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FRONTISPIECE. *Pilobolus* showing phototropism. See page 339.

Physiology *of the* Fungi

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PHYSIOLOGY OF THE FUNGI

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This book is dedicated to the memory of *Leon H. Leonian* and to *Ernst A. Bessey*. The guidance and inspiration of these men in directing our interests to the study of fungi is gratefully acknowledged.

PREFACE

Living fungi are being studied more intensively than ever before. This may be attributed in part to increased interest in the potentialities of the fungi in industry as well as to the greater recognition of fungi as important disease-producing agents of plants and animals and as destroyers of fabrics and other cellulosic materials of commercial importance. This has increased the interest in the cultivation of the fungi and has shown the need for an adequate textbook covering the broad aspects of physiology of the fungi, their growth requirements, and activities. It was the intent of the authors to prepare a textbook which would fulfill the needs of students desirous of some training in this field.

This book is primarily a text for the advanced student and assumes some basic knowledge of the morphology of fungi and of organic chemistry. It had its origin in the lectures and laboratory exercises used for three years by the authors in a course in physiology of the fungi offered to graduate students at West Virginia University. The authors have contributed equally of their time and efforts in the preparation of this text.

For those who are interested or are actively engaged in physiological research on fungi, this textbook may serve as a reference book and as an entry into the literature. The large ever-growing accumulation of literature has also made it desirable to bring together a summary and discussion of the information in this field. However, no attempt has been made at complete documentation of the subjects discussed. Certain particularly important references are marked with a star and are recommended as required reading for students.

For the most part, the scientific names of the fungi are those which were used by the investigators whose work has been cited. No attempt has been made to reduce these names to synonomy. Because of the close relation between fungus physiology and plant pathology, plant pathogenic fungi have been used as examples whenever possible.

Several suggested laboratory exercises with suggested test fungi are included at the end of the text, so that other teachers might profit by the authors' experience in designing and conducting laboratory work in fungus physiology.

All tables, graphs, and photographs not credited to other sources are original.

It is a pleasure to acknowledge our indebtedness to the many individuals

who have aided us with their suggestions. Among our colleagues at West Virginia University who have read portions of the manuscript are: J. G. Leach, C. R. Orton, M. E. Gallegly, R. P. True, W. T. Jackson, B. G. Anderson, R. B. Dustman, and J. H. Hare. In addition, the following have read one or more chapters: B. W. Henry, J. B. Routien, W. D. Gray, and J. B. Conn. We are also indebted to Mrs. H. L. Barnett, who typed most of the manuscript.

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MORGANTOWN, W. VA.
May, 1951

CONTENTS

CHAPTER 1

INTRODUCTION

The primary role of the fungi in nature has been fittingly described in the prophetic statement of B. O. Dodge (1939):

> . . . the fungi are not degenerate organisms which are on their way out in a scheme of evolution, and so of little economic importance and scientific interest. The fungi, on the contrary, are progressive, ever changing and evolving rapidly in their own way so that they are capable of becoming readily adapted to every condition of life. We may rest assured that as green plants and animals disappear one by one from the face of the globe, some of the fungi will always be present to dispose of the last remains.

The most important role of the fungi in the economy of nature is to act as scavengers in disposing of dead and fallen vegetation. In this way the biologically essential elements are released for reuse, and the balance of nature is maintained. However, these are not the only functions of the fungi which are of interest and importance to man. Since the beginning of agriculture fungi have been used to prepare bread and other foods, as well as fermented beverages. Some fungi cause diseases of plants and animals. Knowledge of their role as the causal agents of plant diseases long antedated the recognition of bacterial diseases. While yeasts have long been used to produce alcohol, the vast potentialities of other species for the industrial production of organic acids and antibiotics have been recognized more recently. An understanding of life processes of the fungi is essential whether one wishes to control the fungi which cause disease, to employ them in industry, or to use them in the laboratory to unlock the secrets of nature.

The domain of physiology is the study of functions or life processes. Fungus physiology is the study of *living* fungi, their functions and activities, how they affect their environment and how the environment affects them. Like other branches of science, fungus physiology has four phases of development: (1) the discovery and verification of facts, which are the foundation of any science, (2) the organization of these facts into a systematic and coherent body of knowledge, (3) the dissemination of newly discovered facts, and (4) use of the newly discovered facts and others already known to formulate principles. Facts are the basis of science, but facts alone are sterile unless they are seen in relation to

previous knowledge. Organization and interpretation of facts are equally as important as the experimentation which reveals them.

The fungi as a group are highly responsive to their environment and are thus excellent test organisms for inquiring into the secrets of nature. Nature always answers correctly the questions we ask, and, in this sense, no experiment is a failure, although we may fail to ask the question we intended, or we may misunderstand the answer given. Infinite care is required to frame a question so that a definite answer may be obtained. By observing fungi in nature we are limited to questions asked by nature. Commonly, the environmental and nutritional factors are so complex that the influence of a single variable cannot be evaluated. By controlling the conditions under which a fungus is placed in the laboratory it is possible to ask questions of great precision. Indeed, the number and scope of the questions which we may ask fungi are limited only by the present-day techniques and the curiosity of the investigator.

