there is evidence that glutamic dehydrogenase consists of a number of units, probably identical and six or eight in number, there is no indication of dissociation of either mutant protein at pH 5.8. Although it is believed that complementation enzyme is formed as a result of the exchange of units between the interacting proteins, there is as yet no direct evidence that the complementation enzyme does contain both types of unit.

Genetic maps are linear representations of the relative positions of substitution of nucleotide pairs in a filamentous molecule, deoxyribonucleic acid. They will also be reflected in substitutions of single amino acids at particular sites in another filamentous macromolecule, the polypeptide chain, in the case of missense mutations. In the case of nonsense mutations, the sites of genetic change will represent points where polypeptide formation has ceased; this sort of mutant will usually be incapable of taking part in allelic complementation. On the other hand, complementation maps are representations of matrices which record the pairs of mutants that are able to complement one another. It is believed that the segments shown by a complementation map represent regions of misfolding of the tertiary structure of the protein, due to the genetically determined substitution of a "wrong" amino acid for the "correct" one of the normal. The segments will be separable according to the degree to which misfoldings of different extent and different, overlapping distribution are capable of correction by different regions of the normally folded parts of the complementing protein. Comparison of genetic and complementation maps may be expected to show in a general way how the tertiary folding is organized. If the segments

of the complementation map followed the same order as the sites of mutation in the genetic map, so that the two could be described as colinear, the type of folding would be a fairly simple zigzag. However, the folding is more likely to have a winding character leading to intercalation of segments of the polypeptide chain between other segments which are relatively closer together as measured along the stretched chain. This should be disclosed by the comparisons.

Construction of genetic maps is a much more difficult and time-consuming task than is the construction of complementation maps. Consequently, the available information is limited, in *Neurospora,* to *pan*-2 (Giles, 1959; Case and Giles, 1960), *try*-3 (Suyama *et al.,* 1964), *me*-2 (Murray, 1960, 1963), *pyr*-3 (Suyama *et al.,* 1959), *ad*-8 (Ishikawa, 1962; Kapuler and Bernstein, 1963), *his*-5 (Smith, 1965), *his*-1 (Jessop and Catcheside, 1965), and *his*-3 (Catcheside, unpubl.). The generalizations that may be made and any individual peculiarities that may be noted must be accepted with the reservation that the genetic maps cannot be accepted with complete confidence.

The sites of the noncomplementing mutants are fairly generally distributed along the genetic map, though they do show some clustering and some stretches of the genetic map may have few or none. Exceptions to this general distribution are that (1) in *his*-1 the noncomplementing mutants are in the left quarter of the map, the complementing mutants being in the right three-quarters; (2) in *his*-3 the noncomplementing mutants are in the left half of the map intermixed with mutants defective for the earlier stages of histidine biosynthesis, the mutants defective only for histidinol dehydrogenase being in the right half of the map.

Complementing mutants belonging to the same complementation group are generally situated in the same region of the genetic map and are not intermixed with mutants belonging to other complementation groups which do not overlap it. This is in agreement with the expectation that mutants affecting the same region will usually be close together on the genetic map and not complement one another. Two kinds of exception may be noted. Mutants belonging to complementation groups which do overlap may be intermixed in the genetic map. It appears that the extent of misfolding depends upon the particular mutational change and the site at which it occurs. Secondly, in *pan*-2, mutants belonging to the rightmost complementation group M (with a "short" defect) are widely spread over the right half of the genetic map. They are separated into three groups by two mutants which belong to two overlapping complementation groups (I and J) in the middle of the map. The I and J mutants do not complement one another, but both are complemented by M mutants. The order of the genetic sites is, from left to right, MMMMJMIM. There is a strong indica-

tion that the physical basis of the M complementation group is composed of three separate regions of the polypeptide chain, presumably brought into association by folding.

Genetic maps sometimes appear to be colinear with complementation maps to a first approximation, but there are distinct exceptions. No comparison can be made for *me*-2 and *his*-1 since these do not have unique complementation maps. In *his*-5, mutants belonging to four complementation groups have been mapped. The order of the groups is G, B, H, and I with H and I overlapping one another. The order in the genetic map of the mutants, which belong to these groups, is BBGGGGIH. The two orders are not colinear, but indicate that the polypeptide chain is bent to bring the B region between the G and H-I segments. In *ad*-8, which has a circular complementation map, good correlation with the genetic map may be secured by coiling the latter into a spiral, in which contributions to some complementation groups are made by two more or less remote regions of the polypeptide chain (Kapuler and Bernstein, 1963). However, it cannot be easily supposed that the protein is merely a simple spiral. The clues provided by correlation of the genetic and complementation maps cannot be regarded as providing more than hints of the projection of the polypeptide chain on the complementing surface of the protein unit.

