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THE FUNGI

VOLUME I

The Fungal Cell

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THE FUNGI

An Advanced Treatise

Edited by

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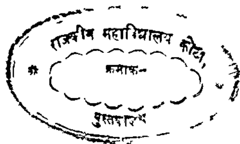
VOLUME I

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 classified as plants, fungi usually receive scant mention, among lower cryptograms, in the average botanical textbook, and in academic texts there is a tendency to give merely a detailed taxonomic account of the group interspersed with phylogenetic speculation While there are satisfactory texts on the physiology of fungi, on fungi as pathogens of plants, animals, and man, and on other aspects of pure and applied mycology, there is no satisfactory modern general treatment, a deficiency which this volume, and its two companions, attempt to correct

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

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

This first volume deals with events at the cellular level, in the two succeeding volumes the fungus organism and fungal populations will be treated A number of topics therefore recur at the different levels of organization, and 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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THE FUNGI

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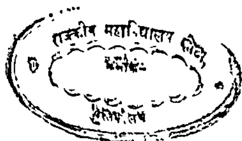

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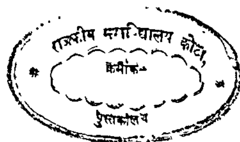

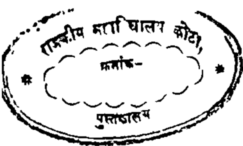

CHAPTER 1

Historical Introduction to Mycology

G C AINSWORTH

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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 through out the world today—the historian of science is becoming an increasingly fashionable figure Although at a first reading histories of science may in duce 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

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 Istvanffi (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 organism o 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

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 Leveille [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 Leveille s paper (Leveille 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

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 30 A 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

self and others (he finally performed key experiments before a Commission of Professors appointed by the University of Pavia) that the muscardine disease of silkworms which for many years had ravaged the silkworm industry of France and Northern Italy was caused by the fungus subsequently named *Beauveria bassiana* He made public the results of his investigations in two monographs published in 1837[1] and 1839 Bassi s findings were confirmed by others and accepted by some, and they must have influenced the views both of those investigating diseases of plants and of those studying diseases of man Mycotic disease in man was firmly established by David Gruby [1810–1898] a Hungarian Jew working in Paris, who in a brilliant series of short papers published during 1841–1844 (Zakon and Benedek, 1944) described the fungi associated with four of the common types 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 systematics 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 interest 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 Desmazieres [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 Kutzing [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 Kutzing'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 1860s and 1870s Another critical stage was reached in 1897 when the chemist E Buchner [1860–1907] of Tubigen 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 Lutjeharms (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)

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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 sub stratum 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 There fore, the reader is referred to books by Alexopoulos (1962) and Gaumann and Dodge (1928), in which the morphology and anatomy of fungi are illustrated

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 con centrated 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 con form 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 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 (Schroter, 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* Schutte (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 Schutte 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 constitution *Heterokaryons* can originate by mutation in a *homokaryon* or by the migration via hyphal anastomoses of nuclei of one mycelium into another They are characterized by variability in the proportions of the two sets of nuclei that exist side by side and provide a unique system of somatic variation

With respect to the mating system which brings together the nuclei in sexual reproduction, fungi can be divided into two groups *homothallic* and *heterothallic* In fungi of the former group, sexual reproduction is accomplished by union of nuclei from one and the same thallus, a process that leads to inbreeding In the heterothallic group sexual reproduction takes place between nuclei from thalli of different mating types Heterothallism is an outbreeding device (Mather, 1942) preventing fusion of gametes from one individual (For a discussion of inbreeding and outbreeding, see Volume II) Two forms of heterothallism occur, isolation of gametes in different individuals and operation of incompatibility genes that allow mating only between nuclei carrying unlike allelomorphs These have been distinguished by Whitehouse (1949 as morphological and physiological heterothallism respectively The interacting thalli of morphologically heterothallic forms bear either male or female reproductive organs, while those of physiologically heterothallic forms differ only in mating type At one extreme, some of these latter lack sex organs completely, at the other, both strains possess both male and female organs (Life cycles and basic patterns of sexuality are reviewed in Volume II)

As a group, fungi display a number of different life cycles (Raper, 1954) centering around sexual reproduction Differing from sexual reproduction is *parasexuality*, demonstrated by Pontecorvo (1956) in members of the Fungi Imperfecti and since found also in other groups (Bradley, 1962) The parasexual cycle involves fusion of haploid nuclei in a heterokaryon, mitotic crossing over, and haploidization Qualitatively it is equivalent to the sexual cycle but differs in the absence of a precise time sequence for its stages For heterokaryotic organisms, with their capacity for rapid adjustment to changing conditions through alteration in the balance of the different types of nuclei in the heterokaryon parasexuality provides in addition the advantages of true sexual reproduction—gene recombination

B Spores

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

active discharge (Ingold 1953) Most are dispersed by wind, often for very long distances (Gregory 1961), or by water insects and other animals 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 and 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

I *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 haploid 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 t ssue by decay or may germinate *in situ* Though feld 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 break down 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 plasmod[illegible] I[illegible] some species, in addition, plasmodia arise by coalescing of man[illegible] o'es

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,

uninucleate, haploid, naked amoebae which multiply by fission and are indistinguishable from amoeboid protozoa No flagellated cells are formed Their distinguishing feature is the aggregation of amoebae to form a *pseudoplasmodium* (Bonner 1959) in which the amoebae do not coalesce but remain as individuals, hence the name cellular or communal slime molds

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

C Labyrinthulales

The Labyrinthulales comprise a small group of marine and freshwater parasites of algae and higher plants The unit of vegetative structure is again a naked, uninucleate cell It is spindle shaped and secretes from each end 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 liberated 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 reproduction has not been observed among these organisms

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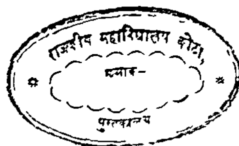

Cell Components

CHAPTER 3

The Cell Wall[1]

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I HISTORICAL RESUME

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

zation 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

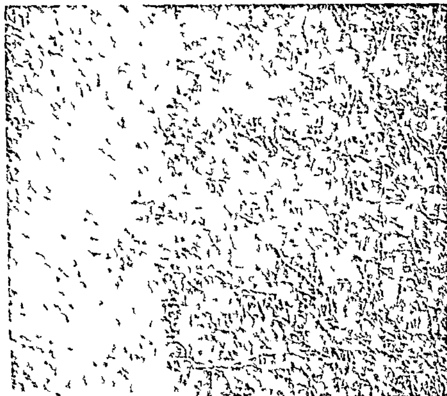


FIG 1 Electron micrograph of a portion of a hyphal wall of *Allomyces macrogynus* showing fibrous texture and masking of fibrils by matrix substances Magnification × 21 000 Reproduced from Aronson and Preston (1960a)

FIG 2 Electron m crograph of the cell wall of *Rl opl yd um spl aerotl eca* Mag n ficat on × 11 570 (Aronson and Preston prev ou ly unpubl shed photograph)

2) The literature on this top c is vast and has been the subject of several reviews (e g Setterfeld and Bayley 1961) The fbrous texture of fungal walls was observed for the first time by Frey Wyssling and Muhlethaler (1950) in the sporangiophore of *Phycomyces* The existence of such fibrous stru tures however had been deduced much earl er through the applica tion of polarization optics (Oort and Roelofsen 1932) and X ray dif fraction (van Iterson *et al* 1936) Indeed prior to pioneering electron micro copy of plant cell walls (e g Frey Wyssling *et al* 1948 Preston *et al* 1948) it was commonplace for investigators to make reference to

chitin chains and their orientation in the cell walls of fungi (e g , Castle, 1942) The principles involved in the application of the techniques referred to above have been treated in sufficient detail by Preston (1952)

Following the initial observations of the fibrillar structure in the *Phycomyces* wall, the presence of a fibrous structure received confirmation through the work of Roelofsen (1951) and Middlebrook and Preston (1952) The scope of observations in this area has since been widened to include many other fungi Houwink and Kreger (1953) demonstrated a fibrous texture in the chemically treated walls of yeasts, and recently, several of the aquatic phycomycetes have been investigated by Aronson and Preston (1960a), leading to the demonstration of a fibrous wall structure in all cases

C. Lamellae

Several electron microscopic analyses have shown that the cell wall is multilaminate, as in the *Phycomyces* sporangiophore (*e g* , Frey-Wyssling and Muhlethaler, 1950), where the primary wall of the growth zone has been resolved into two lamellae that differ in their microfibrillar orientations As the growth potential diminishes below the apical 2 mm of the sporangiophore, an inner lamella, designated as the secondary wall, is apposed to these outer layers of fibrils A considerable amount of discussion in the literature has attempted to reconcile fibril textures with the occurrence of spiral growth in the sporangiophore (Castle, 1942, Roelofsen, 1951, Middlebrook and Preston, 1952) That the cell wall is, in some fashion, implicated in spiral growth in *Phycomyces* most workers would agree, but the mechanism involved remains to be elucidated A recent series of investigations (e g , Probine, 1963) has attempted to explain spiral growth and reversal of spiraling in *Nitella* The basis for the proposed spiral growth theory lies in the observed mechanical anisotropy of the cell walls, and it was pointed out that the same explanation might be applied to the growth of the *Phycomyces* sporangiophore

Distinct lamellae have been observed also in the hyphal walls of *Allomyces* (Aronson and Preston, 1960b) In Fig 3 a bilaminate condition is illustrated The oriented fibrils of the inner layer are aligned parallel to the major axis of the hypha In this respect the wall texture of *Allomyces* shows similarity to the secondary wall of *Phycomyces* Evidence of laminations has not been limited to these observations of fibrous texture' Agar and Douglas (1955) prepared ultrathin sections of cells of *Saccharomyces* which revealed a multilaminate wall structure when viewed in the electron microscope The laminations appeared especially prominent in the bud scar regions Studies by Mundkur (1960) and Vitols *et al* (1961), both deal-

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 poly saccharides to a great extent In this multitude of polysaccharides attention must be focused on the most ubiquitous of all biologically elaborated sub stances—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 cellu lose 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 re ferred to as *micelles* Several workers have written at length on the crystal line properties of cellulose (e g , Preston, 1952), with reference primarily to green plants Nevertheless, these remarks serve to describe fungal cellu lose 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)

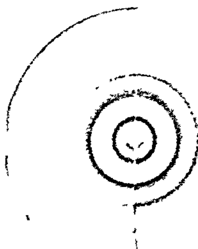


FIG 4 X ray powder diagrams of *Allomyces* chitin (left) and crustacean chitin (right)

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

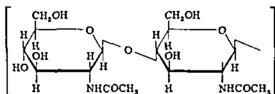
[b] Total ash excluding phosphate

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 $a = 4\ 76$ A, $b = 10\ 28$ A, and $c = 18\ 85$ A (Carlstrom, 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 (Carlstrom 1957)	Chitosan (Bartnicki Garcia and Nickerson 1962)	Cellulose I (Gezelius 1959)	Cellulose II (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 lished This tabulation, admittedly, reflects the author's bias which for 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 A 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

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

3 Other Aminopolysaccharides

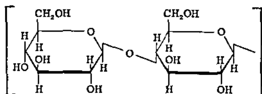
Evidence for other types of nitrogenous polysaccharides has come to light in recent investigations Harold (1962) has isolated from walls of *Neurospora crassa* a polymer of galactosamine which is capable of binding polyphosphate Since the binding of polyphosphate anions would depend upon the cationic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

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

C Nonaminopolysaccharides

1 Cellulose

a Structure and Properties Cellulose is a linear polysaccharide, composed 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 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 Hyph[illegible]hytriales) the number of genera that are known to produce cellulose [illegible] few to allow any far ranging conclusions to be drawn (Table IV) [illegible]ddition to its occurrence in green plants cellulose is known to [illegible]n the bacterium *Acetobacter xylinum* (e g, Hassid, 1962), in amoe[illegible] (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
Acrasiales		
Acytostelium[b]	X-ray	Gezelius (1959)
Dictyostelium[b]	X ray	Gezelius and Rånby (1957)
Hyphochytriales		
Rhizidiomyces	X ray	Fuller and Barshad (1960)
Lagenidiales		
Lagenidium	X ray	Frey (1950)
Saprolegniales		
Saprolegnia	X ray	Frey (1950)
Achyla	X ray	Frey (1950)
Leptomitales		
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

2 *Glucans*

Polysaccharides distinct from cellulose but composed of glucose residues are of common occurrence in fungal walls The most thoroughly studied is yeast glucan, some properties of which have been reviewed by Nickerson *et al* (1961) Yeast glucan is weakly crystalline, but its crystallinity is enhanced by treatment with dilute acid (Houwink and Kreger, 1953) According to Houwink and Kreger (1953), the dilute acid conversion of glucan to what they term "hydroglucan" is accompanied by the formation of aggregates of microfibrils It is significant that yeast glucan may be complexed 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 of yeasts (Houwink and Kreger, 1953, Kreger, 1954) and has also been detected in the walls of *Penicillium notatum* (Kreger, 1954) X-ray data indicate that the derivative, hydroglucan, is similar to, if not identical with, paramylon, the storage glucan found in *Euglena* (Kreger and Meeuse, 1952)

Another glucan that has received considerable attention as a fungal wall constituent is callose, which occurs widely throughout the plant kingdom, but is especially prominent in the phloem of vascular plants In a number of 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

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

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

5 Polyuronides

An unexpected fact of fungal wall chemistry is that uronic acids have never been conclusively demonstrated For many years it has been assumed that fungal walls contain pectic substances The basis for this assumption are results obtained primarily with ruthenium red staining, solubility tests, and qualitative tests on extracts (e g , Graham, 1960, Thomas, 1928, 1942, 1943) Once again the evidence indicates that there is danger in placing too much reliance upon this kind of analysis While the demonstration of uronic acids is attended by technical difficulties, it should be possible to detect them chromatographically in cell wall hydrolyzates, but this has not been accomplished In species of *Phytophthora,* for example, Thomas (1943) reported the presence of pectic substances detected by cytochemical and solubility tests whereas Crook and Johnston (1962), working with formic acid hydrolyzates of isolated walls, found no trace of any uronic acid

D Proteins

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

In studies with yeasts, on the other hand, the results have been much more 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 a significant role to nucleoside diphosphate sugars in polysaccharide synthesis (e g , Hassid 1962) Sugar nucleotides are considered to be precursors of several types of polysaccharides Of particular interest is the work of Glaser and Brown (1957), who found that an enzyme preparation from *Neurospora crassa* would catalyze the incorporation of uridine diphosphate *N*-acetyl D glucosamine into an insoluble aminopolysaccharide The product was undoubtedly a β 1 4 linked polymer of *N* acetylglucosamine and was sensitive to chitinase In this case, the position and stereochemistry of the glycosidic linkages are the principal criteria for assessing the nature of the synthesized product At the very least, however, chitin synthesis probably occurs in two stages The first of these, involving the formation of polysaccharide chains, would seem to be similar to, if not identical with, the *in vitro* synthesis just referred to The second stage, involving the fabrication of chitin fibrils with their concomitant deposition, remains to be elucidated

Until very recently the synthesis of cellulose from uridine diphosphate D glucose (UDPG) had been suspected, but a definitive demonstration of this synthesis had not been accomplished (Hassid, 1962) The inability to demonstrate UDPG participation in cellulose synthesis in green plants now seems to have been explained by the work of Elbein *et al* (1964) In this work, employing extracts of mung bean seedlings, it was shown that guanosine diphosphate D-glucose serves as the glucosyl donor in the *in vitro* synthesis of a β-1,4 glucan The product was indistinguishable from cellulose using various chemical criteria UDPG is inactive in this system, but the incorporation of D-glucose into polysaccharide is markedly enhanced by the presence of guanosine diphosphate mannose (Hassid, 1964)

Work carried out on the cellulose-forming bacterium *Acetobacter xylinum* does not indicate that the above mechanism of glucose activation is operative in this organism (e g , Colvin, 1964) Obviously, the initial phase of cellulose formation in the cellulosic fungi can be known with certainty only when enzymological studies are carried out with this group Whatever the course of glucose activation, the mechanism of fabrication of the physical entities (*viz* , microfibrils) must also be illuminated as was indicated also in connection with chitin formation Colvin (1964) has considered 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

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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

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

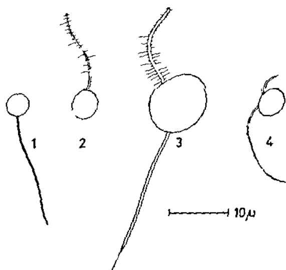


FIG 1 Zoospore types according to flagellation (for explanation see text) Number 2 was redrawn from Couch (1941), the others are original freehand drawings

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

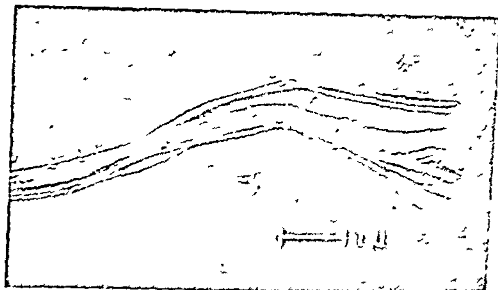


Fig 2 Flagellum of *Pythium aphanidermatum* showing disintegration into eleven fibers

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)

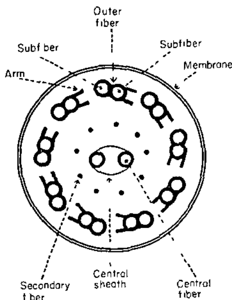


FIG 3 Diagrammatic transverse section of a protozoan flagellum From Gibbons and Grimstone (1960)

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

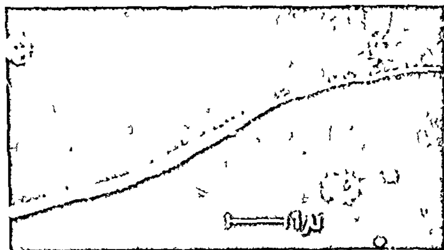


Fig 4 Wh plash endp ece of the fla ellum of *S) l)tr m en lob ot ci m*

end of the whiplash is generally encountered it is considered to be char acteristic Koch (1956) also found this swelling occasionally in chytrids ac cording to Kole (1957) a slight terminal swelling on the end of the whip lash flagellum of *Synchytrium endobioticum* sporangial zoospores is some times 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 Accord ing 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 fla gella 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 prepara tions 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 bi flagellate 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 disintegra tion

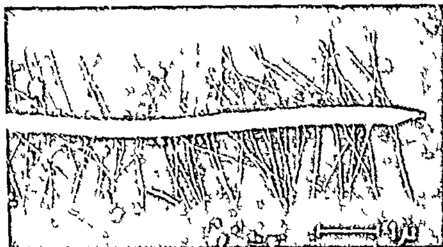
Opinions of the internal structure of the whiplash differ considerably According to Manton *et al* (1952) the whiplash in *O brassicae* is composed of the same eleven strands as the rest of the flagellum, but in a much more destructible condition However both Koch (1956) and Kole (1957) on examining 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-*



F G 5 T nsel type flagellum of *Pl ytopl tl ora nfestans*

matt m latcr l hairs are never found on the other flagellum in *P torulosum* they are sometimes present sometimes not When lateral hairs are en countered they are extremely fine The absence of lateral hairs in these fungi therefore may be due to their early disintegration These observations con f rm Couch s (1941) statement that the tinsels on the anterior flagellum of *P)tl ι m* 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 contra distinction to the great uniformity in the internal structure of flagella and cilia there is remarkable variation in the structures connected with the in sertion 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 investiga tion of motile cells of a number of oomycetes Cotner (1930) came to the conclusion that the zoospores of the species studied all have a def nite basal granule (blepharoplast) at the insertion of each flagellum This blepharoplast is connected with the nucleus by a definite strand (rhizo plast) According to Cotner the basal apparatus is always of nuclear origin and seems to be composed of several chromatic bodies one of which

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 uni flagellate 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 dif ferent 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 fla-gellum 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 be hind 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 encyst-ment the posterior flagellum is dragged along behind as the anterior fla gellum lashes slowly back and forth McKeen (1962) found that detached flagella frequently continue to move for a short time According to Mc Keen 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 de tecting relationships among lower fungi As Sparrow (1958) states " the structure *par excellence* which lies at the very base of any natural sys tem 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, Lep-tomitales, 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 arcestral 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)

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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 light microscope Recently, however, the techniques of electron microscopy have permitted its accurate visualization The intention of this section is to bring the various observations of fungal ultrastructure together in an interpretation believed compatible with previously known fungal cytology Included is a review of certain aspects of the fine structure of cell and nuclear division, sporogenics, interaction of fungi with plant hosts, and of certain 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

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 aecial 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,

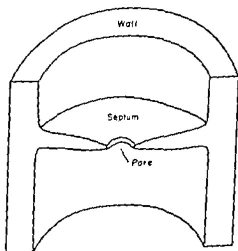

Fig 2 Interpretation of the ascomycete septum Explanation in text Redrawn from Moore and McAlear (1962b)

1963a, 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 the mode of invagination, then the septum in this group of fungi would appear 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 membrane 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 the 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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parently not rigid and, even in old hyphae, will disperse if the confining plasma membrane is ruptured

Both sides of the dolipore in *C solani* are invested by a membraneous septal pore cap—the parenthesome (Moore and McAlear 1962b)—that is confluent with portions of the endoplasmic reticulum that lie next to the main portion of the septum (Figs 4 and 6) The parenthesomes observed in *C solani* are perforated (Fig 6) [as are those in *Polystictus* (Girbardt, 1961)] and offer 'little, if any impedance to protoplasmic streaming , nuclear migration was not reported Similar discontinuous parenthesomes have been observed in association with the dolipore septa of the non-dikaryotic hyphae of *Armillaria mellea* rhizomorphs (Fig 4)

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

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

FIG 3 Ascomycete septum from ascogenous hyphae in *Mollisia* sp Note the plug of 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 inter parenthesome region has a different appearance than the extraparenthesome cyto plasm 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 be tween (*2 2*) and (*3 3*), respectively

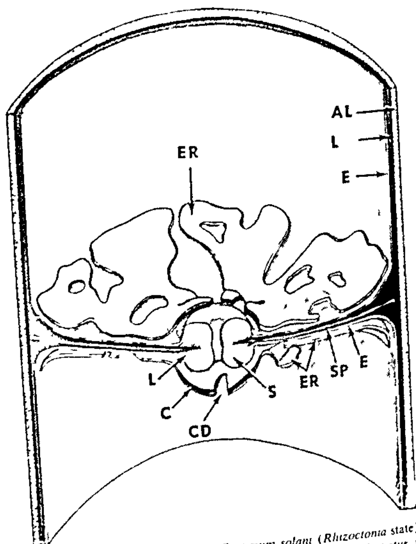


FIG 6 Interpretation of the septum of *Corticium solani* (*Rhizoctonia* state) Similar septa have been observed in *Armillaria mella* rhizomorphs *Polystictus versicolor* hyphae, in early mycelial development in *Calvatia gigantea* and *Schizophyllum commune* basidiocarps From Bracker and Butler (1963) 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

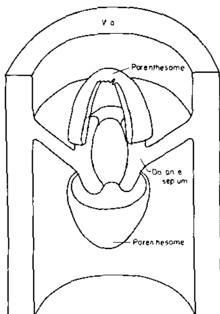


FIG 7 Interpretation of the basidiomycete septum Septa of this form have been reported from basidiocarps of *Auricularia Calvatia Dacrymyces Exidia Merulius* and *Polyporus* and are suggested in mature *Calvatia gigantea* mycelium Redrawn from Moore and McAlear (1962b)

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 dolipore-parenthesome 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 to promote and sustain the dikaryotic condition Such potential differences are clearly evident from a comparison of Figs 4 and 5 and also from Benecke (1963)

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

D Somatic Division of the Nucleus

The nuclei of fungi lie near the limits of resolution of the light microscope and their behavior during somatic division has engendered considerable discussion as to whether it is classically mitotic Whether using time lapse or regular microscopy it is consistantly described that, with the exception of *Basidiobolus* (Robinow, 1963), the nuclei behave amorphously and apparently separate like pulled taffy (see Robinow and Bakerspigel, Chapter 6)

In the electron microscope mitosis is characterized by the dissociation of the nuclear envelope, the condensation of the chromosomes and the attachment of spindle fibers to kinetochores (see Harris and Mazia, 1962) These attributes have not yet been reported for somatically dividing nuclei of fungi Rather, the nuclear envelope is persistant and the electron micrographs of *Saccharomyces* (Hashimoto *et al*, 1958, 1959), *Schizosaccharomyces* (Conti and Naylor, 1960), and *Rhodotorula* (Thyagarajan *et al* 1962) show the nuclei to become dumbbell shaped as the nuclear envelope is drawn out between the separating daughter nuclei (the correlative light micrographs in these reports are quite comparable to similar studies of somatic nuclear division in other fungi) Relating these and other studies with observations of *Cordyceps militaris* Moore (1964a) proposes a model for what he terms karyochorisis (nuclear sundrance) The electron micrographs of *Cordyceps* nuclei suggest that separation is initiated by the invagination of the inner nuclear membrane (Fig 8d) to partition the nucleoplasm into subunits termed karyomes These may number two or more and are bounded by the outer element of the nuclear envelope (Fig 8e) Subsequently, the outer nuclear membrane invaginates to separate the karyomes into daughter nuclei (Fig 8f) This same sequence of events is believed to part the nexus between separating yeast nuclei (Fig 8g)

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

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

III SPOROGENESIS

A Motile Cells

In *Allomyces macrogynus* (Blondel and Turian 1960) prior to initiation of gamete maturation (the 'lipid crown stage of light microscopy) the gametangial cytoplasm contains many scattered small vesicles and cisternae, nuclei that are surrounded by unbounded lipid inclusions, and an abundance of somewhat irregularly distributed ribosomes During the first half-hour of gamete maturation (initiated by immersing the gametangia in a hypertonic solution) the size and number of vesicles increases, the areas of low ribosome density become membrane limited and within the areas of high ribosome density there appear large vesicles In the next quarter hour there develops a concentration of lipid inclusions, mitochondria, ribosomes, and small vesicles in the vicinity of each nucleus Separating these developing cytoplasmic islands are relatively clear areas of protoplasm bounded by plasma membranes that probably arise from vesicle fusion By maturity the ribosomes have become aggregated into the nuclear cap—a crescent-shaped mass in section, partially hooding the nucleus and bounded on its abnuclear side by a double membrane of undetermined origin Outside of this complex are mitochondria, vesicles, and lipid bodies Sections of flagella are most frequently observed in the spaces between gametes, their ontogeny is 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 than that of the general endomembranes The source of these vesicles is presently unknown although the complete absence of a Golgi dictyosome in this 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

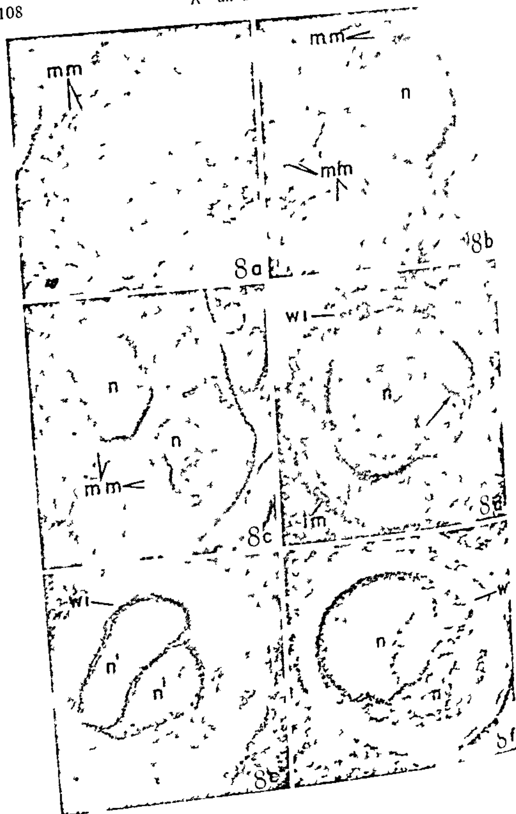
The zoospore of *Blastocladiella emersonii* (Cantino *et al*, 1963) is similar in the structure and relation of the nucleus, nucleolus and nuclear cap but differs in possessing a single large eccentric mitochondrion surrounding the base of the flagellum the presence of small cytoplasmic "gamma" organelles,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

membrane bounding the nuclear-cap as being nonporous and continuous with the outer membrane of the nuclear envelope In contrast, the micrographs of this membrane in the gametes of *A macrogynus* (Turian and Kellenberger, 1956, Blondel and Turian, 1960) and *A javanicus* (Moore, 1964b) suggest only partial confluency with the nuclear envelope

B *Ascospores*

The sequence of events in ascospore formation may be partially reconstructed from a collation of several studies of Euascomycete ultrastructure The whole ontogeny, initiated by karyogamy occurs within a single cell, the ascus The diploid nucleoplasm may appear either homogeneous, as in *Ascodesmis* (Moore, 1963a) or, like the somatic nuclei, composed of light and dark regions, as in *Dasyscyphus* (Moore and McAlear, 1962a), the respective ascal cytoplasms of these species display either a number of vesicles, endoplasmic reticulum, and mitochondria, or appear homogeneous, finally, in *Ascodesmis* there occur endoplasmic continuities with the nucleus, but not in *Dasyscyphus* [Similar continuities have been reported for somatic nuclei in *Mollisia* (Moore and McAlear, 1961c), *Neobulgaria* (Moore and McAlear, 1963a), and *Stilbum* (McAlear and Edwards, 1959) and suggested for mitochondria in *Ascodesmis* (Moore, 1963a) and *Uromyces* (Moore and McAlear, 1963b)] Quite possibly the fusion nucleus ascus shown in *Ascodesmis* represents an earlier stage than the one shown for *Dasyscyphus* Meiosis and a somatic nuclear division immediately follow fusion During the division phase as seen in *Dasyscyphus* the nuclear envelope dissociates and very little fine structure is evident after which nuclear envelopes, endoplasmic reticulum, and electron dense inclusions reappear The re-emergent nuclei have light and dark regions, and before separation opposing pores in the respective daughter nuclear envelopes are evident The 8 nucleate phase representing the end of free nuclear division has been seen in *Ascodesmis*

The development of the ascospore wall is poorly understood In *Cordyceps militaris* the configuration of the endoplasmic reticulum suggests that it may play a role If endoplasmic reticular elements were to form the matrix membranes (*mm* Fig 8a–c) between whose surfaces wall material is produced (similar to the opposing membrane faces during septum formation), it would explain the origin of both the plasma membrane and the investing membrane (*im*, Fig 8d) (and seen also in *Ascodesmis*) of the materializing ascospore The developing spore walls (*wt*, Fig 8d, e) rapidly thicken, apparently as a series of smooth shells, for, again like the septa, they appear layered in section (*w*, Fig 8f), six such layers have been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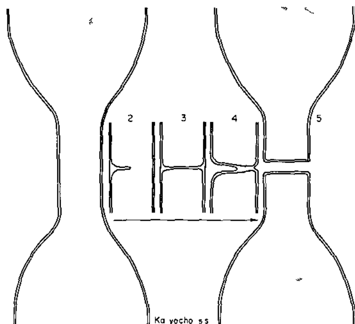

FIG 8g(1–5) Karyochorisis—interpretation of fungal somatic nuclear division 1 Isthmus between separating daughter nuclei Prior to this time or during it the chromatin material (shaded) has replicated and separated and the nucleus is briefly in a genetically $n + n$ state 2 Initiation of invagination 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 (*i i*) 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
9b
10
11
12
13

that the surface pattern may be a reflection of an endoplasmic reticular net pressed against the underlying plasma membrane

The epiplasm, which is the enucleated ascal cytoplasm left after the ascospore nuclei and other organelles are encapsulated, breaks down as the spores mature In *Ascodesmis* it appears to contain only a suspension of ribosomes 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 Muhlethaler (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

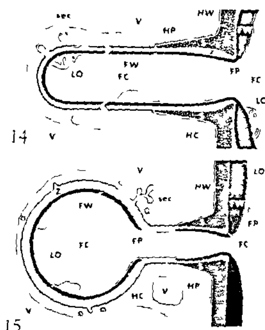
V INTERACTION WITH HOST PLANTS

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 micro morphological 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 (sec) 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 RESUME

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

transition from ascomycetes to basidiomycetes the septa become highly elaborated and the shift is from functional coenocytes to functional diploids Further, the plasma membrane appears to play an active role in the formation of lomasomes—structures presently limited, with two exceptions, to the true fungi and perhaps of phylogenetic significance Moreover, the electron microscopy of dividing nuclei offers support to the hypothesis that fungal nuclei can divide by a nonmitotic process a process described by the term karyochorisis Our information on the fine structure of sporogenesis is very sketchy, but even the little available evidence suggests developmental changes unknown in other major groups Finally the few studies of parasitism and saprobism intimate that these processes differ not so much in microhistology as in the degree of adaptive resistance of the plant partner

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CHAPTER 6

Somatic Nuclei and Forms of Mitosis in Fungi[1]

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and

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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 i
cells of plants and animals preceded recognition of their universal presenc
in fungi by nearly forty years, despite the fact that since 1858 hematoxyli
had been available to help in the search and even then some material:
such as vegetative cells of ascomycetes proved refractory (Schmitz 1879)
It took even longer for the mode of division of fungal nuclei to be under
stood In some instances that problem continues to resist even phase-con
trast microscopy, as for example in *Thanatophorus cucumeris* (Flentj
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 prob-
lems 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 prob-
lems (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 fre-
quent 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 cytologi-
cal studies of *Penicillium notatum* (Pauli, 1956), which is silent about mi-
totic 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 chem-
istry can be properly investigated It is said that the nuclei of the septa-
less 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 2*n* 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),

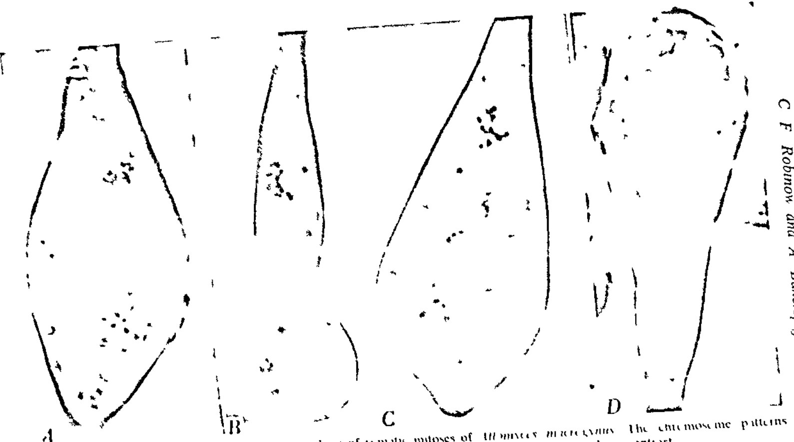


Fig. 1 (A–D) Three metaphases and one anaphase of somatic mitoses of *Allomyces macrogynus*. The chromosome patterns are not unlike those found by Wilson (1952) in meiotic nuclei. Helly, Feulgen acetocarmine phase contrast

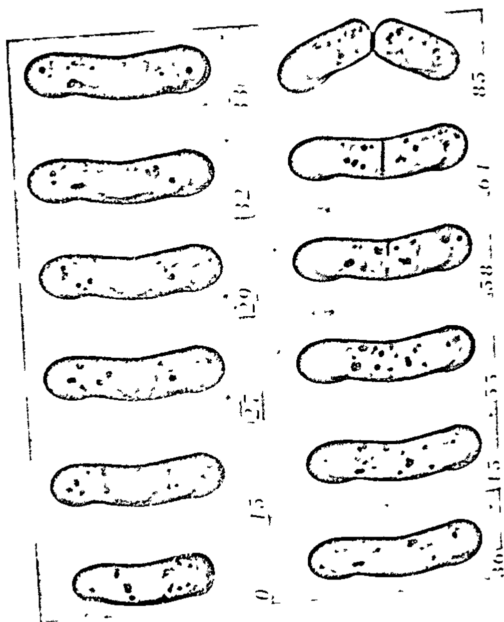
meiotic configurations have been described in conventional terminology, although the behavior of the chromosomes of the same species at mitosis has not (or had not then) been described Where information on both phases is available, as in *Allomyces*, a surprising similarity between them is apparent (Fig 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 place (Wilson, 1952), but it opens the possibility that the process of meiosis in *Allomyces* may not be an entirely conventional one On the morphological 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



lack 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 chromosomes The chromosomes appear to become adsorbed to the cord and to invest it completely at anaphase A Feulgen-positive cord results in which chromosomes can no longer be separately recognized This stretches at anaphase and is broken into two pieces in the manner recently described in *Schizophyllum* (Bakerspigel, 1959a) Numerous illustrations in the literature content themselves with a rendering of this breaking cord as the sole token of mitosis, but true cytology asks for completeness An electron micrograph 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 the first time that a fibrous mitotic motor device in a fungus has been seen in 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)

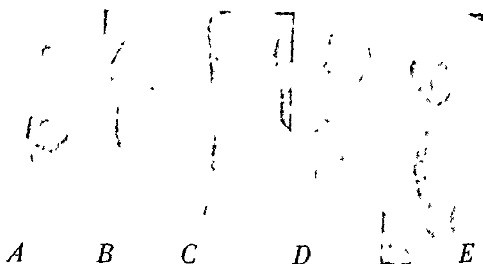

FIG 3 *Schizosaccharomyces versatilis* (A–C) The behavior of the nucleolus during mitosis (D and E) The emergence of the daughter nuclei and the beginning of cell division Helly acetocarmine The technique used here is unsuitable for the demonstration of the spindle fiber in dividing nuclei of this yeast

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-

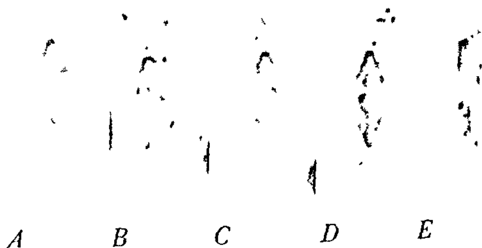

FIG 4 *Schizosaccharomyces versatilis* Disengaging chromosomes at metaphase and anaphase of mitosis Helly HCl Giemsa Magnification × 3600

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

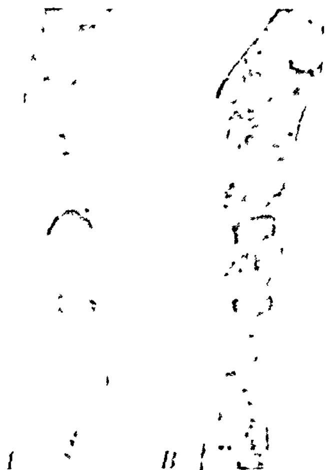

Fig A B *Basidiobolus ranarum* (isolated in Brisbane Australia) Spindles of anaphase Helly bromophenol blue mercuric chloride Magnification × 1800

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 intranuclear 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

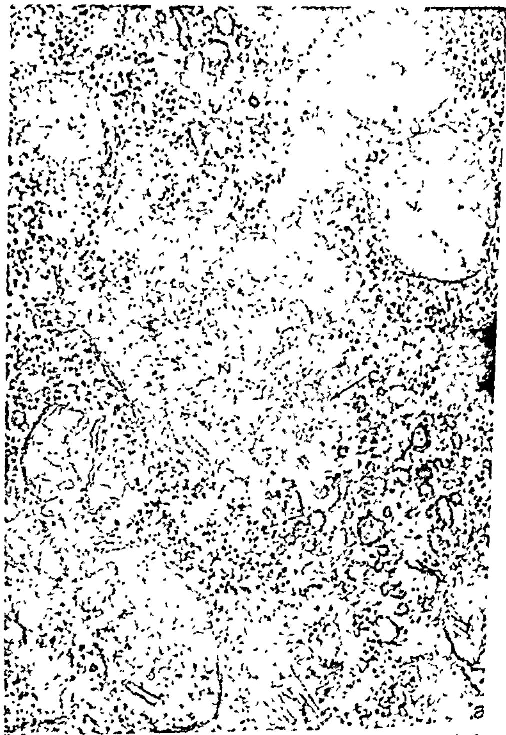

FIG 7a Section of hypha of *Allomyces arbuscula* showing nucleus (N) and centriole (C) This print which appears in Renaud and Swift (1964), was furnished through the kindness of these authors Magnification Approximately × 70 000

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 in compatibility and temperature upon the rate f 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 gr wth which was only 0 13 mm per hour In addition they argued that the high Q of 6 0 for migration indicates that the rate is limited by an unkn wn chemical rather than a physical process

In a more recent report Swiezynski (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 scare Swiezynski 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* by the other the reverse may not be true Obviously this kind of model can be proved only if the factor responsible for the attraction of nuclei is demon strable and if it will function in a predicted manner Thus even though 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 th t of the cell When nuclei of *G tetrasperma* be came freed from th wall to which they appeared to be attached they might be capable of ndependent motility In a later report on nuclear streaming in *Gela inospora* Dowding (1958) described nuclei carried by e streaming cytoplasm at speeds as high as 40 mm per hour On the bas s nese observations eight nuclear migrations were envisaged These might be described as follows

1 Migration of one or more nuclei from a conidiophore into an at a hed 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 "strangulement" 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

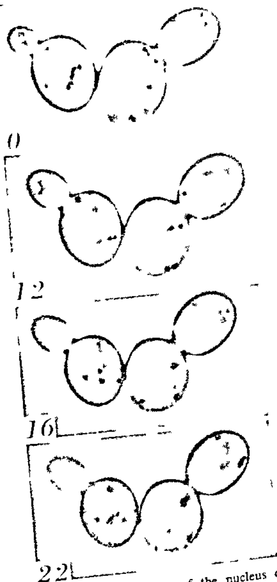

FIG 8 Successive stages of the division of the nucleus of a living yeast cell (*Saccharomyces cerevisiae*) Slide culture in 20% gelatin containing 1% yeast ex tract and 1% glucose Numbers give minutes since the taking of the first photograph At *0* the nucleus an angular body of low density is at the 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 contras 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 p r haps they represent chromosomes (Ramirez and Miller, 1962) But th are very small and their movements are not yet sufficiently understood t

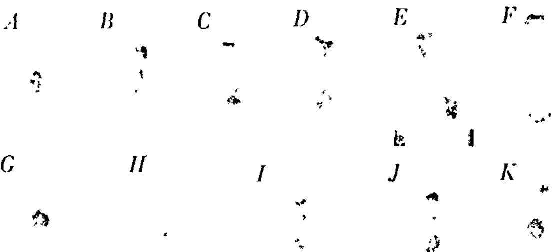

FIG 9 *Saccharomyces cerevisiae* A–F and H K illustrate commonly encountered stages in the direct division of the nucleus The nucleolar moiety of the nucleus is not stained in these preparations Helly, HCl Giemsa Squashed after staining

permit description in terms of a mitotic process According to Moor and Muhlethaler (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)

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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

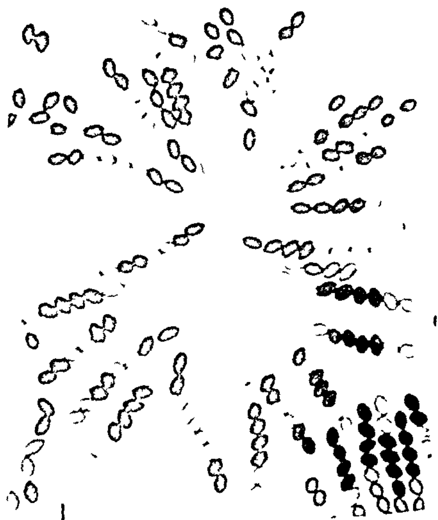

FIG 1 Asci of *Sordaria fimicola* heterozygous for a spore color marker showing first and second division segregation Magnification × 435

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.