VIII. REGULATION OF GENE ACTION

Jacob and Monod (1961) have developed a comprehensive theory of gene action and regulation. They established for bacteria two kinds of gene, structural and regulatory. The latter, it is believed, determine whether and to what extent a given protein shall be formed, in a given set of circumstances, by the specific gene responsible for the enzyme. Cells of bacteria can adapt in enzyme content in two ways in response to environmental changes. One is enzyme induction, in which synthesis of an enzyme is evoked by presence of its substrate, usually an energy source or nutriment which cannot be utilized without the enzyme in question. The other is enzyme repression, in which formation of an enzyme, or a series of them involved in the biosynthesis of a particular metabolite, is repressed by the metabolite. Enzyme repression is distinct from the inhibition of existing enzymes by feedback inhibition. In the latter case, the excess of a metabolite combines with enzymes in the biosynthetic pathway, often the earliest enzyme in the pathway, and prevents it forming more intermediate compound.

However, induction and repression are basically two aspects of the same mechanism. In enzyme induction, there is release of a repression, an inducer antagonizing the action of a repressor, presumably by forming a

complex which is no longer active in preventing formation of the structural enzyme. In enzyme repression, it is the complex of repressor and metabolite that is active in preventing formation of the structural enzyme, subject to repression. The repressors appear to be specific, either for single enzymes or for groups of enzymes involved in the same process. In the latter case, in bacteria, the structural genes for all these enzymes are often in a closely linked group, e.g., the *tryptophan* and the *histidine* genes in *Salmonella,* and show coordinate repression, all being switched on or off together. A group of structural genes subject to coordinate repression by a common repressor is an operon (Jacob and Monod, 1961). Nevertheless, genes which are not closely linked may also be repressed by the same repressor and all freed from repression by mutation of a single gene, e.g., the *arginine* genes in *Escherichia coli,* which show coordinate repression.

The specific repressors are products of genes, recognizable by mutation. The repressors are apparently proteins, though at one time it had been thought that they were not, but instead perhaps ribonucleic acids. Adjacent to the structural gene or genes, subject to repression or to induction, is an operator gene, a segment of the genetic material which normally detects the repressor (or repressor–metabolite complex in the case of repression) and so acts to stop the structural genes of the operon from producing messenger material. The operator may itself mutate to a state in which it is insensitive to the repressor.

The three elements are therefore the structural gene, or genes, the adjacent operator, and repressor genes which need not be closely linked to the operon. So far no corresponding systems have been found in higher organisms, but systematic search needs to be made. It is highly important for several reasons. One is to determine whether the principles of metabolic regulation, elucidated in bacteria, apply to higher organisms. If they do then regulatory circuits of this kind, variously elaborated, would provide workable mechanisms for orderly development and differentiation (Monod and Jacob, 1961) and the disorderly and uncontrolled processes of cancer (Pilot and Heidelberger, 1963).

In *Neurospora,* as in higher organisms generally, there are very few cases of genes belonging to the same biosynthetic pathway being adjacent to one another in the genetic map. Two instances where this may be the case are *arginine*-1 and *arginine*-3 and *adenine*-3A and *adenine*-3B. Most cases of closely linked, biosynthetically related, genes in *Neurospora* show greater dispersion, e.g., the *isoleucine-valine*-1 and *iv*-2 genes which are four units apart in chromosome V and the four *aromatic* genes in chromosome II (Gross and Fein, 1960). Of the *aromatic* genes, *arom*-3 is relatively distant from *arom*-1 and *arom*-4, which are 0.3 units apart. The fourth gene, *arom*-2, does not complement *arom*-1 and *arom*-4 and lacks

four enzymes of the pathway, two of these being those missing in *arom*-1 and *arom*-4, a different enzyme in each mutant. *Arom*-2 may be a deletion covering yet other unknown *arom* genes, all part of the same tight cluster. However, it could also be a mutation in an operator gene controlling a tight cluster similar to the β-galactosidase one in *E. coli*.