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

Kohler (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 Sjowall (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) * 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

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

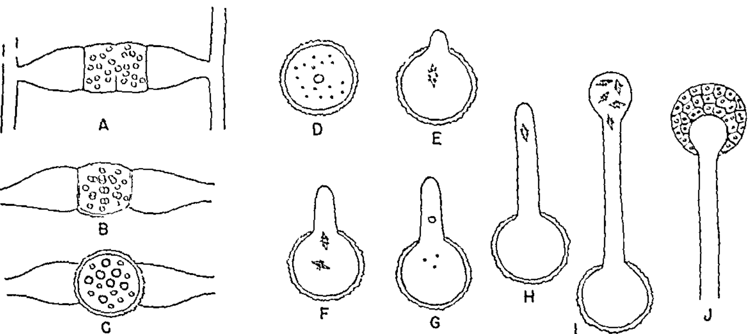

Fig 5 Diagrammatic representation of karyogamy and meiosis in a mucor such as *Rhizopus stolonifer* (A) Plasmogamy (B) karyogamy (C) diploid and haploid nuclei in zygospore, (D) degeneration of all but one diploid nucleus (E) first meiotic division (F) second meiotic division (G) degeneration of all 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

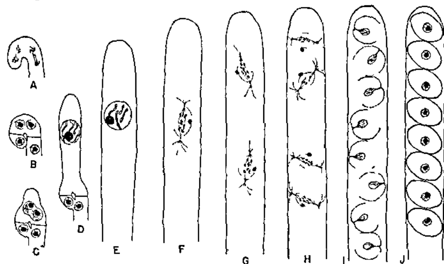

FIG 6 Diagrammatic representation of ascus development in a higher ascomycete (A) and (B) crozier development (C) karyogamy, (D) synapsis, (E) diplotene, (F) anaphase I, (G) anaphase II, (H) anaphase III, (I) ascospore delimitation, (J) 8 spored ascus

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

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 I, 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

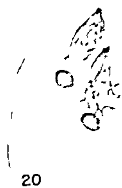
Recent genetic studies on *Sordaria fimicola* (Olive, 1959, Kitani et al 1962) indicate that the completely paired meiotic prophase bivalent should be considered as four chromatids, each in turn composed of two strands that are equivalent to the two strands comprising the double helix of the Watson-Crick 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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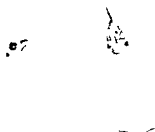
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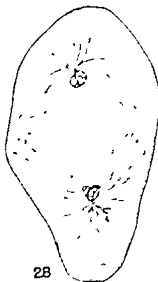
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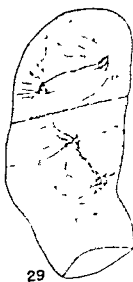
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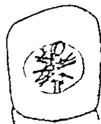

Following telophase II, four nuclei in a row are produced In *N crassa* and most other species with similar cylindric asci, there is little if any spindle overlap in the ascus In a few species however, it is known to occur fairly frequently, in which case the segregation pattern is superficially disturbed 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)

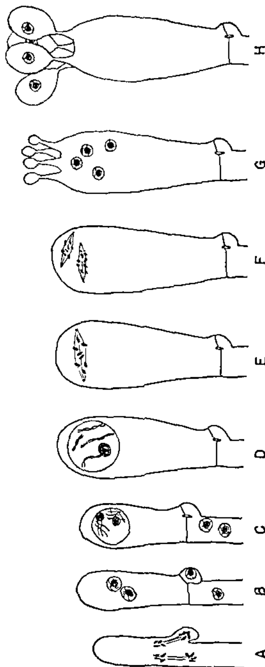
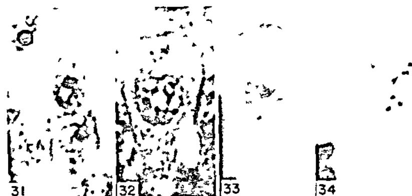


Fig 30 Diagrammatic representation of basidial development in an autobasidiomycete (A) Conjugate mitoses associated with clamp formation, (B) young binucleate basidium, (C) early fusion nucleus, (D) early diplotene, (E) and (F) meiotic divisions G) basidium with a tetrad of haploid nuclei, (H) basidium with 4 haploid basidiospores

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 intra nuclear 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

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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)

to 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, including 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 lDb 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

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 the 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 *Pilobolus* 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 $\mu g/50$ 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 hyponitrite reductases

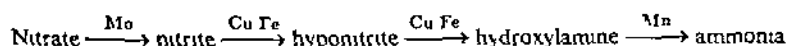
E Molybdenum

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 Et

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:



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-

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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.

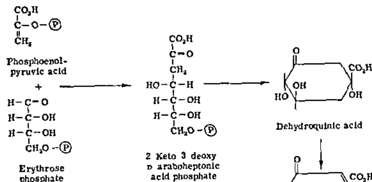
A. The Shikimic Acid Pathway

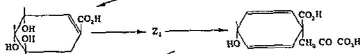
This pathway was elucidated mainly by the work of Beadle, Bonner, Davis, Tatum, and their collaborators, reviewed by Davis (1955). The organisms chiefly employed were the mold *Neurospora crassa* and the bacteria *Escherichia coli* and *Aerobacter aerogenes*. Mutant strains of these organisms were obtained, 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.

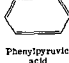
In this scheme phosphoenol pyruvate and D-erythrose 4-phosphate derived via sedoheptulose diphosphate from the pool of reactions associated with carbohydrate metabolism, are condensed to 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate. This by rearrangement and aldol-like condensation is transformed 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 spec-







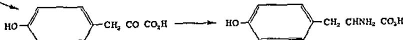


FIG 1 Shikimic acid pathway Ⓟ = $-PO(OH)_2$

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-

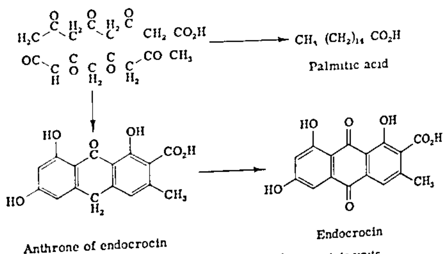


FIG 2 Biosynthesis of endocrocin from acetate units

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

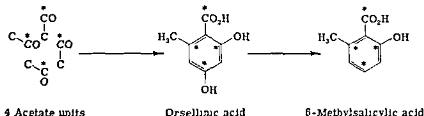


FIG 3 Biosynthesis of 6 methylsalicylic acid from acetate units

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

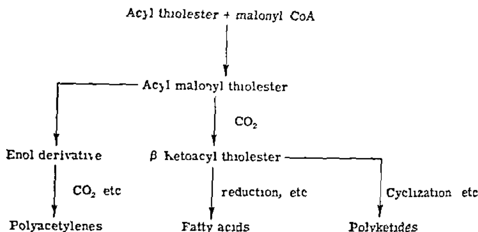


FIG 4 Scheme of biosynthesis from acetate malonate units

(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

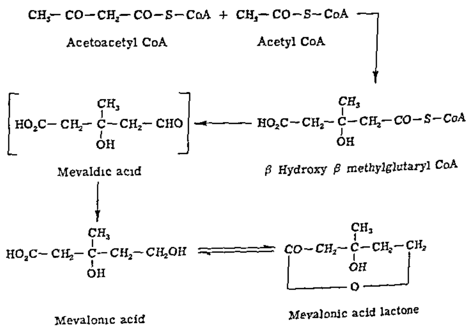


FIG 5 Biosynthesis of mevalonic acid.

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)

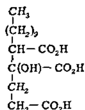
Hydroxy acids are not uncommon, for example, itatartaric acid, $HO_2CCH_2C(OH)(CH_2OH)CO_2H$ was isolated from cultures of *Aspergillus terreus* (Stodola *et al* 1945) The acids α β dihydroxyisovaleric, $CH_3C(OH)(CH_3)CH(OH)CO_2H$, and α β dihydroxy-β methylvaleric, $CH_3CH_2C(OH)(CH_3)CH(OH)CO_2H$, both isolated from a *Neurospora crassa* mutant by Sjolander *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

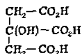
saturated acids myristic, palmitic stearic arachidic behenic and lignoceric acids (39%) together with even larger amounts of the unsaturated acids hexadecenoic, oleic, linoleic linolenic, and C acids (61%) The predominating acids are palmitic (21%) stearic (16%) oleic (40%), and linoleic (17%) (Singh *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*

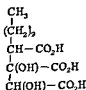
Certain hydroxylated tribasic acids are closely related to citric acid as regards the functional groups, but they contain in addition a saturated chain of ten to sixteen carbon atoms Such are spiculisporic and minioluteic acids from *Penicillium* agaricic acid from *Fomes officinalis*, and caperatic 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



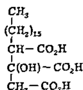
Spiculisporic acid (hydrate)
Penicillium spiculisporum
(Clutterbuck *et al* , 1931
Asano and Kameda, 1941)



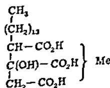
Citric acid



Minioluteic acid (hydrate)
P miniolutetum
(Birkinshaw and Raistrick, 1934)



Agaricic acid
Fomes officinalis
(Thoms and Vogelsang, 1907)



Caperatic acid
Parmelia caperata
(Asano and Ohta, 1933, 1934)

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

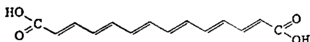
1 Ethylene and Polyenes

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

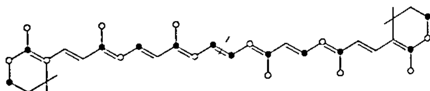
The simplest example of the isolated double bond is, of course, ethylene, $CH_2=CH_2$ It is evolved by respiring plant tissue and promotes the ripening of fruit This property led to its detection as a product of *Penicillium digitatum* by Hall (1951), when he grew the fungus on glucose or arabinose-agar 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 Butler, 1956) Mevalonic acid is an



Corticrocin
Corticium croceum
(Erdtman 1948 B L Shaw and Whiting 1954)



β Carotene
○ Carbon atom from the methyl group of acetate
● Carbon atom from the carboxyl group of acetate

FIG 7 Polyenes

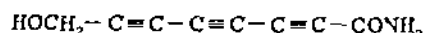
effective precursor of carotene for certain fungi (Braithwaite and Goodwin, 1957, Grob, 1957), as might be anticipated from the fact that the skeleton can be constructed from eight isoprene units linked in appropriate fashion

2 Acetylenic Compounds, Polyynes, Polyenynes

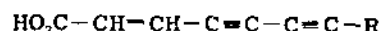
The triple bond as present in acetylene occurs in a number of fungal products, particularly those derived from the higher fungi This type of linkage is almost always conjugated with other triple bonds or with double bonds, forming long unbranched chains When both types of bond occur in the same molecule 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

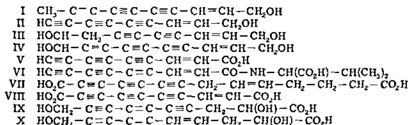
With improvements in the techniques of isolation and identification of the polyacetylenic metabolites, the recording of new discoveries shows no signs of abatement Thus Cambie *et al* (1963) have recently reported the



Agrocybin
Agrocybe dura
(Anchel, 1952 Bu'Lock *et al*, 1954)



Diatretyne I R CONH2
Diatretyne II R CN
Clitocybe diatreta
(Anchel, 1953, 1955 Ashworth *et al* 1958)



Polyacetylenic metabolites of *Poria sinuosa*
(Cambie *et al*, 1963)

FIG 8 Polyacetylenes

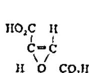
isolation and characterization of ten polyacetylenic compounds from *Poria sinuosa* The structures (I–X) (Fig 8) illustrate the variety and range of polyacetylenes derivable from a single species All these with the exception of (I), (II), and (IV) are new The alcohol compound (I) had previously been 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).

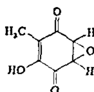
C. Oxygen Heterocycles

1 *Epoxides*

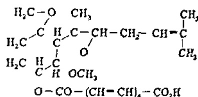
Only a few examples of the epoxide group are known in fungal metabolic products (see Fig 9) The simplest case, *l-trans* oxidoethylene-α,β-dicarboxylic acid (epoxysuccinic acid) has been obtained from the culture filtrates of *Monilia formosa, Penicillium viniferum*, and *Aspergillus fumigatus* Terreic 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

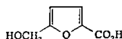
2 Furan Rings

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)

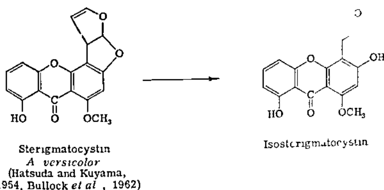
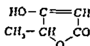


Fig 10 Furan rings

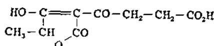
obtained from *Penicillium charlesii*, which also yields several other tetronic acids Further examples are derived from other fungal sources From an examination of the structures (Fig 11) it is evident that substitution occurs at two points in the tetronic acid ring, the α-position which may have acyl substituents and the γ-position which is variously substituted by $-CH_3$, $-CH_2-CO_2H$, and $=CH$. 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

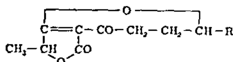
Ascorbic and penicillic acids, although structurally similar to the tetronic acids, are biosynthesized by other pathways Ascorbic acid arises from D-glucuronic acid through L gulonolactone and 2-keto-L-gulonolactone (Sastry and Sarma, 1957), whereas penicillic acid is formed from orsellinic acid by cleavage of the aromatic ring as shown in Fig 12 (Mosbach, 1960,



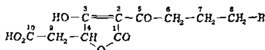
l-γ-Methyltetronic acid
Penicillium charlesii
(Clutterbuck *et al*, 1935c)



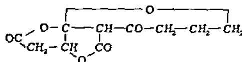
Carolinic acid
P. charlesii
(Clutterbuck *et al*, 1935a)



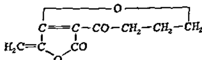
Carolic acid, R = H
Terrestric acid, R = C_2H_5
P. charlesii, P. terrestre
(Clutterbuck *et al*, 1935a,
Birkinshaw and Raistrick, 1936)



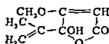
Carlosic acid, R = H
Viridicatic acid, R = C_2H_5
P. charlesii, P. cinerascens, P. viridicatum
(Clutterbuck *et al*, 1935b, Bracken and
Raistrick, 1947, Birkinshaw and Samant, 1960)



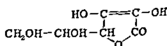
Carlic acid
P. charlesii
(Clutterbuck *et al*, 1935b)



Dehydrocarolic acid
P. cinerascens
(Bracken and Raistrick, 1947)

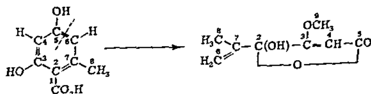


Penicillic acid
P. puberulum, P. cyclopium
(Alsberg and Black, 1913,
Birkinshaw *et al*, 1936)



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 ... have already been mentioned The γ pyrone ring is exemplified by k ... product formed in high yield by the *Aspergillus flavus A oryzae* ... A related product is patulin which contains an unsaturated γ lact ... K jic acid appears to be synthesized directly from glucose by oxid ... elimina tion of water without cleavage of the 6 carbon chain ... tein and Bentley, 1953), whereas patulin is probably produced ... of an aromatic precursor such as gentisaldehyde which is derived ... shikimic acid pathway (Fig 13)

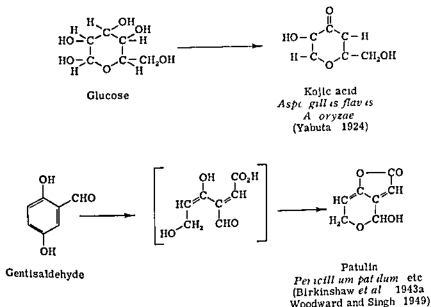


FIG 13 B osynthesis of koj c c d and patul n

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, $C_{14}H_{12}O_7$, *from Chaetomium indicum* is also of this nature (Fig 14) The

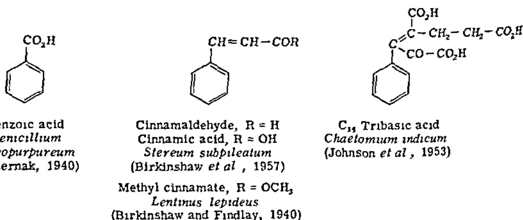
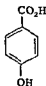


FIG 14 Benzene derivatives

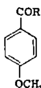
cinnamic acid derivatives have the C_6–C_3 structure which relates them to the shikimic acid pathway through which they are *formed* Benzoic acid may be a product of further oxidation of cinnamic acid

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

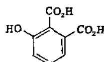


p-Hydroxybenzoic acid
Penicillium patulum
(Bassett and Tanenbaum, 1958)

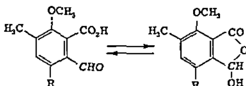


Anisaldehyde, R = H
Daedalea juniperina
(Birkinshaw and Chaplen, 1955)

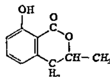
Methyl anisate, R = OCH_3
Lentinus lepideus
(Birkinshaw and Findlay, 1940)



3-Hydroxyphthalic acid
Penicillium islandicum
P patulum
(Gatenbeck, 1957)



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)

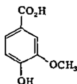


Ochracein (Mellein)
Aspergillus ochraceus
A melleus
(Yabuta and Sumiki, 1933, 1934, Blair and Newbold, 1955)

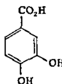
FIG. 15 Phenol derivatives

3 Pyrocatechol Derivatives

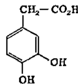
Only a few examples of this arrangement are known (Fig 16) Vanillic and protocatechuic acid were obtained from a mutant of *Neurospora crassa* having strict nutritional requirements Protocatechuic acid is produced also



Vanillic acid



Protocatechuic acid
Phycomyces blakesleeanus
(Schröter, 1956)



Homoprotocatechuic acid
Polyporus tumulosus
(Ralph and Robertson, 1950)

Neurospora crassa
(Metzenberg and Mitchell, 1958)

FIG 16 Pyrocatechol derivatives

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

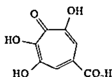
The close relationship between citrinin, formed by *A terreus* wild type and the isocoumarin derivative produced by a mutant, suggests a similar origin for these two products Further, Birch *et al* (1958a) found that the carbon atoms marked with an asterisk in the formula shown differ from others in 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 $C_{11}H_{10}O_5$ (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

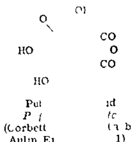
Gibberellic acid is a representative of a group of related substances known as gibberellins, which are produced by *Gibberella fujikuroi* Although strictly out of place among the polycyclic structures of Fig 21, which are partly or mainly aromatic in nature, gibberellic acid is nevertheless included 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

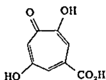
Several fungal metabolites contain the tropolone nucleus, which consists of a seven-membered carbocyclic structure, to which the special system of conjugated double bonds imparts aromatic properties Some of these structures are illustrated in Fig 22 The actual method of biosynthesis of the tropolone 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



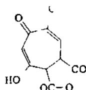
Puberulic acid
Penicillium puberulum etc
(Birkinshaw and Raistrick 1932
Corbett *et al* , 1950a b)



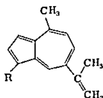
Pul id
P (*tc*
(Corbett (a b
Aulin Er 1)



Stipitatic acid
P stipitatum
(Birkinshaw *et al* 1942c
Corbett *et al* , 1950c)



St pitato ic id
I st p t t
(Segal 195 1959)

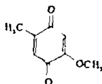


Lactarazulene R CH_3
Lactaroviolin R CHO
Lactarius deliciosus
(Willstaedt and Zetterberg 1946
Sorm *et al* 1953 Heilbronner and Schmid 1954)

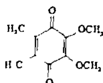
FIG 22 Tropolones

V QUINONES AND QUINONOID PRODUCTS

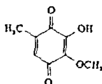
The quinones are closely related to the quinols which have already been discussed as belonging to the aromatic series The quinols are readily con vertible by atmospheric oxidation to the quinones and the reverse change can readily be accomplished by reducing agents The fungi are capable of inducing these changes so that a quinol and its corresponding quinone may in some cases be isolated from the same fungal culture Thus gentisyl



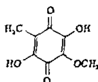
2 Methoxy 5 methylbenzoquinone
Coprinus similis
(Anchel *et al* 1948)



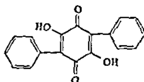
Aurantiogliocladin
Gliocladium sp
(Vischer 1953)



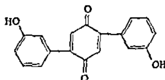
Fumigatin
Aspergillus fumigatus
(Anslow and Raistrick 1938
Baker and Raistrick, 1941)



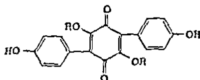
Spinulosin
Penicillium spinulosum
(Birkinshaw and Raistrick, 1931)



Polyporic acid
Polyporus nidulans
(Kögl 1926)

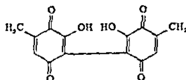


Volucrisporin
Volucrispora aurantiaca
(Divekar *et al* , 1959)

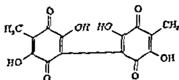


Atromentin R = H
Polyporus nidulans
(Kogl and Becker 1928)

Aurantiacin *R* = C_6H_5CO
Hydnum aurantiacum
(Gripenberg 1956)



Phoenicin
Penicillium phoeniceum
(Posternak 1938)



Oosporein
Oospora colorans
(Kogl and van Wessem 1944)

FIG 23 Benzoquinones.

alcohol (Fig 18) and its quinone have been obtained f [illegible] *nicillium patulum* as a deep violet colored complex (Engel and Br [illegible] 947)

The substituents of the quinone nucleus are in gen [illegible] similar to those found in the phenolic derivatives and include [illegible] droxyl and methoxyl groups The carboxyl group is of infrequ [illegible] nce It cannot be a substituent of the quinone ring but ma [illegible] d on an aromatic nucleus fused with the quinone ring as in th [illegible] quinone series All the known fungal quinones are *p* quinones [illegible] mplex structures (diquinones) in this series are represented by [illegible] n 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 [illegible] nting 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 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

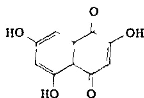
B Naphthoquinones

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

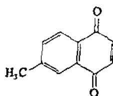
C Anthraquinones

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*

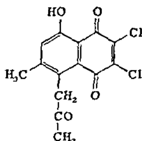
gramineum was found to consist of a mixture of polyhydroxyanthraquinones The fungal anthraquinones, as illustrated in Fig 25, contain from one to five hydroxyl groups, one of which may be methylated A β-methyl group is almost always present as such or in one of its oxidized forms (CH_2OH, CHO, or CO_2H) 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



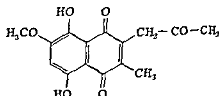
Flaviolin
Aspergillus citricus
(Davies *et al*, 1955)



6-Methyl-1,4-naphthoquinone
Marasmius gramineum
(Bendz, 1948)



Mollisin
Mollisia caesia
(Van der Kerk and Overeem, 1957)

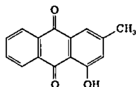


Javanicin
Fusarium javanicum
(Arnstein and Cook, 1947, Ruelius and Gauhe, 1950)

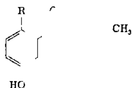
FIG 24 Naphthoquinones

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

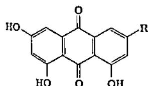


Pachybasin
Pachybasium candidum
(Shibata and Takido, 1955)



Chrysoph[illegible]
Islan[illegible]
Penicillium
(Howard and Raist

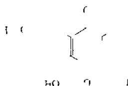
Helminthosporin
Helminthospor[illegible]
(Charles *et al*



Emodin, R = CH_3
Cortinarius sanguineus
(Kogl and Postowsky, 1925)

ω Hydroxyemodin, R = CH_2OH

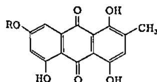
Emodic acid, R = CO_2H
Penicillium cyclopium
(Anslow *et al* , 1940)



Physcion, R [illegible] CH_3
Aspergillus glaucus
(Ashley *et al* 1939)

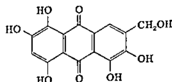
Teloschistin, R = CH_2OH
Teloschistes flavicans
(Seshadri and Subramanian, 1949)

Fallacinal R CHO
Xanthoria fallax
(Murakami 1956)



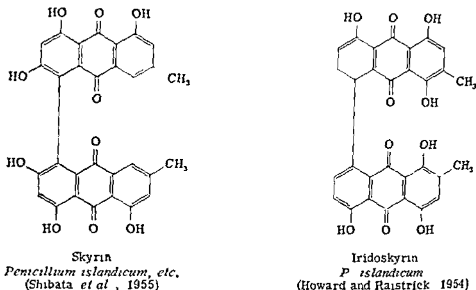
Catenarin, R H
Helminthosporium catenarium
(Anslow and Raistrick, 1940)

Erythroglaucin R = CH_3
Aspergillus glaucus
(Ashley *et al* , 1939)



Asperthecin
Aspergillus quadrilineatus
(Howard and Raistrick 1955,
Birkinshaw and Gourlay, 1961)

Fig 25 Anthraquinones



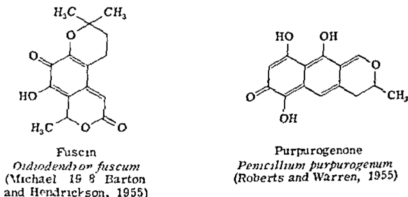
Skyrin
Penicillium islandicum, etc.
(Shibata *et al.*, 1955)

Iridoskyrin
P. islandicum
(Howard and Raistrick 1954)

FIG 26 Dianthraquinones

D Hemiquinones

Some of the fungal metabolites possess a hemiquinonoid (or methylene quinone) 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

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

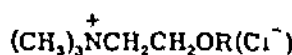
2 *Quaternary Ammonium Compounds*

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)

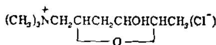
3 *Unusual Nitrogen Groups*

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-

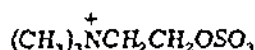
thesized from aspartic acid by monodecarboxylation and conversion of the amino to the nitro group (Birch *et al* 1960)



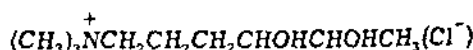
Choline, R = H
Acetylcholine R = $COCH_3$
Ergot
Claviceps purpurea
(Ewins, 1914)



Muscarine



Choline sulfate
Aspergillus sydowii
(Woolley and Peterson, 1937)

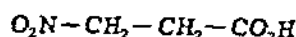


Muscaridine
Amanita muscaria
(Kogl *et al*, 1957, 1960)

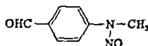
FIG 28 Quaternary ammonium compounds

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)



β-Nitropropionic acid
Aspergillus flavus
Penicillium atrovenetum
(Bush *et al*, 1951 Raistrick and Stössl, 1958)



p-Methylnitrosamine benzaldehyde
Clitocybe suaveolens
(Herrmann, 1960)

FIG 29 Unusual nitrogen groups

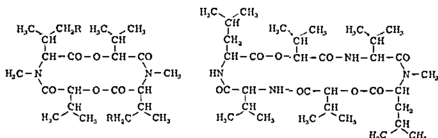
B. Oligopeptides

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

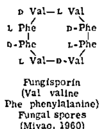
The simplest fungal oligopeptide containing only two building units, is DL fumarylalanine, $HO_2C—CH=CH—CO—NH—CH(CH_3)—CO_2H$, obtained from *Penicillium resticulosum* (Birkinshaw *et al*, 1942b) It is evidently formed by peptide linkage between DL-alanine and one of the carboxyl groups of fumaric acid 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

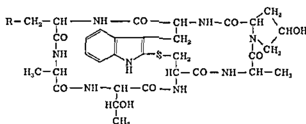


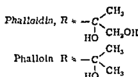
Enniatin A R = CH_3
Enniatin B, R = H
Fusarium sp
(Plattner *et al*, 1948)

Sporidesmolide I
Pithomyces chartarum
(Russell, 1960)



Fungisporin
(Val valine
Phe phenylalanine)
Fungal spores
(Miyao, 1960)





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 $C_{24}H_{42}O_6N_2$ 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

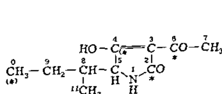
Fungisporin a sublimable component of the spores of some fungi is a cyclic polypeptide containing equal numbers of molecules of valine and phenylalanine

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

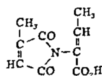
C Heterocyclic Nitrogen Compounds

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

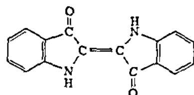
Only relatively simple indole derivatives are mentioned in this section, more complex representatives are considered in other groups

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)

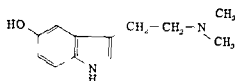
Bufotenin (5-hydroxy-*N*,*N*-dimethyltryptamine) is a constituent of the skin secretion of toads It has been obtained by Wieland by extraction of *Amanita mappa* [A citrina] and has also been detected in other *Amanita* species

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

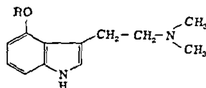
The indole nucleus is also present in the large group of ergot alkaloids derived from *Claviceps purpurea* The natural ergot alkaloids and those obtainable by laboratory culture of the fungus are all related to lysergic acid Some are peptides, others differ in substituents and in configuration Other fungi, e g, *Aspergillus fumigatus*, also produce good yields of indole



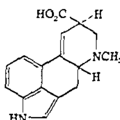
Indigo
Schizophyllum commune
(Miles *et al*, 1956)



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

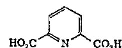
alkaloids It has been found that tryptophan can act as precursor of lysergic acid (Mothes *et al* 1958), and the remainder of the molecule appears to arise by incorporation of mevalonate

3 Pyridine Derivatives

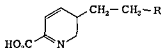
The pyridine derivatives nicotinic acid and pyridoxine are vitamins They are of widespread occurrence in living cells since they function as coenzymes and act as prosthetic groups in a variety of enzymatic reactions

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

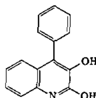
An example of a quinoline metabolite is viridicatin, $C_{15}H_{11}O_2N$, derived from *Penicillium viridicatum* and *P cyclopium* It can also be obtained by mild acid hydrolysis of the more complex structure cyclopenin, $C_{17}H_{14}O_3N$ also produced by *P cyclopium* The degradation products of cyclopenin, in



Dipicolinic acid
Penicillium sp
(Ooyama *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)



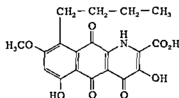
Viridicatin
Penicillium viridicatum,
P cyclopium
(Cunningham and
Freeman, 1953
Bracken *et al* , 1954)

FIG 33 Pyridine and quinoline derivatives

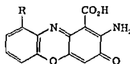
addition to viridicatin are methylamine and carbon dioxide The structure of cyclopenin is still under investigation

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
(Kogl and Quackenbush, 1944,
Birch *et al*, 1964)



Cinnabarin R CH_2OH
Cinnabarinic acid R CO_2H
Polystictus sanguineus
(Cavill *et al* , 1959,
Gripenberg, 1958)

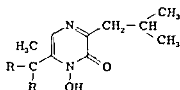
FIG 34 Tricyclic nitrogen derivatives

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

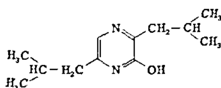
related to the insect pigments known as ommochromes and to the actinomycins obtained from *Streptomyces* species

5 Pyrazine Derivatives

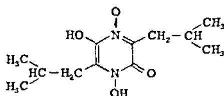
Aspergillic acid and hydroxyaspergillic acid are products of *Aspergillus flavus* The first indication of the presence of aspergillic acid in the culture filtrate was given by its antibiotic activity Muta aspergillic acid and flavacol have also been obtained from the same mold The four structures (Fig 35)



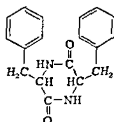
Aspergillic acid, $\begin{cases} R = C_2H_5 \\ R = H \end{cases}$
Hydroxyaspergillic acid, $\begin{cases} R = C_2H_5 \\ R = OH \end{cases}$
Muta-aspergillic acid, $\begin{cases} R = CH_3 \\ R' = OH \end{cases}$
Aspergillus flavus
(Glister, 1941, Dutcher, 1958, Nakamura, 1960, 1961)



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

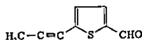
CH_3S-SCH_3 Dimethyl disulfide

$(CH_3)_2S$ Dimethyl sulfide

Schizophyllum commune
(Birkinshaw *et al* , 1942a
Challenger and Charlton, 1947)

$(CH_3)_2SO_2$ Dimethyl sulfone

Cladonia deformis
(Bruun and Sorensen, 1954)



Junipal
Daedalea juniperina
(Birkinshaw and Chaplen, 1955)

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

Penicillium chrysogenum, after brief incubation with C^{14}-valine, a tripeptide having the structure δ (α aminoadipoyl)cyst(e)inylvaline (Fig 37) α Aminoadipic acid was also obtained chiefly in the L form The possible biogenetic relationship with cephalosporin N, another antibiotic, is immediately evident The pathway for penicillin biosynthesis proposed by Arnstein and Morris (1960) involves the conversion of the tripeptide to cephalosporin N by appropriate ring closures The final stage requires exchange of the α-aminoadipoyl side chain of cephalosporin N for a phenacyl group

Another antibiotic from a *Cephalosporium* species, cephalosporin C, has a structure which could also arise from the tripeptide δ (α aminoadipoyl)cysteinylvaline by suitable oxidation of the *gem* dimethyl group and a different type of ring closure to form a six-membered dihydrothiazine ring in place of the five-membered thiazolidine ring of penicillin

A further member of this group, gliotoxin, derived from several fungal species, has bacteriostatic and fungicidal properties Radioactive tracer studies have shown that gliotoxin is biosynthesized by *Trichoderma viride* from one molecule each of phenylalanine and serine, which combine to form an anhydropeptide ring, with addition of two sulfur atoms to form a disulfide bridge Since *m*-tyrosine can act as precursor, it appears that hydroxylation can occur before cyclization of the aliphatic side chain of phenylalanine

VIII METABOLITES CONTAINING CHLORINE

Chlorine is a constituent of a number of fungal metabolites, some of which have already been mentioned, e g, geodin (Fig 21) and mollisin (Fig 24) Organically bound chlorine is not a necessary precursor in their biosynthesis since these products are readily formed in synthetic media in which the chlorine is present as chloride ion When the fungus is deprived of chloride, it may produce the dechloro analog of the metabolite In most cases the chlorine is attached to a carbon atom of a carbocyclic ring of aromatic type Exceptionally, caldariomycin, a dihydroxycyclopentane derivative, carries two chlorine atoms attached to the same carbon atom

In studying the synthesis of caldariomycin, P D Shaw *et al* (1959) have found that the formation of the carbon-chlorine bond is catalyzed by an enzyme which is present in *Caldariomyces fumago* The enzyme promotes the conversion of β-ketoadipic acid and chloride ion into δ chlorolevulinic acid The latter, when labeled with Cl^{36}, is converted by the mold into labeled caldariomycin The reactions are presumed to follow the scheme shown in Fig 38

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 Pornidulin (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

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CHAPTER 10

Carbohydrate Metabolism

I 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 Buchner 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 d phosphopyridine 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 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 phosphoryla tion 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,

1963) Lest anyone should assume that pathways involving phosphorylated intermediates are the only ones it may be noted that three pathways for carbohydrate catabolism that do not involve phosphorylated sugars have already been discovered in bacteria These are the conversion of glucose to α-ketoglutarate by *Acetobacter melanogenum* and the conversion of L-arabinose to α ketoglutarate and of D-arabinose to glycolate and pyruvate by *Pseudomonas saccharophila* (cf Hollmann, 1964) The chances are very great that there are still undiscovered pathways for carbohydrate metabolism in fungi Furthermore, 'Whereas the tell tale evidence of existence of new metabolic pathways in the great majority of other organisms cannot be foretold without laborious studies, it seems to be uniquely characteristic of fungi that they virtually advertise the presence of new metabolic pathways by the piling up of compounds representing one or more stages in a pathway" (Foster, 1958)

II METHODOLOGY

The classical procedures for the investigation of carbohydrate catabolism have not been very successful when applied to fungi The many advances in our understanding of fungal glycolytic processes during the past decade have resulted primarily from studies both with isolated enzymes and with radioisotopic techniques applied to intact cells Consequently, emphasis will 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 can 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 high 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-

nate aerobically but not anaerobically, glucuronic acid, glucose 6 P or fructose-1,6 di-P were not oxidized These results, as well as supplementary manometric studies employing metabolic poisons and isotopic glucose, led to the conclusion that this fungus employs a modified ED pathway Negative data obtained by this technique, or any other, must be interpreted carefully Many compounds particularly the phosphorylated sugars involved in the EM, HMP, or ED pathways, are usually impermeable to cells In addition, phosphatases often are located on the cell surface (cf Rothstein, Chapter 15) and can hydrolyze the phosphate ester so that it is the free sugar that actually is being metabolized This problem can become even more complicated if the phosphatase has some degree of specificity, as in the case of a highly purified acid phosphomonoesterase from *Neurospora crassa* which hydrolyzes phosphorylated sugars at different rates (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 measured 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 situation 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 phosphofructokinase 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 accumulation 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: Trivial name(s)	Enzyme: Systematic name	Pathway(s) involved
Glucose + ATP	Glucose 6-P + ADP	Hexokinase	ATP D Hexose 6-phosphotransferase	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	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 + 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

of evidence used by Ramachandran and Gottlieb (1963) to determine that glucose was catabolized by the ED pathway in *Caldariomyces fumago*

Cycloheximide (Actidione) is another agent that can be used to cause the accumulation of α keto acids in fungi The action of the cycloheximide can be reversed in *Neurospora sitophila* by the addition of thiamine (Seydoux and Turian, 1962)

Useful information may sometimes be secured by means of studies on the mechanism of resistance to a poison For example, in a study on the resistance of *Aspergillus niger* to the fungistatic action of *m* dinitrobenzene, Higgins and Chambers (1963) reported that some important changes in carbohydrate metabolism had accompanied the acquisition of resistance The resistant strain could metabolize glucose anaerobically whereas the parent strain could not and the former could no longer oxidize gluconate

In addition to studies of the types just described, poisons are used to cause the accumulation of intermediates which can then be identified by various chromatographic techniques (cf Godin 1955), It is not always necessary to add poisons, however, to induce the accumulation of intermediates Duff and Webley (1959) were able to accomplish this with HMP pathway components from soil nocardias simply by keeping the pH near neutrality or by using gluconate as the carbon source rather than glucose

The compounds found in the medium will usually be dephosphorylated by cell phosphatases, so in order to obtain phosphate esters it is necessary to extract the mycelium Thus, Moses (1959) used this technique to study the effect of azide on the intracellular intermediates formed during glucose metabolism in *Zygorhynchus moelleri*

The effect of respiratory poisons, such as cyanide, on fungi has been considered by Lindenmayer in Chapter 12 In addition, Hochster and Quastel (1963) have written a comprehensive treatment of metabolic inhibitors

E Mutants

Although *Neurospora* mutants have been used for the determination of certain biochemical pathways, particularly those involving amino acids, they have not been employed for the investigation of glycolytic mechanisms Strauss and Pierog (1954) did, however, report an apparent increase in the utilization of the HMP pathway as a result of a mutation in *N crassa* that nearly completely blocked its pyruvate metabolism

Mutations affecting glycolytic enzymes were apparently unknown before the recent report of a mutant of *Salmonella typhimurium* deficient in glucosephosphate isomerase (Fraenkel *et al*, 1963) Because of this defect, glucose, but not fructose, must be glycolyzed in this bacterium by means 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 (Horvath 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 thr [illegible] nt media (Tsao, 1962) It is possible that the isozymes are repr [illegible] of glycolytic sequences from different cellular locations (cf Z [illegible] ter 14)

The successes associated with the demonstration of the [illegible] zymes in molds have paralleled the general rapid rise in enzyme te [illegible] A list of the EM enzymes and some HMP enzymes that have be [illegible] representative fungi is presented in Table III The phosphor [illegible] age of fructose-6-P or xylulose-5 P that is catalyzed by the [illegible] spho ketolase in certain bacteria (Horecker 1962a) has not b [illegible] ted in fungi

The preparation of active cell free extracts has been [illegible] d 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 [illegible] ars to demonstrate fungal glycolytic enzymes was due in part to the [illegible] ry 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	+	+	+	+	+	+		+	+	+	+	+	Horvath and Szentirmai (1962)
Tilletia caries	+	+		+							+	+	Newburgh *et al* (1955)
Ustilago maydis, mycelium				+		+	+				+		McKinsey (1959)
Ustilago maydis, spores	+	+	+	+	+	+	+	+	+	+	+	+*	Gottlieb and Caltrider (1963)

* 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 phospho gluconate 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

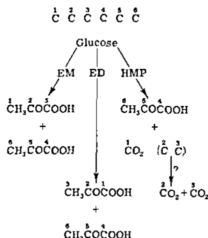

FIG 1 Distribution of the carbon atoms of glucose in pyruvic acid formed by the Embden Meyerhof (EM) Entner Doudoroff (ED) and hexose monophosphate (HMP) pathways of glucose catabolism

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 $C^{14}O$ 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 $C^{14}O$ 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

$$G_p = \frac{G - G}{G_T - G_T} \qquad (1)$$

and

$$G_e = 1 - G_p \qquad (2)$$

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 $C^{14}O$ 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

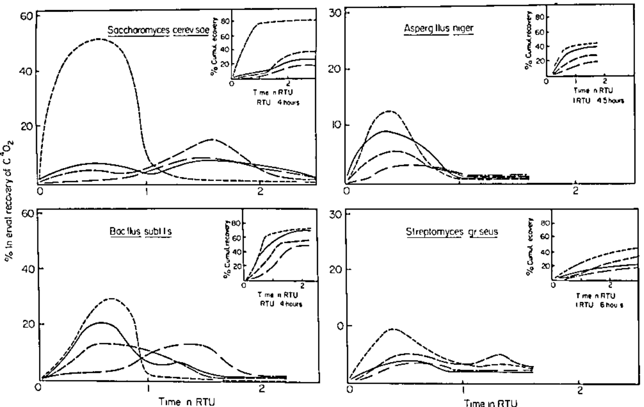

FIG. 2. Time course plots of interval radiochemical recoveries from proliferating microorganisms metabolizing specifically labeled glucose. Legend: Glucose-1-C^{14} ———, 2-C^{14} — — —, 3,4-C^{14} - - - - - -, 6-C^{14} — — —. The insets show the cumulative recoveries of $C^{14}O_2$.