Fairly recently, the *histidine*-3 region in *Neurospora* has been interpreted as an operon (Giles, 1963). Certainly the gene governs at least two enzymic functions, viz., histidinol dehydrogenase and one or more of the stages by which phosphoribosyl adenosine triphosphate is converted to phosphoribosyl formimino aminoimidazole carboxamide ribotide. However, it is as yet unknown whether these functions are borne by the same protein or by separate ones. So far, however, no operator nor repressor mutants have been recognized. The interpretation is based in part on the position in the genetic map of the noncomplementing mutants and in part on other mutants which appear to show a polarity with respect to the regions for which they are defective.

In *Neurospora,* quantitative effects are produced by some genes. Thus in the case of tyrosinase (Horowitz *et al.,* 1960), there are several alleles of *T*, producing structurally different enzymes, with characteristically different properties, especially of thermostability and electrophoretic mobility. In addition, there are recessive genes, *ty*-1 and *ty*-2, not allelic to one another nor to *T*, which drastically reduce the amount of tyrosinase, but without affecting its specific structure. Thus strains pure for *ty*-1 or *ty*-2 can be induced to form tyrosinase by addition of an aromatic acid to the culture medium, and in these conditions the tyrosinase produced depends upon the *T* allele present. The mutants *ty*-1 and *ty*-2 could be regarded as producing repressor substances, blocking activity of the *T* gene, unless an inducer compound (such as an aromatic acid) is present to complex with them and render the repressors inactive.

Metzenberg (1962) has described a case in which the levels of production of several enzymes has been affected by what appears to be a single gene alteration. In the wild type, invertase and trehalase are repressed to different degrees by different monosaccharides, mannose being the most effective. In the derepressed mutant, the specific activities of these enzymes are several times higher; smaller effects of the gene are shown in the amounts of maltase.

REFERENCES

Ahmad, M., and D. G. Catcheside. (1960). *Heredity* **15**:55–64.
Atwood, K. C., and F. Mukai. (1953). *Proc. Natl. Acad. Sci. U.S.* **39**:1027.
Barratt, R. W., and W. N. Strickland. (1963). *Arch. Biochem. Biophys.* **102**:66.
Bauerle, R. H., and H. R. Garner. (1963). *Genetics* **48**:882.