When the magnitude of G_t is small, as it is usually assumed to be, Eq. (1) becomes

$$G \quad G \quad G \tag{3}$$

If necessary, the magnitude of the term G' can be estimated by determining the amount of C^{14} in cellular constituents using glucose-U-C^{14} or glucose-3,4-C^{14}. 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^{14} along with the glucose-1- and -6-C^{14} has recently been reported (Wang and Krackov, 1962). If A_t is equal to the $C^{14}O_2$ yield observed at time *t* following the metabolism of a given amount of gluconate-6-C^{14}, then Eq. (1) becomes

$$G = \frac{G - (G - G_p A_t)}{G_T - G_T} \tag{4}$$

and Eq. (3) becomes

$$G = \frac{G - G}{1 - A} \tag{5}$$

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 $C^{14}O_2$ from glucose-6-C^{14} 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 estimation of pathways will be discussed in the next section However, a new method based on the release of CO_2^{18} from glucose 1- and 6 O^{18} has appeared (Rittenberg and Ponticorvo, 1962) By means of this technique, it was estimated that *Escherichia coli* utilized between 10 and 30% of the glucose via the HMP pathway This compares with a minimal value of 28% obtained by Wang *et al* (1958a) Since there are some major differences in the assumptions on which the two different CO methods are based, it is encouraging to see relatively good agreement in the estimates Evidence for a similar concurrence of pathway estimates based on the isolation of C^{14} intermediates and the $C^{14}O_2$ yields in the same experiment gives still more confidence in these techniques

2 Methods Based on the Specific Activities of C^{14} Labeled Intermediates

A procedure has been devised for the estimation of the relative extents of participation of the EM and HMP pathways based on the following assumptions (a) that these pathways are the only ones involved and (b) that trioses, or 3 carbon compounds derived therefrom, formed via the EM process arise equally from glucose carbons 1 to 3 and 4 to 6, whereas those derived via the HMP pathway arise only from carbons 4 to 6 (Blumenthal *et al*, 1954) These relations can be seen in Fig 1 Experimentally the procedure consists of carrying out simultaneously, with the same cell preparation, experiments with glucose 1 C^{14} and glucose-U C^{14} A 3 carbon compound such as lactate or pyruvate or a 2 carbon compound derived therefrom, e g acetate and/or ethanol, is isolated from each and is purified and assayed for radioactivity It is unnecessary to recover the intermediates quantitatively When an appropriate intermediate does not accumulate, one can be "trapped" by the addition of some of the unlabeled substance *in* question, or an inhibitor, such as arsenite, can be added The data from glucose-1-C^{14} provide an indication of the relative incorporation of this carbon in the intermediate, and the data from glucose U-C^{14} provide a correction factor for the endogenous metabolism, whereby "true" values may be 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 the 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 $(1/4 - 1/6) \times 100 = 150$ 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

$$\text{RSA(corrected)} = \frac{\text{RSA(observed) for acetate from glucose 1 } C^{14}}{\text{RSA(observed) for acetate from glucose U } C^{14}} \quad (7)$$

Since acetate formed via the EM would have a RSA(corrected) of 150

$$\%\ \text{Acetate via EM} = \frac{\text{RSA(corrected)}}{150} \times 100 \quad (8)$$

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

$$\%\ \text{Glucose via EM pathway} = \frac{\%\ \text{acetate via EM}}{200 - \%\ \text{acetate via EM}} \quad (9)$$

$$\%\ \text{Glucose via HMP pathway} = 100 - \%\ \text{glucose via EM} \quad (10)$$

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 or 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 [?] 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 $C^{14}O$ 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 $C^{14}O$ 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 (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 phosphofructo kinase 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 (%)	Hexose monophosphate (%)	Entner-Doudoroff (%)	Method	References
Aspergillus niger	75		—	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	—	$C^{14}O$ 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 albo-atrum	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] Major	Minor	References
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)
Claviceps 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 Koffler (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	(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 albo-atrum	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.

thereby 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 controversa* 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

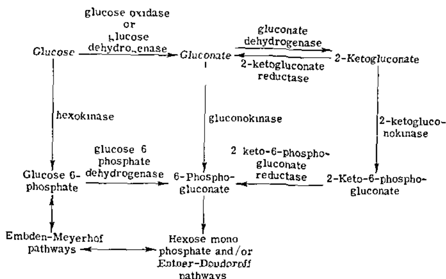

FIG 3 Interrelationships in the metabolism of hexoses and hexonic acids

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 as 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 $C^{14}O$ 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

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 transket l e and trans aldolase reactions The results of experiments with spe c lly labeled glucose indicate that both arms of the HMP cycle c ib te to the ribose P of nucleotides and ribonucleic acid

Erythrose 4 P is a second important component for yn reactions that is provided by the HMP pathway This intermed it n he HMP pathway is an important precursor in the biosynthesis of r ti t c 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 metabolism are not known although great advances can be expected in the near future The general problem of metabolic regulation is covered by Z[illegible]r (Chapter 14) and has been the subject of symposia (cf Holzer [illegible] 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* microconidia 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 $C^{14}O$ from resting suspensions of these cells was measured in the presence or absence of suffi

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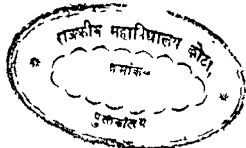
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CHAPTER 11

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	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-

cinic acid accumulated Finally if citrate was acting catalytically some mechanism must exist through which it could eventually be regenerated A variety of intermediates were examined but only oxalacetic acid gave rise to citric acid and this could be demonstrated only anaerobically (i e under conditions that arrested further citrate oxidation) From these experiments and the data of previous investigators a cyclic mechanism for the pathway of carbohydrate oxidation in animal tissues was envisaged (Fig 1) The citric acid cycle was shown to be operative in a variety of animal tissues but was reported absent in yeast and bacteria in these studies (Krebs and Johnson 1937) and also in later work by Krebs (1943)

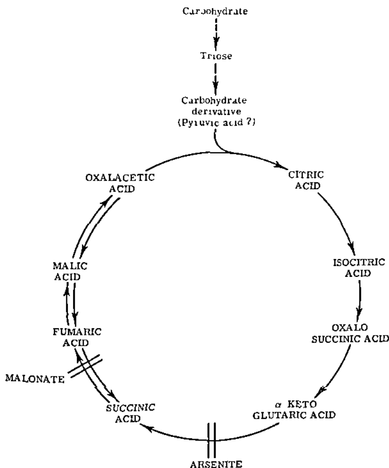


Fig 1 Citric acid cycle (TCA cycle) of Krebs and Johnson

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-

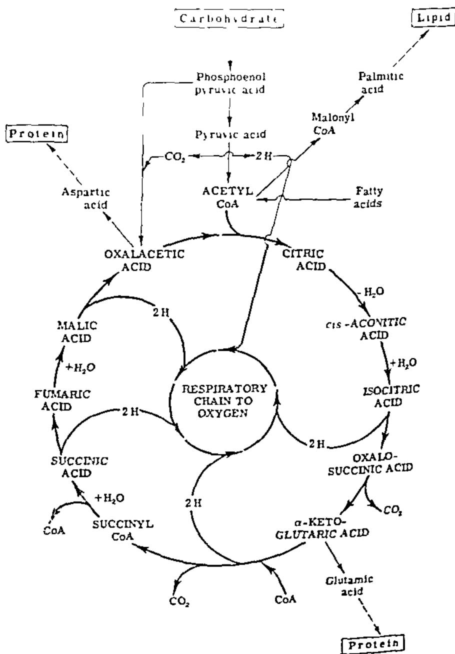


FIG 2 Tricarboxylic acid (TCA) cycle as currently conceived

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 can not 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 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* KBI 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

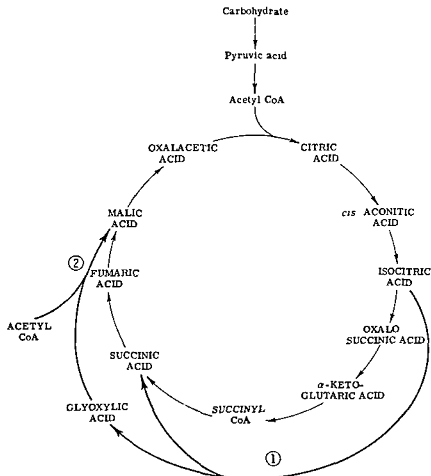


FIG 3 Relationship of TCA cycle to glyoxylate pathway Enzymes catalyzing numbered reactions are (*1*) isocitritase and (*2*) malate synthetase

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.

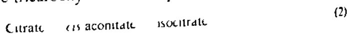
Condensing enzyme. The formation of citrate by an aldol condensation involving acetyl coenzyme A and oxalacetate is mediated by the condensing enzyme (Eq. 1).

$$\text{Acetyl coenzyme A} + \text{oxalacetate} + H_2O \rightleftharpoons \text{citrate} + \text{coenzyme A} + H^+ \quad (1)$$

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

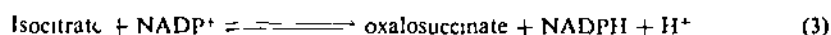
Aconitase The enzyme aconitase is concerned primarily with the interconversion of the three tricarboxylic acids (Eq 2)


Citrate cis aconitate isocitrate (2)

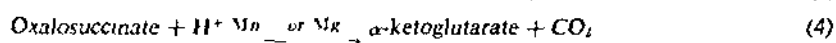
Aconitase has been partially purified from animal tissues (see Anfinsen 1955) and is activated by ferrous ions and reducing agents (Dickman and Cloutier 1951, Morrison, 1954) Cofactor requirements of this type have likewise been demonstrated for aconitase preparations from *A niger*, *Penicillium purpurogenum* 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

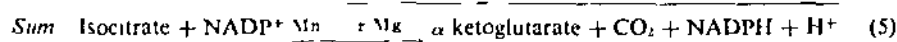
dehydrogenases are distinguished by their cofactor specificities and other important features The NADP linked enzyme is described here, for it appears to be widely distributed in fungi In addition the latter activity occurs largely in the soluble, extramitochondrial fraction of the cell and may play an important role in the maintenance of reducing power (Lowenstein, 1961) The reaction sequence catalyzed by the NADP specific isocitric dehydrogenase is as follows



$$\text{Isocitrate} + \text{NADP}^+ \rightleftharpoons \text{oxalosuccinate} + \text{NADPH} + \text{H}^+ \qquad (3)$$



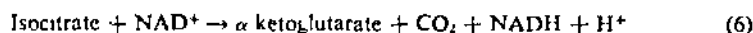
$$\textit{Oxalosuccinate} + H^+ \xrightarrow{Mn \text{ or } Mg} \alpha\textit{-ketoglutarate} + CO_2 \qquad (4)$$



$$\textit{Sum} \quad \text{Isocitrate} + \text{NADP}^+ \underset{}{\overset{Mn \text{ or } Mg}{\rightleftharpoons}} \alpha \text{ ketoglutarate} + CO_2 + \text{NADPH} + \text{H}^+ \qquad (5)$$

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

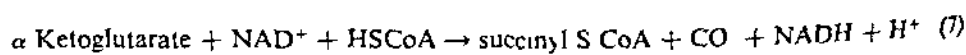


$$\text{Isocitrate} + \text{NAD}^+ \rightarrow \alpha \text{ ketoglutarate} + CO_2 + \text{NADH} + \text{H}^+ \qquad (6)$$

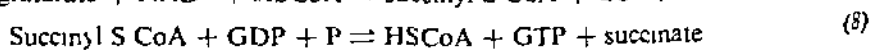
While the NADP-linked enzyme occurs largely in the cytoplasm the NAD-specific isocitric dehydrogenase appears to be bound to the mitochondria

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



$$\alpha \text{ Ketoglutarate} + NAD^+ + HSCoA \rightarrow \text{succinyl S CoA} + CO + NADH + H^+ \quad (7)$$

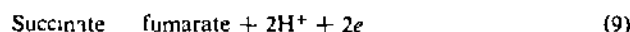


$$\text{Succinyl S CoA} + GDP + P \rightleftharpoons HSCoA + GTP + \text{succinate} \quad (8)$$

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:



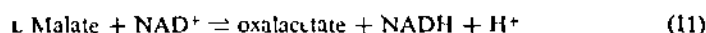
$$\text{Succinate} \rightleftharpoons \text{fumarate} + 2H^{+} + 2e \qquad (9)$$

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

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.



$$\text{L-Malate} + NAD^+ \rightleftharpoons \text{oxalacetate} + NADH + H^+ \quad (11)$$

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 Sclerotinia 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	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

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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

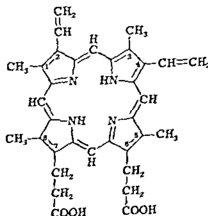


FIG 1 Protoporphyrin 9

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—CH₂—R₁	—CH=C(R₂)(R₃)	—C(H)=O
B	Protoporphyrin iron	—CH=CH₂	—CH=CH₂	—CH₃
C	Substituted mesoporphyrin iron with covalent porphyrin protein linkages	—CH(CH₃)—S—R	—CH(CH₃)—S—R	—CH₃
D	Dihydroporphyrin iron	1 vinyl (or alkylvinyl) and 1 α hydroxy alkyl side chain		—CH₃

[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 $Fe^{++} \rightleftharpoons Fe^{+++}$ 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 mostly 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) Smith (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

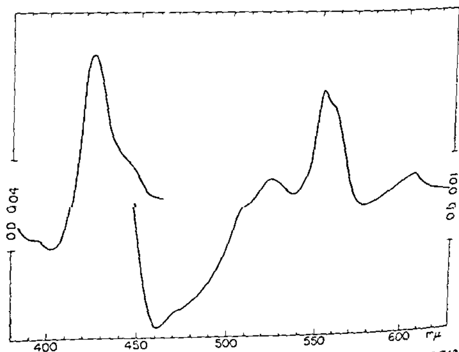


FIG 2 Difference spectrum of aerobically grown bakers' yeast (strain LK2G12) Reference cuvette was aerated the other cuvette anaerobic Optical density scale is different on right and left hand side of figure, as indicated on margins From Lindenmayer and Smith (1964).

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-

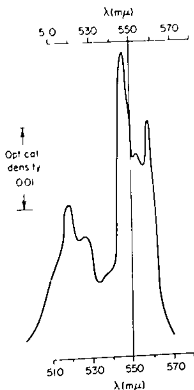


FIG 3 Absolute absorption spectrum of commercial bakers yeast at the temperature of liquid air From Lindenmayer and Estabrook (1958)

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 Tissieres 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* [*Cerebella puteana*]
Absidia orchidis	*Cyathus striatus*
Achlya radiosa	*Fomes annosus*
Allomyces javanicus	*Hypholoma fasciculare*
Cunninghamella echinulata	*Marasmius androsaceus*
Mucor hiemalis	*Marasmius graminum*
Mucor ramannianus	*Marasmius peronatus*
Phycomyces nitens	*Polyporus betulinus*
Pythium ultimum	*Polystictus versicolor*
Rhizopus stolonifer	*Sphaerobolus stellatus*
Saprolegnia sp	*Tricholoma nudum*
Syncephalastrum spinosa	*Trametes rubescens*
Thamnidium elegans	
Zygorhynchus moelleri	Deuteromycetes
Zygorhynchus vuillemini	*Aspergillus nidulans*
Ascomycetes	*Aspergillus niger*
Gelasinospora tetrasperma	*Aspergillus versicolor*
Neurospora crassa	*Botrytis allii*
Sordaria fimicola	*Cladosporium fulvum*
	Cytosporina sp
Mycelia sterilia	*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 Dohn (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 sauerstoffubertragendes Ferment ist die autoxydable Haminverbindung die sich

(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 × 10^5 (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 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^{-3} *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 Tissieres, 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 cyto chromes 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 Paleus 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 ± 0 05, and its redox potential at pH 6 4 is E_o = +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 $m\mu$. 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 (Boreı and Sjoden, 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 (Ycas 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 (Ycas 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 $m\mu$ 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 $m\mu$. 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

studied by Ball and Cooper (1957), Bernstein and Wainio (1960), Bomstein *et al* (1961), and Orii *et al* (1962)

The 554-mμ low temperature band was observed in bakers yeast by Lindenmayer and Estabrook (1958) "Frozen steady-state' spectra of bakers' 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 function the subject of the most controversies (cf Slater and Colpa Boonstra, 1961) Purified preparations of cytochrome b have been obtained from bovine heart and studied by Hubscher *et al* (1954), and Doeg *et al* (1960), those of cytochrome b and c_1 by Takemori and King (1962) No work has come to my attention concerning the isolation of cytochrome b from yeast or any other fungus

The absorption bands of the mammalian pigment in the reduced state are at 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 react 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 catal se f hcr eradi h peroxidases and of methemoglobin (oxidized hemoglobin fr m b d and of their various compounds, are very similar to each ther K il n 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 m 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

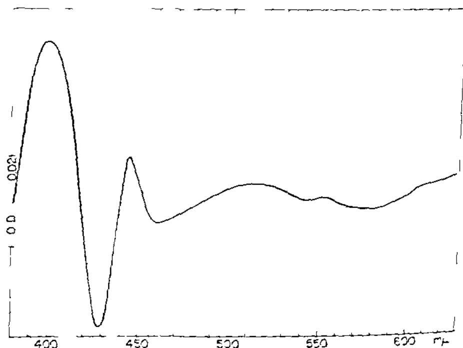


Fig 4 Cyanide difference spectrum of commercial bakers' yeast. Both cuvettes were anaerobic, cyanide was added to one Previously unpublished experiment of Lindenmayer and Smith

of the reduced band of cytochrome a 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 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 Tissieres (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 O_ itself) with H O₂, 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 cyto chrome deficiency) The absorption spectra of normal and *petite* yeast grown anaerobically or oxygen adapted are shown in Fig 5 The spectra of

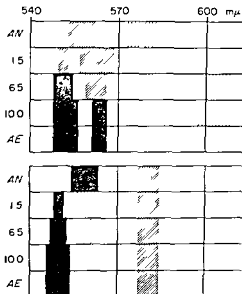


FIG 5 Absorption spectra in the visible region of wild type (top of figure) and *petite* (bottom of figure) yeast *AE* aerob cally grown cells *AN* anaerobically grown cells The other rows show spectra of anaerobically grown cells after they have been aerated for the given number of hours in glucose 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 Tissieres *et al* (1953) Keilin and Tissieres (1953) Tissieres and Mitchell (1954) and Pittenger (1956)

The difference in cytochrome composit on 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

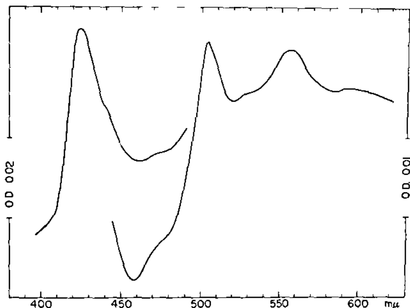


FIG 7 Difference spectrum of anaerobically grown bakers yeast (strain LK2G12) Reference cuvette was aerated, the other cuvette anaerobic Optical density scale is different on right- and left hand side of figure From Lindenmayer and Smith (1964) *The peak at 503 mμ is due to an unidentified pigment present 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. 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:

uroporphyrinogen III $\longrightarrow$ coproporphyrinogen III $\xrightarrow{+O_2}$
$\longrightarrow$ protoporphyrinogen $\xrightarrow{+O_2}$ protoporphyrin 9 $\xrightarrow{+Fe^{++}}$ heme
protoporphyrinogen $\xrightarrow{Fe^{++}\text{, cys and polypeptides}}$ cytochrome c

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

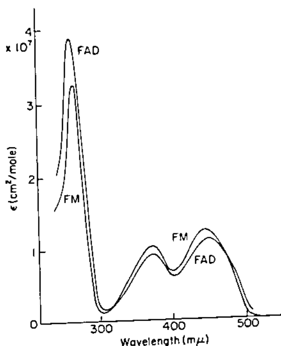


FIG 8 Absorption spectra of flavine mononucleotide (FM) and of flavine adenine dinucleotide (FAD) in the oxidized state Reproduced, by permission from Dixon and Webb (1958)

(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-

tivity Similarly, Linnane *et al* (1962) reported succinate ferricyanide and NADH ferricyanide oxidoreductase activities in the same organism

An FMN enzyme was isolated from yeast (cf Haas 1955) which catalyzes the oxidation of NADPH by cytochrome c directly, but it has not been shown whether this enzyme occurs in the mitochondria or in the cytoplasm A soluble succinate phenazine methosulfate oxidoreductase also was found in *Claviceps purpurea* by McDonald *et al* (1963)

Another set of flavoproteins the lactate oxidizing enzymes of yeast, some of which occur in the mitochondria, is discussed in the next section

Many other flavine enzymes have been described from yeast and other fungi, including various dehydrogenases, nitrate and nitrite reductases, glucose and L-amino acid oxidases, but none of these are involved in particulate electron transport systems Riboflavine is known to be widespread among fungi and abundant in some species, in fact it is from this source that 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 (L(+) lactate cytochrome c oxidoreductase), a flavohemoprotein also called cytochrome b_2 This enzyme is partly soluble and partly particulate (Vitols and Linnane, 1961), and its ability to react with cytochrome c increases markedly as it becomes solubilized (Somlo, 1962) It has been studied very extensively in recent years, mainly because of the great interest in 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 single-stranded DNA (consisting of 33 nucleotides) is regularly associated with this enzyme, even through crystallization However, the DNA is not essential for activity The prosthetic groups are iron protoporphyrin, FMN, and magnesium The electron (or hydrogen) donors are L lactate, L-α hydroxybutyrate, and glycolate, and the electron acceptors include cytochrome 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

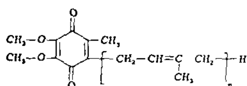
(Chance, 1961a) or in isolated electron transport particles of yeast unless exogenous cytochrome c is added to them (Mahler *et al* 1964a) Furthermore, Slonimski (1953) was not able to demonstrate such participation in aerobically grown *petite* cells although there was lactate cytochrome c reductase activity in their extracts Also Gregolin and Ghiretti-Magaldi (1961) succeeded in isolating normal cytochrome b from *petite* cells These and other findings suggest that cytochrome b 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

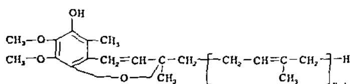
In anaerobically grown bakers' yeast, on the other hand, there is a fourth lactate oxidizing enzyme which oxidizes D(−)-lactate and other D α-hydroxy acids with ferricyanide, methylene blue, and 2,6 dichlorophenolindophenol, but not with cytochrome c as acceptor (Lindenmayer and Smith, 1957, Slonimski 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

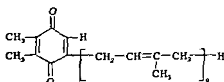
Ubiquinone (UQ_n) $6 < n < 10$



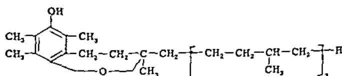
Ubichromenol (UC_n) $6 \quad n \quad 10$



Plastoquinone (PQ_9)



Vitamin E (α tocopherol)



Vitamin K_1

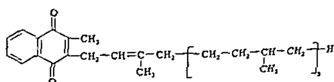


Fig. 9 Quinones

TABLE III

UBIQUINONE IN THE FUNGI

Organism	Type of UQ	Amount (μmole/g dry wt)	Ref.
Mucor Absidia corymbifer	UQ_9	0.70	Lester and Crane (1959); Lester *et al.* (1958, 1959); Crane or *et al.* (1958)
Saccharomyces cerevisiae			
Grown aerobically	UQ	0.35	Lester and Crane (1959); Lester *et al.* (1958, 1959); Crane or *et al.* (1958)
Grown aerobically	UQ	0.14	Mahler *et al.* (1964b)
Grown aerobically	UQ	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	Tetrahydro UQ_{10}	—	Lavate *et al.* (1962)
Aspergillus terreus	Tetrahydro UQ_{10}	—	Lavate *et al.* (1962)
Penicillium stipitatum	Tetrahydro UQ_{10}	—	Lavate *et al.* (1962)
Neurospora crassa	Tetrahydro UQ_{10}	—	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	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 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 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 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 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-

tion does not hold, however because there are some organisms that contain polyunsaturated fatty acids but no mitochondria (the blue green algae in their chromatophores), and there are also organisms which have sterols but apparently no mitochondria (the red algae) The polyunsaturated fatty acids of yeast and other fungi except *Phycomyces blakesleeanus*, are primarily linoleic and α linolenic acids, which compounds they share with the blue green, red and green algae as well as with all of the higher plants The animals, and the ciliated and amoeboid protozoa on the other hand, share the γ-linolenic acid derivatives, which have also been found in *P blakesleeanus* (Bernhard and Albrecht, 1948) Some unusual polyunsaturated fatty acids have been found in *Dictyostelium discoideum* (Davidoff and Korn, 1963) the synthesis of which does not correspond to 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 monounsaturated fatty acids from saturated ones takes place by oxidative desaturation only in the fungi, animals, and in some of the algae and bacteria Other bacteria are able to accomplish the latter transformation by anaerobic means, while the green plants seem to have a third mechanism still, requiring oxygen (Erwin and Bloch, 1964) The biosynthesis of sterols also requires molecular oxygen (Goldfine and Bloch 1963)

The inability of bakers yeast to grow under prolonged anaerobiosis, unless ergosterol and oleic acid (or other monounsaturated fatty acids) are added to the usual growth factors, was demonstrated by Andreasen and Stier (1953, 1954, 1956) and Harris (1956) These findings helped eventually to elucidate the sterol and unsaturated fatty acid synthetic pathways 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 requires 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

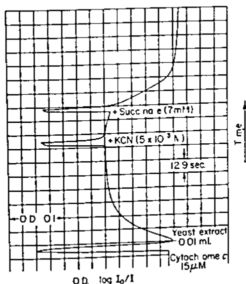


FIG 10 Ox dat on of reduced cytochrome c by cytochrome c ox dase of yeast extract followed by reduction of the cytochrome c by the yeast reductase in the presence of endogenous substrate then after add t on of succinate Cytochrome c concentrat on in test was 15 μM buffer was 0 015 M phosphate pH 7 0 final con centration of succinate was 7 mM Yeast extract was prepared from starved com merc al bakers yeast Sharp peaks in the trace result from the introduct on of stirring rod and mark the time of add t on of reagents Reproduced by perm ssion from Smith (1954a)

strate—B—C—A—O has been postulated at an early stage and still ap pears to be valid For a while doubts were cast on the role cytochrome c played in the system and it has even been called an artifact but there seems to be general agreement now concerning its participation The above se quence was borne out by the redox potentials of the various cytochromes (Falk and Perrin 1961) which are increasingly positive in the postulated order Kinetic studies concerning the time sequence at which the different cytochromes become oxidized on admission of oxygen or reduced on addi tion of substrate corroborate this order (Chance and Williams 1956)

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, Tissieres and Mitchell, 1954, Tissieres, 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)

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)

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*

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ADDENDUM TO SECTION VII

Further extensive work on the various yeast mutants unable to utilize nonferment able 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 Labbe *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*

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	43
1×10^{-2}	0.48	60	85
5×10^{-2}	0.30	75	90

* 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

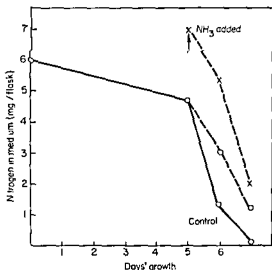


FIG 1 *Scopulariopsis brevicaulis* Effect of addition of ammonia on the uptake of *nitrite from the medium* O, Nitrite nitrogen, X, ammonia nitrogen, —, culture in nitrite only - - - culture in nitrite and ammonia From Morton and MacMillan (1954)

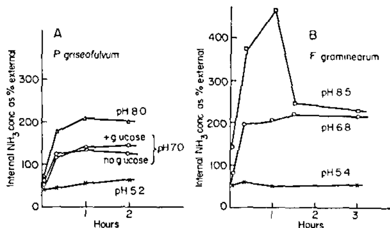


FIG 2 Ammonia in mycelium of (A) *Penicillium griseofulvum*) and (B) *Fusarium graminearum* shaken in buffers containing 0 4 mg of ammonia nitrogen per milliliter From MacMillan (1956)

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)[a]

Parameter	Nitrogen source				
	KNO_3	NH_4NO_3	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + tartrate	$(NH_4)_2SO_4$ + 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).

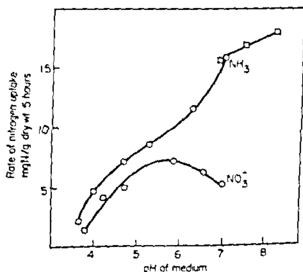


FIG. 3. *Scopulariopsis brevicaulis*. Rate of uptake of ammonia and nitrate in relation to pH of medium. O—O, 0.1 M citrate phosphate buffers; □—□, 0.1 M pyrophosphate buffer (separate experiments). From Morton and MacMillan (1954).

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-

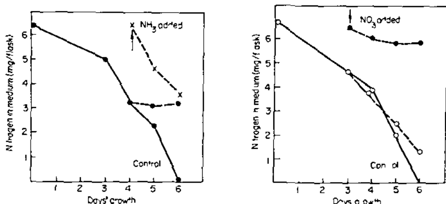


FIG 4 *Scopulariopsis brevicaulis* (*Left*) Effect of addition of ammonia on the uptake of nitrate from the medium — Culture in nitrate only – – culture in nitrate and ammonia ● nitrate N X ammonia N (*Right*) Effect of addition of nitrate on the uptake of ammonia from the medium — Culture in ammonia – – culture in ammonia and nitrate ● nitrate N O 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*

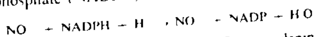
When nitrate grown mycelia of *A niger* *B allii* *P chrysogenum* and *Trichoderma viride* was transferred to nitrate, ammonium nitrate and ammonium media, the rate of nitrate uptake was reduced by 42–82% in the presence of ammonia whereas the rate of ammonia uptake was reduced by only 6–11% in the presence 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

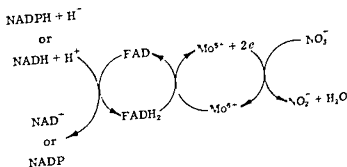
A Nitrate Reductase

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

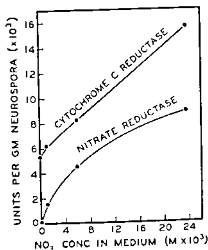


The enzyme which was shown to contain flavine adenine dinucleotide (FAD), was inhibited by cyanide and various metal-chelating agents Although a metal requirement was inferred the authors obtained no response with several metals including molybdenum Subsequently it was established that molybdenum is 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 facili tate the oxidation reduction between flavine and nitrate It is known that the chemical reduction of these anion–molybdate complexes is more read ily 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 molyb denum is involved in electron transfer in the enzyme has come from recent studies with electron paramagnetic resonance studies (EPR) with the puri fied 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 re ductase activity closely paralleled that of nitrate reductase during purifica tion (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 ni rate



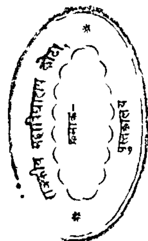


FIG 5 Effect of nitrate concentrat on on the adaptive formation of n trate and cytochrome c reductase act vity *Net rospora* was grown at room temperature without forced aerat on in basal growth medium containing the following (grams per l ter) NH Cl 1 0 KH PO 1 0 MgSO 0 5 NaCl 0 2 CaCl 0 2 sucrose 20 0 sod um tartrate 5 0 biotin 5 μg trace element solution 5 ml Fifty m lliliters of med um 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)

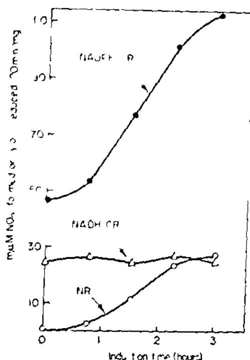


FIG 6 Time course of enzyme induction in *Neurospora* Mycelial sections were incubated for various times in the complete induction medium containing nitrate Sections were then harvested and dried extracts were prepared and assayed for nitrate reductase (NR) NADPH cytochrome c reductase (NADPH CR) and NADH-cytochrome 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 were induced in parallel fashion in *Neurospora* as illustrated in Fig 6

Sorger (1963) found that sucrose density gradient preparations of crude extracts of *Neurospora* had nearly identical activity profiles for the two activities From genetic studies with mutants of *Neurospora* and *Asper*

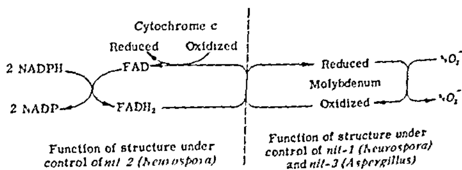


FIG 7 Functional model of nitrate reductase in *Neurospora* and *Aspergillus*

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)

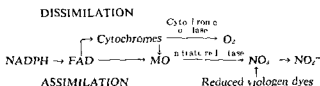


FIG 8 *Alternative pathways of electron transfer to nitrate and oxygen in fungi*

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

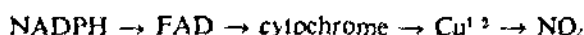
Nitrate reductases so far examined in fungi are induced by their substrate, nitrate The presence of ammonia in the nutrient often reduces enzyme activity in the mycelium Thus in *Scopulariopsis brevicaulis* the enzyme is suppressed by ammonia even when nitrate is provided (Morton, 1956) This effect occurs *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



$$NADPH \rightarrow FAD \rightarrow cytochrome \rightarrow Cu^{+2} \rightarrow NO_2$$

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 denitrifying bacteria (Chung and Najjar, 1956a, b, Fewson and Nicholas, 1960), and there is evidence that the purified enzyme from *Neurospora* also yields nitric oxide (Walker and Nicholas, 1961c) It has also been shown that under strict anaerobic conditions, nitric oxide is readily taken up by a range of microorganisms including fungi Iron deficiency depressed the uptake of nitric oxide by these organisms Although it is clear that nitric oxide is an intermediate in bacterial denitrification, there is insufficient evidence as yet that nitric oxide, or a compound with which it equilibrates, is en route to ammonia during nitrate assimilation in fungi

D Hyponitrite Reductase

An enzyme system has been found in *Neurospora* which catalyzes the reduction of hyponitrite to ammonia This enzyme, which requires NADH,

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)

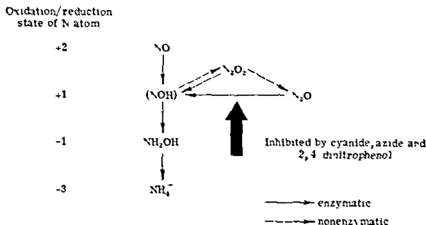


FIG 9 Scheme for the nitrate reduction with nitroxyl (NOH) as an intermediate

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

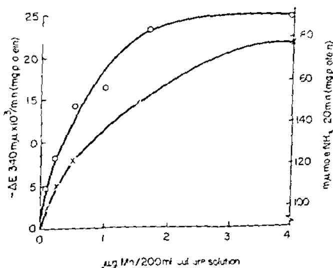


Fig 10 Effect of manganese content of culture solution on hydroxylamine reductase in cell free extracts of *Neurospora* measured by two independent methods NADH oxidation at 340μ O—O and NH_3 production in Conway Units X—X From Nicholas (1959b)

reduced in extracts of mycelium deficient in manganese or in magnesium as shown in Figs 10 and 11 The effects of metal deficiencies on nitrite, hy-

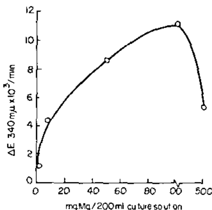


FIG 11 Effect on magnesium content of culture solution on hydroxylamine reductase in cell free extracts of *Neurospora* measured by NADH oxidation at 340 mμ

ponitrite, and hydroxylamine reductases in *Neurospora* based on ammonia production are shown in Table III

TABLE III

EFFECTS OF METAL DEFICIENCIES ON NITRITE HYPONITRITE AND HYDROXYLAMINE REDUCTASES IN *Neurospora crassa* (BASED ON AMMONIA PRODUCTION)[a] [b]

Reductase enzymes	Omit Fe	Omit Cu	Omit Mn	Omit Mo	Omit Zn
Nitrite	22	36	53	100	68
Hyponitrite	51	53	60	100	100
Hydroxylamine	100	100	57	100	95
Weight 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 the hydroxylamine or hyponitrite systems would also inhibit nitrite reductase, provided the reductive pathway to ammonia involved these intermediates Thus a deficiency of manganese that reduced hydroxylamine reductase also depressed nitrite and hyponitrite reductases A deficiency of magnesium was also found to restrict hydroxylamine reductase (Nicholas, 1959a,b) Although manganese and magnesium were necessary for the formation 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 (Burstrom, 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-

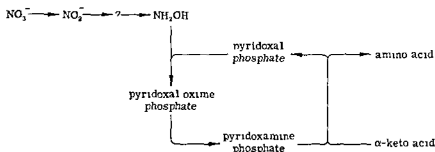
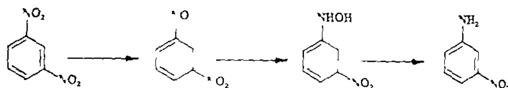


FIG 12 Scheme of nitrate reduction postulated by Silver and McElroy (1954)

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 salt 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

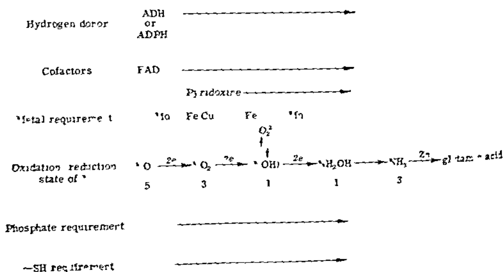


Fig 13 Inorganic reductive pathway for nitrite

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 hydro lyzed and the hydroxylamine formed is reduced to ammonia by hydrox ylamine 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 *Penicillium* 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 ni trite 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 am monium salt with alumina prior to inoculation with the fungus inhibited nitrite or nitrate formation but not that of bound hydroxylamine The ac tivity was restored by returning cerium chloride (10 *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 am monia 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 hydrox ylamine to nitrite and of nitrite to nitrate is mediated by their respective cytochrome c reductases The addition of NADP also stimulated the oxida tion 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 atrovenatum* suggest that an inorganic route involving hy droxylamine similar to the one in nitrifying bacteria is the main pathway for ammonia oxidation in these heterotrophic fungi

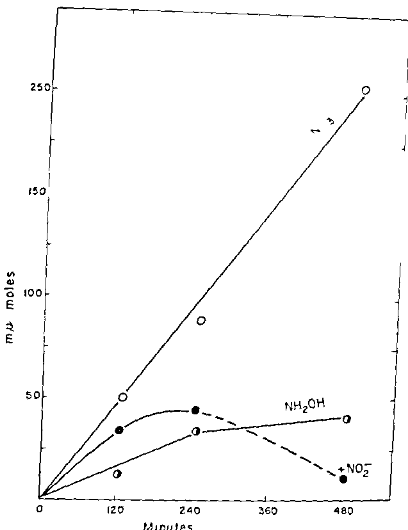


FIG 14 The pattern of ammonium oxidation by cell free extracts of *Aspergillus wentii* Reaction mixtures contained 2 0 ml of cell free extracts 0 2 ml of 2% cytochrome c 0 1 ml of NADPH (3 mg ml) 10 μmoles of $(NH_4)_2HPO_4$ and 0 1 M Tris buffer pH 8 0 to make a total volume of 10 ml Samples of 0 5 ml were taken every 120 minutes and tested for the formation of NH_2OH (◑) NO_2^- (●) and NO_3^- (O) From Aleem *et al* (1964)

V AMINO ACID METABOLISM

The uptake of amino acids by fungi has not been extensively studied Mathieson and Catcheside (1955) found that the growth of a histidine requiring mutant of *Neurospora crassa* was inhibited by certain other amino acids in combination with lysine and arginine The addition of the keto acid derivative of the required amino acid was found to overcome the inhibition by certain other amino acids in *Neurospora* (Brockman *et al*

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

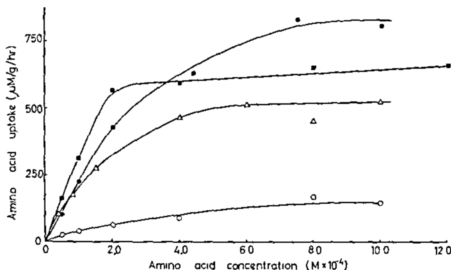


FIG 15 The effect of amino acid concentration on rate of uptake by *Botrytis fabae* Mycelial pellets were suspended in amino acid solutions of the indicated strengths and the rate of uptake was measured △ Glycine ● L valine ■ L lysine ○ L tryptophan From Jones (1963)

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 phycomycete 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*[a]

Inhibitor	Concentration	Inhibition (%)
2 4-Dinitrophenol	10^{-5} M	30
2 4-Dinitrophenol	10^{-4} M	96
Na azide	10^{-2} 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 available suggests that they are incorporated directly into protein and are not degraded first to ammonia Free amino acids are readily detected in fungi Glycine, asparagine, glutamic acid, and aspartic acid in well buffered culture media usually support good growth of a variety of fungi although they are not equally effective Leucine, although utilized by *Trichophyton persicolor*, is not a generally good source of nitrogen, neither is tryptophan which supports a partial growth of the actinomycete *Streptomyces coelicolor*

In many fungi growth is often best on a mixture of amino acids, and others grow well when amides are supplied Thus asparagine serves better than aspartate for *Tricholoma imbricatum Pyricularia oryzae, Leptographium* sp , and *Phycomyces blakesleeanus* Whether this results from the extra nitrogen in the amide or from better buffering capacity of the medium in its presence is not known There is evidence that some fungi utilize the amide nitrogen of asparagine or glutamine more readily than the amino nitrogen Glutamine is readily utilized by *Tricholoma gambosum*

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 Redrawn 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

$$H_2N\,CH_2\,CH_2\,CH_2\,COOH + HOOC{-}CH_2\,CH_2\,CO\,COOH \rightarrow \underset{\text{L glutamic acid}}{HOOC\,CH_2\,CH_2\,CH(NH_2)\,COOH} + \underset{\text{butyric acid}}{CH_3\,CH_2\,CH_2COOH}$$

It is beyond the scope of this chapter to consider the biosynthesis of amino acids so brilliantly exploited by the use of biochemical mutants of fungi, e g , *Neurospora* and *Aspergillus* Adelberg (1953) has concluded however, that present day techniques with isotopes, isolated enzymes, and biochemical mutants which are blocked at various points in a biosynthetic pathway, cannot establish unequivocally that a given metabolite is an intermediate in a vital process

Some of the interconversions studied by means of biochemical mutants of *Neurospora crassa* and *Aspergillus* species may be mentioned briefly

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*

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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

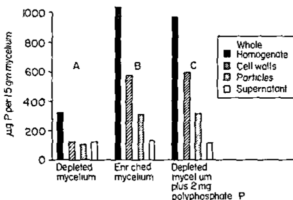


FIG 1 Distribution of insoluble polyphosphate among centrifugal fractions of *Neurospora* (A) mycelium depleted of polyphosphate by growing it in medium devoid of phosphate (B) Mycelium enriched in polyphosphate by the addition of phosphate to the medium (C) Depleted mycelium to which 2 mg polyphosphate 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 (Tissieres *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 1