Beadle, G. W., and E. L. Tatum. (1945). *Am. J. Botany* 32:678.
Benzer, S. (1956). *In* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 70. Johns Hopkins Press, Baltimore, Maryland.
Bernstein, H., and A. Miller. (1961). *Genetics* 46:1039.
Bonner, D. M., Y. Suyama, and J. A. de Moss. (1960). *Federation Proc.* 19:926.
Brenner, S. (1959). *In* "Biochemistry of Human Genetics" Ciba Foundation Symposium (G. E. W. Wolstenholme and C. M. O'Connor, eds.), p. 304. Churchill, London.
Brockman, H. E., and F. J. de Serres. (1963). *Genetics* 48:597.
Case, M. M., and N. H. Giles. (1960). *Proc. Natl. Acad. Sci. U.S.* 46:659.
Catcheside, D. G. (1951). "The Genetics of Microorganisms." Pitman, New York.
Catcheside, D. G. (1960a). *Symp. Soc. Gen Microbiol.* 10:181.
Catcheside, D. G. (1960b). *Proc. Roy. Soc.* B153:179.
Catcheside, D. G. (1962). *Sci. Basis Med., Ann. Rev.* 9:140.
Catcheside, D. G., and A. Overton. (1958). *Cold Spring Harbor Symp. Quant. Biol.* 23:137.
Crick, F. H. C., and L. E. Orgel. (1964). *J. Mol. Biol.* 8:161.
Davis, R. H. (1960). *Proc. Natl. Acad. Sci. U.S.* 46:677.
Davis, R. H. (1961). *Science* 134:470.
Davis, R. H. (1962a). *Genetics* 47:351.
Davis, R. H. (1962b). *Arch. Biochem. Biophys.* 97:185.
Davis, R. H. (1963). *Science* 142:1652–1654.
Davis, R. H., and W. M. Thwaites. (1963). *Genetics* 48:1551.
Davis, R. H., and V. W. Woodward (1962). *Genetics* 47:1075.
De Moss, J. A., and D. M. Bonner. (1959). *Proc. Natl. Acad. Sci. U.S.* 45:1405.
De Moss, J. A., and J. Wegman (1964). Bact. Proc.: 93.
de Serres, F. J. (1963). *Genetics* 48:351.
de Serres, F. J. (1964). *O.R.N.L. Symp.* Molecular Action of Mutagenic and Carcinogenic Agents, *J. Cell. Comp. Physiol.* 64, Suppl. 1:33.
Dixon, M. (1949). "Multi-Enzyme Systems." Cambridge Univ. Press, London and New York.
Emerson, S. (1947). *J. Bacteriol.* 54:195.
Emerson, S. (1950). *Cold Spring Harbor Symp. Quant. Biol.* 14:40.
Fincham, J. R. S. (1957). *Biochem. J.* 65:721.
Fincham, J. R. S. (1959a). *Proc. 10th Intern. Congr. Genet. Montreal, 1958* Vol. 1, p. 335. Univ. of Toronto Press, Toronto, Ontario, Canada.
Fincham, J. R. S. (1959b). *Ann. Rev. Biochem.* 28:343.
Fincham, J. R. S. (1959c). *J. Gen. Microbiol.* 21:600.
Fincham, J. R. S. (1960). *Advan. Enzymol.* 22:1.
Fincham, J. R. S. (1962). *J. Mol. Biol.* 4:257.
Fincham, J. R. S., and P. A. Bond. (1960). *Biochem. J.* 77:96.
Fincham, J. R. S., and A. Coddington. (1963). *J. Mol. Biol.* 6:361.
Fincham, J. R. S., and P. R. Day. (1963). "Fungal Genetics." Blackwell, Oxford.
Fischer, G. A. (1957). *Biochim. Biophys. Acta* 25:50.
Garnjobst, L. (1953). *Am. J. Botany* 40:607.
Gibson, M. I., and F. Gibson. (1964). *Biochem. J.* 90:248.
Giles, N. H. (1951). *Cold Spring Harbor Symp. Quant. Biol.* 16:283.
Giles, N. H. (1959). *Proc. 10th Intern. Congr. Genet., Montreal, 1958* Vol. 1, p. 261. Univ. of Toronto Press, Toronto, Ontario, Canada.
Giles, N. H. (1963). *Proc. 11th Intern. Congr. Genet., The Hague, 1963.*