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	[illegible] and Chargaff 1948
Candida	Mitochondria	Respiratory enzymes		[illegible] 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* 1961
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 Necas (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 Kuster, 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 (enzymic system) detected	Method used	Fungus	References
Iron	Ferricyanide	[illegible]	Ritchie 1947
Polyphosphates	Lead and benzidine metachromatic stains	*Various fungi of all systematic groups*	Ebel *et al* 1958
	Neisser reagent	*All*	Turian 1958
	Metachromatic stains	[illegible]	Wiame 1947
Organic phosphorus	Molybdate of ammonia	*All*	Turian 1958
Glycogen	Bauer's reaction	*Ne[illegible]p[illegible]*	Zalokar 1959b 1960a
Glycogen	Iodine	*All myc[illegible]*	Turian 1958
Polysaccharides	Periodic acid Schiff (PAS)	*All myc[illegible]*	Turian 1958
	PAS	*Penicillium*	Stout and Koffler 1951
	PAS	*Saccharomyces*	Mundkur 1960
	PAS	*Neurospora*	Zalokar 1961b
Fat droplets	Sudan IV	*Saccharomyces*	Lindegren and Rafalko 1950
Lipids	Sudan IV OsO	*Neurospora*	Zalokar 1960a
Proteins	Pauly Sakaguchi SH groups (Seligman)	*Neurospora*	Zalokar 1959b 1960a
—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 1960a
	Triphenyl tetrazolium	*Candida*	Seyfarth 1957 Nickerson 1954
	Triphenyl tetrazolium Nadi Janus Green	*Saccharomyces*	Mudd *et al* 1951
Cytochrome oxidase	Nadi	*Rhodotorula*	Marquardt and Bautz, 1954
	Nadi	*Saccharomyces*	Bautz and Marquardt 1953 Bautz 1956
	Nadi	*Allomyces*	Turian 1958
	Nadi	*Blastocladiella*	Cantino and Horenstein 1956
	Nadi	*Neurospora*	Zalokar 1959b 1960a
Peroxidase	Benzidine	*Neurospora*	Zalokar 1959b 1960a
	Gomori	*Allomyces*	Turian 1958
Alkaline phosphatase	Gomori Meinheimer and Seligman	*Neurospora*	Zalokar 1959b 1960a

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*	Necas 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 $(H_2PO_4)n$], 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 Å 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) Tremothecium* 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

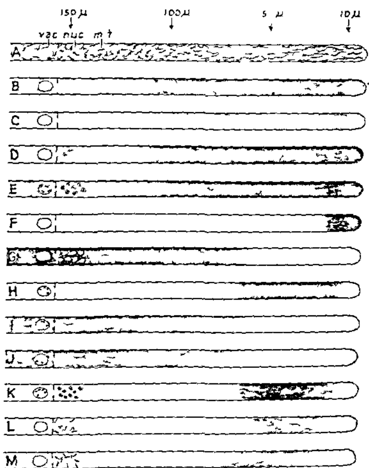
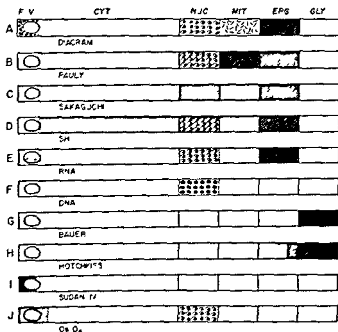


FIG 2 Schematic presentation of the results of cytochemical tests on *Neurospora* Shading indicates relative intensity of the reaction Nuclei mitochondria and vacuolar inclusions are drawn only when they give a significant reaction (A) Cytology of hyphal tips (first 100 μ) and young hypha (at the left) *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's 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 Kuster, 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 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

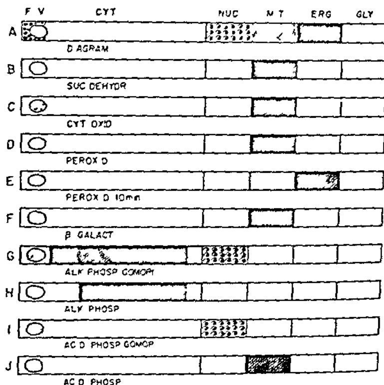


FIG 4 (A) Same as Fig 3A. (B) Succinic dehydrogenase (tetrazolium) (C) *Cytochrome oxidase* (Nadi) (D) *Peroxidase* (benzidine) (E) Peroxidase after 10 minutes (F) β-Galactosidase (o-nitronaphthyl galactoside) (G) *Alkaline phosphatase* (Gomori adenine 3 phosphate) (H) Alkaline phosphatase (β-naphthyl 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 th s oxid it on vi i icet ildehyde nd acetate (Holzer and Goedde 1957)

Succinic dehydrogenase—mitochondria exclusively by both centr fugation and cytochemical methods Any differing reports could be traced to artifacts or uncritical application of the method

Cytochrome oxidase—same as succ nic 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 (Maru yama 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 re duced disulfide linkages in cell wall protein (Nickerson and Falcone 1956a b)

DPN cytochrome red ictase and *TPN cytochrome reductase*—mainly in mito chondria 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 mito chondria

Alkaline phosphatase—Gomori reaction for alkaline phosphatase is positive in the cytoplasm and strongly stains mitochondria (Turian 1958 Zalokar 1959b) In cen trifuged 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—D ffuse 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 phospha tase Since these granules have not yet been found in fungi the localization in m to chondria 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 phos phatase is located near the cell membrane (Schm dt *et al* 1963) Both phosphatases are absent in nuclear cap of *Allo nyces* (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 pre sumably endoplasmic membranes

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 mito chondria 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, Schul man 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

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* (Schulman 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 nucleo-cytoplasmic 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 Halvorsen, 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.

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 Buchner 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 Heineman (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 15 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, 1959 a 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., Schroder, 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 (Muller, 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 contra diction 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 con stituent parts and, by combining certain of these, reconstitute some of the original function Similarly, the succinic oxidase system could be recon-stituted 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 de-rived according to Porter (1961) Ribosomal RNA originates in nuclei, as demonstrated in *Neurospora* and there is no self replication of ribo somes 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 for-mation 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 Guilliermond 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

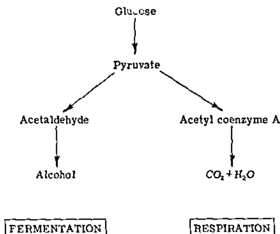


FIG 5 Branching of glucose breakdown in yeast at the pyruvate level from Holzer (1961)

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

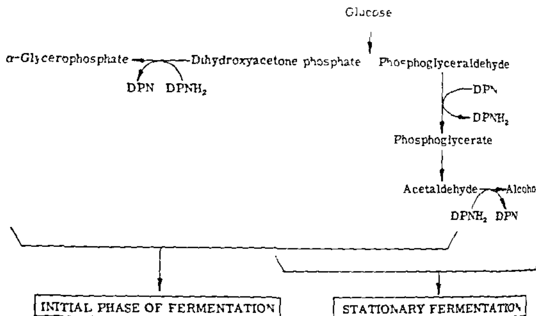


FIG 6 Scheme of initial phase of fermentation (*Angärung*) and stationary phase of fermentation in yeast from Holzer (1961)

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 process 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 drop lets 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 re arrangements 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

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Nutrition and Growth of Cells

CHAPTER 15

Uptake and Translocation

1 Uptake[1]

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I 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-

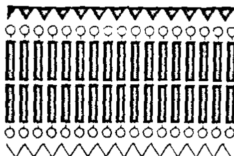

Fig 1 Highly schematic diagram of the unit membrane structure The jointed circles and bars represent lipid molecules and the zigzag lines represent nonlipid molecules designated X and Y From Robertson (1961)

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-

ments of water (Solomon, 1961) Thus, the movement of labeled water (tritium) in a system which is in osmotic equilibrium, follows a typical diffusion pattern The tritium-labeled molecules are relatively few in number relative to unlabeled water, and behave like the diffusion of solute in a solvent If, however, the water movements are measured in terms of net change driven by an osmotic gradient (a difference in the activity of water on the two sides of the membrane), then it is the solvent itself that moves rather than a solute The apparent permeability may be three or more times higher The water behaves as though it is moving in bulk rather than as individual molecules in random motion The kinetics follow the flow equations (Poiseuille's law) rather than diffusion equations (Fick's law) From the data an average equivalent pore radius can be calculated For several kinds of cells it is of the order of 4–5 A Two other consequences 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 the phenomenon of "solvent drag " The latter phenomenon involves movements 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

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

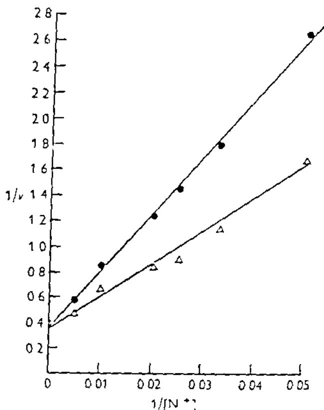

FIG 2 Demonstration of competitive inhibition of uptake of sodium by potassium during fermentation ∆ sodium acetate alone • sodium acetate with 5 mM potassium acetate From Conway and Duggan (1958)

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

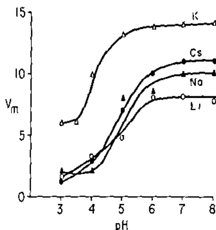

Fig 3 The effect of pH on the maximal rate (V_m) of transport of the alkali metal cations by yeast From Rothstein (1964)

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

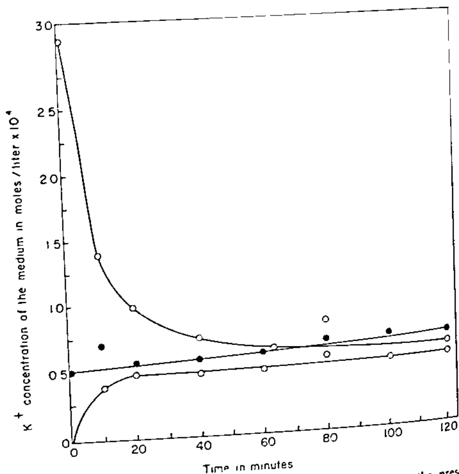

FIG 4 Changes in the potassium concentration of the medium in the presence of fermenting yeast The yeast concentration was 10 mg/ml glucose 0 1 M pH 4 5 with TST buffer From Rothstein and Bruce (1958)

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 in organic polyphosphates 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 environ ment 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 by 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

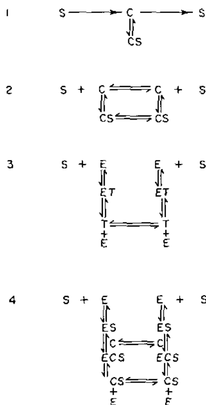

FIG 5 Schemes of carrier models From Cirillo (1961)

out that the above results can be predicted from a model in which the carrier moves across the membrane with the sugar The phenomena listed above have been observed in sugar transport in the yeast cell (Cirillo, 1961) as well as in animal cells

The specificity of sugar transport is much broader than that of sugar-enzyme reactions The enzymes are group specific whereas the transport seems to depend predominantly on the shape of the sugar molecule (LeFevre, 1961)

The disaccharides can be utilized by cells in two ways (de la Fuente and Sols, 1962) (1) They can be split by saccharases located on the outer surface of the cell to monosaccharides The products are then transported into the cell by the carrier systems for monosaccharides (2) Some disaccharides are moved into the cell by specific membrane transport systems For example, in *Saccharomyces cerevisiae* sucrose is split to glucose and fructose by invertase on the outer surface of the cell (Rothstein,

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

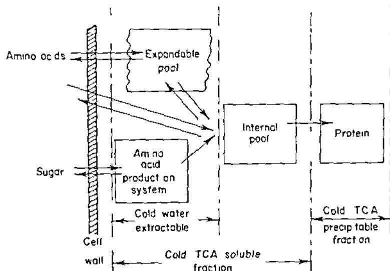

FIG 6 Carbon flow in yeast From Halvorson and Cowie (1961)

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

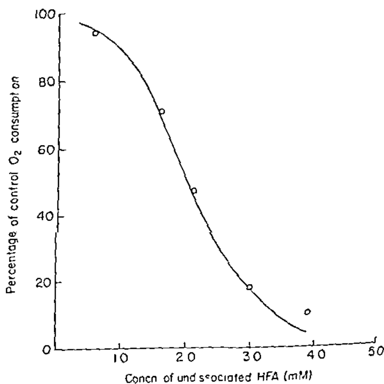

FIG 7 Inhibition of oxygen consumption of yeast cells by HFA, plotted as a function of the concentration of undissociated HFA in the suspension medium Concentration of HFA $5 \times 10^{-3} M$, pH varied from 2 15 to 3 50 Duration of experiment 90 minutes, temperature 25° From Aldous and Rozee (1956)

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,

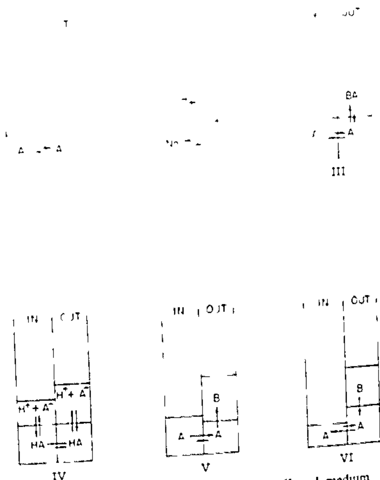

FIG 8 Distribution of solutes between cell and medium

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)

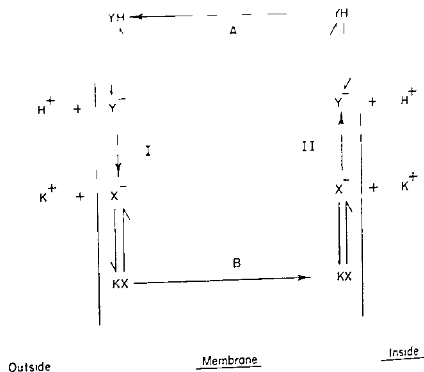

FIG 9 Diagram of carrier system for K–H forced exchange X is a K specific form Reactions I and II are coupled to metabolism, for example ATPase or redox Reactions A and B represent the translocation through the membrane, the mechanism is unknown at the present time

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

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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

Schutte (1956) employed a technique of qualitatively determining trans location 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* (Schutte, 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 Schutte (1956) in basidiomycetes Both employed various dyes as a marker of transpirational flow Schutte 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 Kogane, 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 Schutte'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 over grow 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 Shutte s dish technique to demonstrate that of the 20 species representing all classes of fungi, 10 were translocating fungi as defined by Shutte 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 Shutte'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 Kogane, 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

colonies with special reference to phosphorus metabolism *J* [illegible] *Appl Microbiol (Tokyo)* 9 313–330

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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 d um 20 manganese 0 iron 2 aluminum 3 8 copper 0 08 and bor n 6 Agar contains organic nutrients as the growth of fungi on water a a h s

C Synthetic Media

The ideal synthetic medium is one in which every const tu nt and its concentration is known This ideal situation is never attained n 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 mall 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 es sential 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 de termined 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 considera tion 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)

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 ephemerus* 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

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	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 am monium 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 non essential 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)

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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
α Aminobutyric acid	Creatine	Glycine	Ornithine	Tryptamine
Amygdalin	Cysteine	Hippuric acid	Phenaceturic acid	Tryptophan
Anthranilic acid	Cystine	Histamine	*Phenylalanine*	Tyramine
Arginine	Diaminopimelic acid	*Histidine*	Phenylglycine	Tyrosine
Asparagine	Djenkolic acid	Isoleucine	*Proline*	Valine
Aspartic acid	*Ephedrine*	Leucine	Sarcosine	
	Ethionine	*Lysine*	Serine	
		Methionine		
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* butyl sebacate	Lactic acid	Phenylpyruvic acid	Triolein	
Ethyl acetate	Lauric acid	Pimelic acid	Tristearin	
Ethanol	Linoleic acid	Propionic acid	Valeric acid	
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

Fermentation period (days)	High yielding citric producer: Sucrose used (gm l)	High yielding citric producer: Mycelium formed (gm l)	High yielding citric producer: Economic coefficient	Low yielding citric producer: Sucrose used (gm l)	Low yielding citric producer: Mycelium formed (gm l)	Low yielding citric producer: 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): Zn absent	Glucose used (gm/100 ml): Zn present	Efficiency of fumaric acid production[b]: Zn absent	Efficiency of fumaric acid production[b]: Zn present	Economic coefficient[c]: Zn absent	Economic coefficient[c]: 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	Sorbose	Glucose + sorbose	Maltose	Maltose + sorbose
Aspergillus niger	6	936	344	1136	826	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)

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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 1

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 *max*imum 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

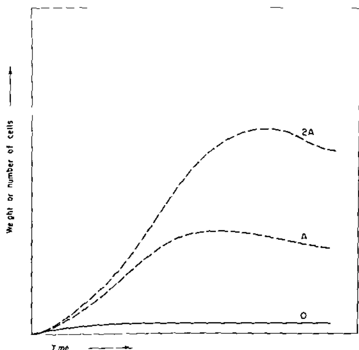


FIG 1 Action of an *indispensable* growth factor on the growth of a fungus. The curves show the growth without the factor (O) and with suboptimal amounts added (*A* and 2*A*). Growth stops when growth factor is consumed

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

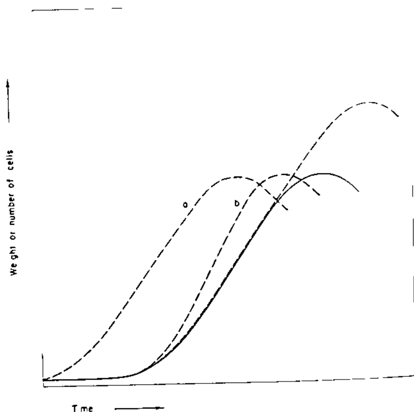


FIG 2 Action of a dispensable but *promoting* growth factor on the growth of a fungus The solid line (O) shows the growth without and the dashed lines (*a* *b* and *c*) the growth with the factor added Three different types of response are illustrated (*a*) abbreviated lag phase (*b*) increased growth 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 1 7) 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* (Sundstrom, 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	Riboflavine	Nicotinic acid	Pantothenic acid	Pyridoxine	Biotin	PABA	Inositol	Other growth factors	References[a]
Phycomyces blakesleeanus	+									1
Trichophyton equinum			+							2
Brettanomyces bruxellensis					+					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), Kreneta-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 Kallstromer, 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 Sundstrom (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* (Sundstrom, 1964)

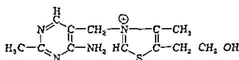
Efforts have also been made to use the type and degree of auxoheterotrophy for the elucidation of phylogenetic relationships among genera and among other taxa of higher orders (Cantino, 1955)

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

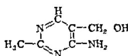
auxoautotrophic just as are many ubiquitous saprophytes like the penicillia On the other hand, fungi living on exactly the same substrate, e g , pine wood, often exhibit auxoheterotrophy, as well as complete auxoautotrophy Some obligate parasites, like Erysiphales and Uredinales still cannot be cultivated 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 Lochhead 1959)

V THE GROWTH FACTORS

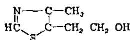
A. Thiamine



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* (Sundstrom

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* (Area 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 (Area 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* (Muller and Schopfer 1937) *Stereum frustulosum* (Noecker and Reed 1943), *Endomyces magnusii* (Meissel, 1947), and *Trichophyton concentricum* (Georg and Camp 1957)

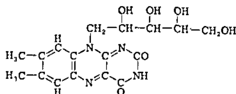
The biosynthesis of thiamine is usually assumed to take place by the condensation of the two above-mentioned precursors the pyrimidine and the thiazole However, there is some evidence in favor of another pathway where pyrimidine is first coupled to a thiazole precursor and the compound thus formed is 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

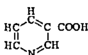


Riboflavine

(IV)

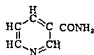
Poria vaillantii is the only fungus so far found which requires an external supply of riboflavine (plus thiamine, biotin, and adenine Jennison *et al*, 1955) No further particulars of this case have been published However, the fact that many bacteria with this requirement are known and that riboflavine-less 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)

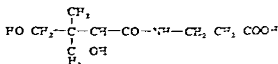
Species requiring nicotinic acid (= niacin V) or nicotinamide (VI) occur in almost all the main groups of fungi. Among phycomycetes, *Phlyctorhiza variabilis* (Rothwell 1956), *Blastocladia pringsheimii* (Cantino 1948; Crasemann 1957) and *B. ramosa* (Crasemann 1957) require this vitamin together with 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* (Area Leao 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).

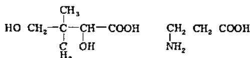
In contrast to many induced niacin-less mutants, the niacin-heterotrophic species usually cannot substitute tryptophan or other indole derivatives for niacin. *Trichophyton equinum* constitutes an exception since it is able to utilize tryptophan to some degree (Georg 1949; Drouhet and Mariat 1953). However, 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).

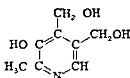
Pantothenic acid, in itself an intermediate in the formation of coenzyme A, is normally synthesized in the cell from the two precursors pantoic acid (VIII) and β-alanine (IX) At least some yeasts, e g, *Schizosaccharomyces pombe* (McVeigh and Bracken, 1955), can do with β-alanine alone, the synthesis 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

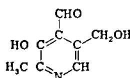
amides act as antagonists to β alanine but not to pantothenic acid (Betz, 1960).

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.

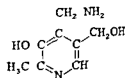
E. Pyridoxine (Vitamin B_6)



Pyridoxine (X)



Pyridoxal (XI)



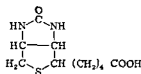
Pyridoxamine (XII)

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.