Giles, N. H., C. W. H. Partridge, and N. J. Nelson. (1957). *Proc. Natl. Acad. Sci. U.S.* 43:305.
Gross, S. R. (1962). *Proc. Natl. Acad. Sci. U.S.* 48:922.
Gross, S. R., and A. Fein. (1960). *Genetics* 45:885.
Haas, F., M. B. Mitchell, B. N. Ames, and H. K. Mitchell. (1952). *Genetics* 37:217.
Haskins, F. A., and H. K. Mitchell. (1952). *Am. Naturalist* 86:231.
Hawthorne, D. C., and R. K. Mortimer. (1963). *Genetics* 48:617.
Henning, U., and C. Yanofsky. (1962). *Proc. Natl. Acad. Sci. U.S.* 48:1497.
Hogness, D. S., and H. K. Mitchell. (1954). *J. Gen. Microbiol.* 11:401.
Holloway, B. W. (1955). *Genetics* 40:117.
Horowitz, N. H. (1945). *Proc. Natl. Acad. Sci. U.S.* 31:153.
Horowitz, N. H. (1947). *J. Biol. Chem.* 171:255.
Horowitz, N. H., and M. Fling. (1953). *Genetics* 38:360.
Horowitz, N. H., and M. Fling. (1956). *Proc. Natl. Acad. Sci. U.S.* 42:498.
Horowitz, N. H., M. Fling, H. L. Macleod, and N. Sueoka. (1960). *J. Mol. Biol.* 2:96.
Houlahan, M. B., and H. K. Mitchell. (1947). *Proc. Natl. Acad. Sci. U.S.* 33:223.
Houlahan, M. B., and H. K. Mitchell. (1948). *Proc. Natl. Acad. Sci. U.S.* 34:465.
Ishikawa, T. (1962). *Genetics* 47:1147.
Jacob, F., and J. Monod. (1961). *J. Mol. Biol.* 3:318.
Jessop, A. P., and D. G. Catcheside. (1965). *Heredity*. In press.
Kaplan, S., Y. Suyama, and D. M. Bonner. (1963). *Genet. Res.* 4:470.
Kapuler, A. M., and H. Bernstein. (1963). *J. Mol. Biol.* 6:443.
Lacy, A. M., and D. M. Bonner. (1961). *Proc. Natl. Acad. Sci. U.S.* 47:72.
Lewis, D. (1963). *Nature* 200:151.
Loper, J. C. (1961). *Proc. Natl. Acad. Sci. U.S.* 47:1440.
Manney, T. R. (1964). *Genetics* 50:109.
Mathieson, M. J., and D. G. Catcheside. (1956). *J. Gen. Microbiol.* 13:72.
Metzenberg, R. L. (1962). *Arch. Biochem. Biophys.* 96:468.
Mitchell, M. B., and H. K. Mitchell. (1952). *Proc. Natl. Acad. Sci. U.S.* 38:205.
Monod, J., and F. Jacob. (1961). *Cold Spring Harbor Symp. Quant. Biol.* 26:389.
Munkres, K. D., and F. M. Richards (1965). *Arch. Biochem. Biophys.* In press.
Murray, N. E. (1960). *Heredity* 15:207.
Murray, N. E. (1963). *Genetics* 48:1163.
Myers, J. W., and E. A. Adelberg. (1954). *Proc. Natl. Acad. Sci. U.S.* 40:493.
Newmeyer, D., and E. L. Tatum. (1953). *Am J. Botany* 40:393.
Pateman, J. A. (1957). *J. Genet.* 55:444.
Pateman, J. A., and J. R. S. Fincham. (1958). *Heredity* 12:317.
Pilot, H. C., and C. Heidelherger. (1963). *Cancer Res.* 23:1694.
Pritchard, R. H. (1955). *Heredity* 9:343.
Rachmeler, M., and C. Yanofsky. (1961). *J. Bacteriol.* 81:955.
Roman, H. (1956). *Cold Spring Harbor Symp. Quant. Biol.* 21:175.
Roper, J. A. (1952). *Experientia* 8:14.
Smith, B. R. (1965). *Heredity* In press.
Stadler, D. R. (1963). *Proc. 11th Intern. Congr. Genet., The Hague, 1963.*
Strauss, B. S., and S. Pierog. (1954). *J. Gen. Microbiol.* 10:221.
Suskind, S. R. (1957). *J. Bacteriol.* 74:308.
Suskind, S. R., and L. I. Kurek. (1959). *Proc. Natl. Acad. Sci. U.S.* 45:193.
Suskind, S. R., and C. Yanofsky, and D. M. Bonner. (1955). *Proc. Natl. Acad. Sci. U.S.* 41:577.

Suskind, S. R., D. Ligon, and M. Carsiotis. (1962). *In* "The Molecular Basis of Neoplasia," p. 307. Univ. of Texas Press, Austin, Texas.
Suyama, Y. (1960). *Biochem. Biophys. Res. Commun.* 3:493.
Suyama, Y., K. D. Munkres, and V. W. Woodward. (1959). *Genetics* 30:293.
Suyama, Y., A. M. Lacy, and D. M. Bonner. (1964). *Genetics* 49:145.
Tatum, E. L., D. Bonner, and G. W. Beadle. (1944). *Arch. Biochem.* 3:477.
Wagner, R. P., C. E. Somers, and A. Bergquist. (1960). *Proc. Natl. Acad. Sci. U.S.* 46:708.
Webber, B. B., and M. E. Case. (1960). *Genetics* 45:1605.
Woodward, D. O. (1959). *Proc. Natl. Acad. Sci. U.S.* 45:846.
Woodward, V. W. (1962). *Proc. Natl. Acad. Sci. U.S.* 48:348.
Yanofsky, C. (1952). *Proc. Natl. Acad. Sci. U.S.* 38:215.
Yanofsky, C. (1960). *Bacteriol. Rev.* 24:221.
Yanofsky, C., D. R. Helinski, and B. D. Maling. (1962). *Cold Spring Harbor Symp. Quant. Biol.* 26:11.
Yourno, J., and S. R. Suskind. (1963). *Genetics* 48:917.
Yura, T. (1959). *Proc. Natl. Acad. Sci. U.S.* 45:197.

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