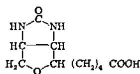
Pyridoxine is only one of the three active, unphosphorylated forms of vitamin B_6 that occur in nature, the other two being pyridoxal (XI) and pyridoxamine (XII). Bacteria are known to respond differently to these three forms and to their phosphorylated derivatives. Very little is known about fungi in 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

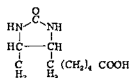
F Biotin



Biotin
(XIII)



Oxybiotin
(XIV)



Dethiobiotin
(XV)

Like pantothenic acid, biotin (XIII) was first recognized as a growth factor for certain yeasts (Kogl and Tonnis, 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* (Area Leao 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, Wiken 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

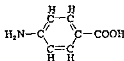
Another biologically active biotin derivative is biocytin which was isolated from yeast by Wright and collaborators (Wright et al 1951) and identified as ε-N biotinyl L lysine *Isaria cretacea* responds to biocytin (Taber and Vining, 1959), and *Saccharomyces carlsbergensis* to biocytin as well as to some other derivatives, viz, biotinamide, N biocytin p amino benzoic 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)

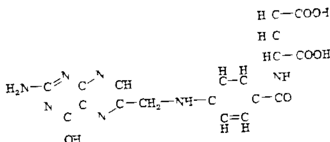
The natural biotin molecule has a side chain containing 4 methyl groups The two analogs, norbiotin, with 3, and homobiotin, with 5 methyl groups seem to be inactive for most fungi as substitutes for biotin e g in *Claviceps purpurea* (Taber and Vining, 1957) *Isaria cretacea* (Taber and Vining, 1959), 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

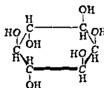
Fries, 1962) This probably means that the biosynthesis of these substances is controlled by folic acid, of which PABA constitutes an essential structural unit

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, Dulaney 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 (Braekkan 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 $\gtreqless 1 \mu g/ml$

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 Sisco, 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 (~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 responsible 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)

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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 Danielli, 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} (μg/gm)
Atropine	Man	1 4
Botulinum toxin	Mouse	$0\ 23 \times 10^{-6}$
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 sub*stances, 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 (Hors fall, 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 fungi cide 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 influ ences 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 car ried out on the possible use of *systemic* fungicides, which act within the plant tissues So far only limited success has been achieved, and no com mercial 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 (1967)

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) (Hartel, 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 di sulfide), 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

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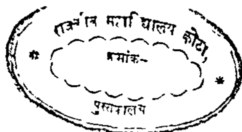

CHAPTER 21

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

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 duponti* 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

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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 Heden, 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 Hohnk (1956) was able to demonstrate the occurrence of fungi in North Sea sediment cores from depths of 1150 and 1250 m Hohnk (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 reaction 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

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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 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 Delbruck and co workers (cf Delbruck, 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 I) 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 (Delbruck 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 Delbruck 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 Delbruck 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 More over 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 melano genesis 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 × 5 μ and predominantly mycelial, compared to dark-grown ones which averaged 13 × 14 μ 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/mm²/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

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 reac*tion 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 as 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 (Delbruck 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 sporan-

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 Delbruck (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 (Delbruck and Varju, 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

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

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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

II 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

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 yield 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 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 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 Muller (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 ethylenediaminetetra acetic acid (EDTA) increased the radiation sensitivity of *S cerevisiae* in terpreted 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, Kafer, 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 radia tions 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

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

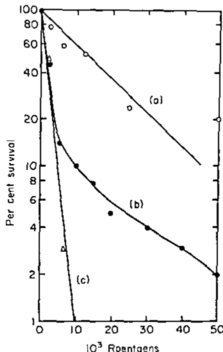

FIG 2 X ray survival curves by microcolony counting procedure survival criterion >40 cells Age of culture prior to irradiation 4 days ● Whole survival, △ single cells only ○ budding cells only From Beam *et al* (1954)

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

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 A, 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 A, 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*] (Taguena 1959) Recently Svihla et al (1960) described in some detail the sequence of morphological events that occurred in *Candida utilis* after ultraviolet (2650 A) 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 observat ons with *S cerevis ae* It seems l kely that the exact details of the sequence of events described above may reflect in part at least particular experimental conditions since the description pro vided for ultraviolet treated commercial bakers yeast by Townsend and Sarachek (1953) is quite different The irradiated bakers yeast was re

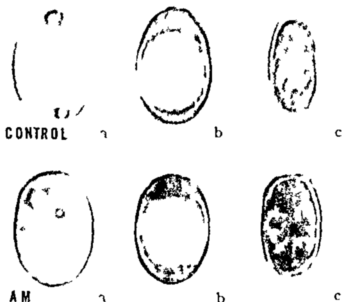

FIG 3 C d da u l s Ultraviolet (265 mμ) photom crographs of control and S adenosylmeth on ne enr ched cells show ng the effects of ultrav olet (265 mμ) ir rad at on Sequent al p ctures were obta ned at 0 2 and 4 m nutes n each case *a* Normal cell *b* enlarged vacuole *c* collapsed cell From Sv hla *et al* (1960)

ported to develop a granular cytoplasm giant cells and an unusual dumb bell 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 stolonifer	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 wave length range about 2600 A 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

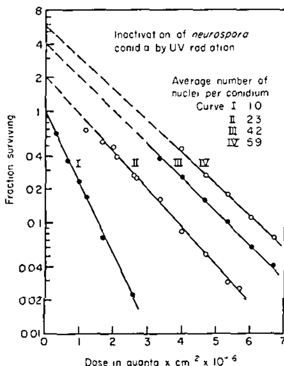

FIG 4 The inactivation of *Neurospora* conidia by ultraviolet radiation The wavelength is 254 mμ From Norman (1954)

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

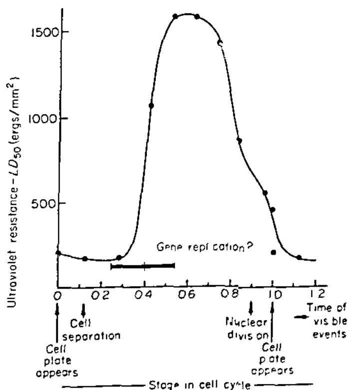

FIG 5 Resistance to killing by ultraviolet rays and the timing of various events in the cell cycl From Swann (1962)

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 A by light of about 3300–4800 A (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 A 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

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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, con*tinuous 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 , O), 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

$$\frac{dN}{dt} = kN \quad (1)$$

where N is the number of cells t is time and K is a proportionality constant By integrating

$$\log N = kt + c \quad (2)$$

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

$$N = ce^{kt} \quad (3)$$

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

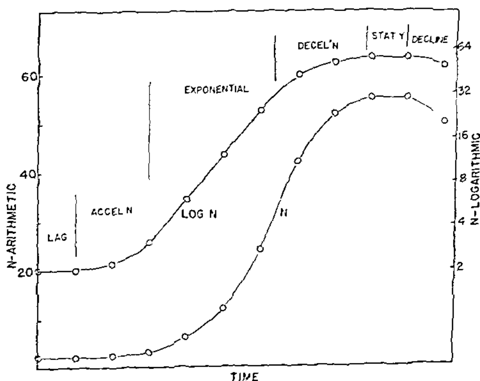

FIG 1 Idealized representation of exponential growth and of the phases of the growth curve

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 measurement 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

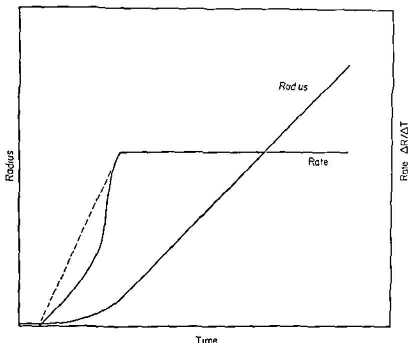

FIG 2 Idealized representation of linear growth The dashed line (— — —) indicates the linear increase in rate to a constant level as shown for *Botrytis* by Smith (1924)

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*

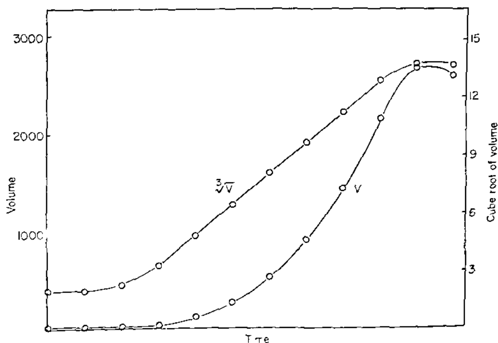

FIG 3 Idealized representation of cubic growth relations for spherical mycelial pellets in liquid culture

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

$$R \quad \frac{dy}{dt} = Kt + b$$

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 develop ment of a better undertsanding of the variables involved in determining the extent of hyphal branching may reveal more definable growth patterns

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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 result in regrowth by a narrow extension from the very tip of the hypha It is true that no one has demonstrated a localization of extension in the subapical region in vegetative hyphae although very clear subapical extension zones have been demonstrated in sporangium bearing fungi such as *Phycomyces blakesleeanus* and *Thamnidium elegans* (Castle 1942, Lythgoe 1961), and Castle (1937) has shown that in vegetative hyphae of *Phycomyces*, although the extreme tip is the main region of growth in area of the primary wall, extension takes place over a larger part of the apex decreasing in amount farther away from the tip

B Nature of the Wall at the Apex

Mycologists have always had difficulty in visualizing the way in which fungal hyphae mold and shape themselves at the tip, where accretion of the structural material is taking place We clearly need electron microscopic evidence for the wall structure at the apex, but there are certain aspects we can ascertain by a consideration of the evidence before us If the hypha consists of a rigid tube with viscous contents that emerge from the end of the tube, we should expect the viscous material to assume a spherical surface 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 1952, Robertson, 1958, Zalokar, 1959) shows a narrowing of the hyphal apex (Fig 1B), which suggests that the hyphal apex cannot be considered as a simple tube with viscous contents This is made more plain when one considers that the system shows turgor pressure equivalent to 6–7 atmospheres (atm), e g , in *Neurospora* When the apex is placed in water, the turgor increases and the apex is seen to stretch and eventually to burst It very rarely shows the spherical deformation that we might expect if it were entirely viscous Instead we find that the expanded apex assumes a diamond shape (see Robertson, 1958, for figures)

We must assume, therefore, that this apical region which contains a viscous cytoplasm is bordered by a wall of such structure that an increase in internal pressure causes little stretching or obvious deformation of the extreme apex, but does cause substantial irreversible stretching in the subapical shoulder The simplest explanation of such behavior would be found in a model of wall formation such as that postulated by Preston (1952) for the alga *Valonia*, where a tight spiral of wall material over the apex, and presumably continually generated from within, may be interpreted as being slowly uncoiled backward by the extension of the apex, and helices, as they become progressively further apart, are crossed by

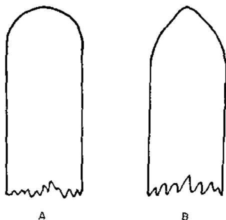

FIG 1 Hyphal tips For explanation see text

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-

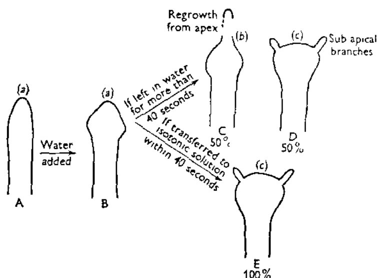

FIG 2 Diagrammatic explanation of behavior of hyphal apexes of *Fusarium oxysporum* in water and isotonic solutions (A) Normal hypha (B) Hypha showing arrestment and beginning of swelling (C) Hypha showing regrowth from the apex after immersion in water (D) Hypha which has swelled and branched at the 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

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

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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, Bunning, 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 (Bunsow, 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 fun n?

Among the fungi, different processes show rhythm which often a endogenous, such as protoplasmic streaming in the plasmodium of *Ph sarum polycephalum* (Kamiya *et al* 1957) the discharge of ascospor in *Daldinia concentrica* (Ingold and Cox 1955) or of sporangia in *Pilol olus* (Schmidle, 1951, Lebelmesser 1954) and even the intensity luminescence in certain basidiomycetes (Berliner 1961) However th most common periodicity involves the development on solid media of series of regularly spaced concentric circles of growth which are calle zonations These zonations can affect a single process as for example th thickness of the mycelium of *Ascochyta chrysanthemi* (Stevens and Hal 1909), or the distribution of spores of *Penicillium* and *Aspergillus* (Galle maerts, 1911, Hedgecock, 1906 Munk, 1912) and pycnidia of *Ascochyt 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 fructicol* (Hall, 1933, Sagromsky, 1959a) and pigmentation and formation of fertil and sterile aerial hyphae in *Alternaria tenuis* (Jerebzoff, 1961b) Thus, tw types of hyphal growth can be observed, one characterized by monopodia ramification, and the other by the formation of a cyme, as in the cases o *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 laterium* 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 ricl and not permit too much thickening of the mycelium, in order to avoid th coalescence of the zonations, all conditions that might disturb growth, and prevent the mycelium that is sensitive to excitation from being in an ever 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 h ve been used, the content of phosphate has been shown to have a m ke effect on the zonations, as in *Ascochyta rabiei* and in *Fusarium*

III ENDO OUS RHYTHMS

A Action 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 *A pergillus ochraceus* and *A niger* are sensitive also to the action of thermocycles (Munk, 1912)

Group 3 In this group weak physical excitation 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 ≥ 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 ≥ 6 if manganese is absent and at a pH ≤ 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

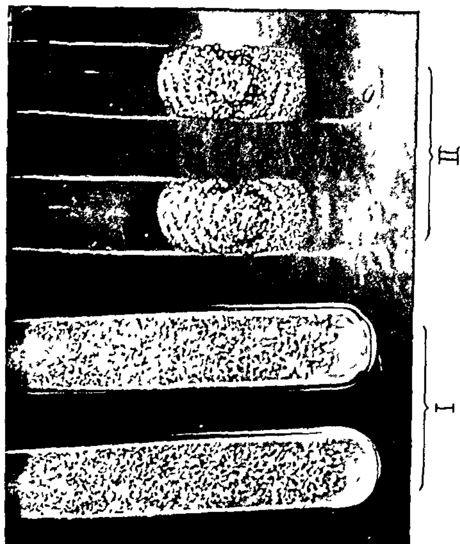

Fig 2 Appearance of *Sclerotinia fructicola* under uniform conditions in the dark at 23°C (I) Grown on a synthetic medium (II) Grown on the same medium to which the "basic" fraction of yeast extract was added

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 zonations 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 *Aspergillus* 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 attenuation (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)

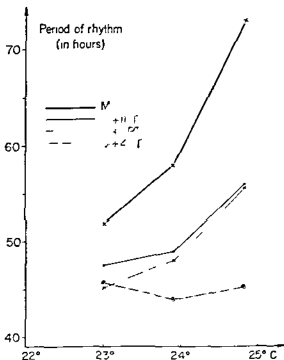

Fig 3 Duration of the endogenous period of *Alternaria tenuis* in the dark as a function of the temperature and composition of the medium $M = 5$ gm of malt extract per liter $PT =$ water extract of 12 5 gm (*1*) 25 gm (*2*) and 50 gm (*4*) of peeled potatoes per liter

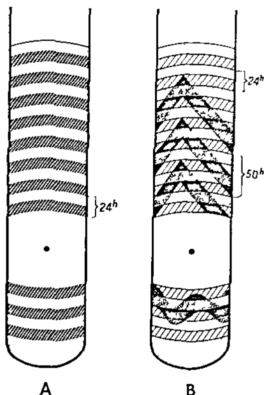

FIG 4 Simultaneous evocation of exogenous and endogenous zonations in *Alternaria tenuis* grown on malt extract (10 gm/liter) and receiving daily 12 hour photoperiods When the stimulus is 250 lux only an exogenous 24-hour rhythm appears (A) If the stimulus is 100 lux the preceding rhythm still is present 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 24 hours which results from the application of physical stimuli to cultures of *A tenuis* in malt extract can not, 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* (Buhnemann, 1955) but not in higher plants like *Phaseolus* where only red light is effective in starting the endogenous diurnal rhythm (Bunning 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* (Bunsow 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 rhythm 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 (Bunning, 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* (Faure Fremiet, 1948, 1950, 1951) show endogenous "tidal rhythms " Moreover, diverse systems with diastatic capacity are capable of functioning rhythmically (Bunning, 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

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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, syn*chronization 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*

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 (Sylven *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 (Guttes *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

$$\frac{1}{n}\frac{dn}{dt} = \alpha - \beta \qquad (1)$$

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

$$\frac{dc}{dt} = (a - c)\beta - kn\alpha \qquad (2)$$

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

$$c = a - kn \qquad (3)$$

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

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Gene Action

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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[*] 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

[*] 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 com plementation 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 diplo dy 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 hetero* zygote $\left(\frac{m^1\ m^2}{+\ +}\right)$ or heterokaryon $[(m^1\ m^2) + (+\ +)]$ as well as the usual repulsion (or *trans*) phase heterozygote $\left(\frac{m^1\ +}{+\ m}\right)$ 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 avail *able 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

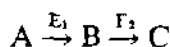
Each group of mutants differentiated by the physiological test appears *usually to be concerned in one physiological function in the organism* Where a number of functions appear to be affected, it is possible in some cases to demonstrate a single underlying cause, it is reasonable to assume such singularity 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

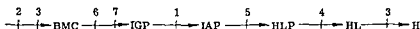


FIG 1 Pathway of biosynthesis of histidine showing positions of action of the seven genes in *Neurospora crassa* Abbreviations *BMC* Bratton Marshall compound *IGP*, imidazole glycerol phosphate *IAP* imidazole acetol phosphate *HLP* histidinol phosphate *HL* histidinol *H* histidine

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

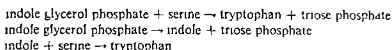
Yura (1959) showed that the *proline*-1 mutant (21863) of *Neurospora crassa* produced a pyrroline 5-carboxylate reductase with about 0 2–0 4% *of the activity typical of the normal wild type The mutant enzyme has an abnormally low thermostability and a decreased catalytic efficiency The latter is shown 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* 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*[r1] (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*[r8] 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 cofactors.
 (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 hypothes 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 tryrosine 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 path ways 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 succinylcarboxamide) 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* th s b f nctional enzyme is *composed of two distinct polypeptides each determined by a distinct gene ma* 1 and *ma* 2 respectively Both enzymic activities rema n together in constant proportions during purification f the prote n I e protein has been shown to consist of three units of the kind determ ned by one gene combined with one unit of the kind determined by the ther 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 th t 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

V I GENE INTERACTION

Genes control steps ir 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 inhib tor which tends to accumulate because of the primary lesion

A famous and intricate case is presented by the suppressor f *pyrimidine* 3 discovered by Houlahan and Mitchell (1947) This w s found t be specific in its action to some but not all *pyr* 3 mutants The combin tion *pyr* 3ᵃ *su pyr* 3 was able to grow without uridine but this relief of the requirement of the metabolite was reversed by additi n f arginine to the medium Later further complexities were disclosed incl ding 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 sensi tive 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 Wood ward 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 ureido succinic acid from aspartic acid and carbamyl phosphate is catalyzed (Davis 1960) All of the members of class II possess aspartic acid trans carbamylase and it is these that are suppressible by *su pyr* 3 It has become clear that these are impaired in the production of carbamyl phos phate 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 spe cifically 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 ex*tinguished 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

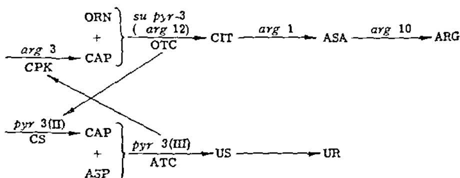


Fig 3 Interaction of arginine and uridine biosyntheses The arrows indicate the mutant genes which are suppressed and the mutant genes which suppress them Abbreviations Substrates *ORN* ornithine *CAP* carbamyl phosphate *CIT* citrulline *ASA* argınino succinic acid *ARG* arginine *ASP* aspartic acid *US* ureidosuccinic acid *UR* uridine Enzymes *CPK* carbamyl phosphate kinase *OTC* ornithine transcarbamylase *CS* carbamyl synthetase *ATC* aspartic acid transcarbamylase

It has been noted that suppressors are generally specific for each allele, a given suppres or 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

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

C *Interaction between Alleles*

In this section there will be considered interactions which may occur between similar products of allelic genes probably as a rule between proteins but perhaps also between the nucleic acid systems responsible for protein synthesis Broadly two kinds of such interaction have been detected but only one of them allelic complementation is known to occur in fungi in which it was discovered

The proportions of primary products in the form of enzyme or protein may not always be equivalent to the proportional representation of different alleles in the nuclei and cells This has been observed with certainty only in relation to the formation of hemoglobins in man Thus the allelic genes for A and S hemoglobins respectively the normal and sickle types are in the ratio of 1 1 in heterozygotes but there is 60% of A and 40% of S in the hemoglobin The disproportion is not always in favor of the normal e g the A I heterozygote shows only 30% of A compared with 70% of I hemoglobin The mechanism of this interaction is obscure

Secondly in many cases pairs of alleles each producing inactive enzyme, may together produce an active enzyme (Catcheside, 1962) This is the complementation between alleles noted at the beginning of this chapter, and for which complementation maps representing some kind of functional relationship, may be drawn

The principles may be illustrated by reference to a particular example, namely *tryptophan* 1 (Ahmad and Catcheside, 1960 Catcheside, unpubl) Some 120 *Neurospora crassa* mutants at this locus have been classified by

	A	B	C	D	E	F	G	H	I	% 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 mutants 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

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

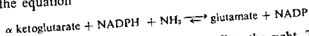
Construction of a detailed hypothesis of complementation is handicapped by the general lack of knowledge of the architecture of proteins *which show the effect Our only model is the hemoglobin molecule* and if its type of symmetry is general the usual elements of symmetry would be axes of rotation Crick 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



$$\alpha \text{ ketoglutarate} + NADPH + NH_3 \rightleftharpoons \text{glutamate} + NADP$$

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-

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 com parison 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 (Pitot 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 How ever, 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 re- gions 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 in duced 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 com pound (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 produc tion 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

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