Since most of our knowledge of the physiology of the fungi has been gained from laboratory investigations, the experimental approach will be emphasized in the discussions which follow. However, this choice is not meant to minimize the importance of and need for critical observations in nature. By emphasizing the results of careful laboratory research, we are better able in the following chapters to present the facts necessary for an understanding of the vital principles of fungus physiology, and also to show that these principles, theories, and hypotheses are founded upon experimental evidence.

FUNGUS PHYSIOLOGY IN RELATION TO OTHER SCIENCES

Physiology is that branch of science which deals with the life processes or the activities of organisms. The activities of the whole organism or of any of its parts may be limited by its form or structure. Both the activity and the form of an individual are determined to a great extent by its genetic constitution and are modified by the environment to which the organism is exposed. Physiology, therefore, is not an independent subject. An understanding of physiological principles is based, in part, upon facts and theories from many other fields of science, such as chemistry, physics, anatomy, cytology, bacteriology, and genetics.

Many of the physiological principles which have been established for one group of organisms apply equally well to other groups. The vitamins essential to the normal growth of the fungi are the same as those required by man, animals, and the higher plants. The general functions of these vitamins appear to be the same in all organisms. The difference in the vitamin requirements seems to lie in the different abilities of these groups of organisms (or individuals within the group) to synthesize these necessary compounds. As Schopfer (1943) has pointed out, the

vitamin problem is common to many branches of science. Many other problems investigated in fungus physiology are likewise common to other related fields of study.

In a similar way, a better understanding of certain related fields is gained by knowledge of fungus physiology. The plant pathologist commonly finds it necessary to study the living parasitic fungus apart from its host and must know something of the cultural methods and the specific nutritional requirements of the fungus at hand. The mycologist and plant pathologist are faced with numerous unsolved problems which must be investigated by physiological methods. One of the most challenging problems is the cultivation of certain fungi now classed as obligate parasites on synthetic media of known composition. Until this is accomplished, the nutritional requirements of these fungi cannot be fully determined. Such knowledge would without doubt lead to a better understanding of parasitism and resistance.

The taxonomic mycologist uses morphological characters almost exclusively in his identification and classification studies, while the bacteriologist, being unable to use distinct morphological features to any great extent, emphasizes the physiological characters in classifying bacteria. Much more information is needed before it can be determined whether any physiological characters are sufficiently valuable and uniform to be used to supplement morphological characters in taxonomy of fungi. It seems logical that such physiological differences between groups of fungi do exist, and that the main problem lies in the discovery and recognition of these characters and their application to taxonomy. On the other hand, caution must be observed, for nutritional and environmental conditions are known to affect, to a certain extent, some morphological characters used in classification.

The geneticist and the biochemist may find the fungi interesting and suitable subjects for the study of their respective problems, while the bacteriologist finds many points of similarity between the physiology of the bacteria and that of the fungi. Industry has used many species of fungi to its own advantage for many decades. Yeasts were used long before the physiology of the fungi became an organized study, but the search for superior strains of yeast continues. The widespread use of antibiotics has brought under laboratory study many species of fungi which would otherwise have been ignored. This has created many new problems of nutrition, especially with regard to large-scale cultivation of these fungi.

Thus, knowledge of the life activities of the fungi is important and useful in many related fields of science, just as some knowledge of these related fields is essential to an understanding of the fungi. The study of fungus physiology is justified as a separate field in which the basic or fundamental

principles are the aim, or as a study closely integrated with the fields of science concerned with more practical problems. Often, the most valuable results are obtained when research is not restricted by the boundaries of practical application.

AIMS

This book is a discussion of living fungi, of their life processes and the factors which influence them. It is written primarily for the student who is acquainted with the structure of fungi and who is beginning the study of their activities. From the discussions which follow the student should gain a knowledge and understanding of the basic principles of fungus physiology. To this end a considerable amount of factual material concerning the behavior of specific fungi under specific conditions is cited. The secondary aim of this book is to present a limited number of selected references which may be of use to the student or investigator who wishes more detailed information. Where possible, review articles have been included. Complete documentation is impossible because of the tremendous volume of literature. However, becoming familiar with the literature is an essential part of a student's education.

SCOPE

As a text this book must cover many phases of the subject. One of the first problems to be considered is the choice and preparation of suitable media for growth and sporulation of the fungi under study. Since there is no universal medium suitable for all fungi, a wise choice of media for the purpose at hand is of fundamental importance in any investigation.

Before a fungus can be studied in any great detail in the laboratory, it is necessary to determine the conditions which affect growth. Growth is a complex phenomenon, and some discussion of the phenomenon itself and the ways of measuring growth is necessary for the understanding of these conditions. Nutritional factors, such as source of nitrogen, source of carbon, the presence of essential elements and vitamins, and the pH of the substrate, affect growth in interrelated ways. Each of these factors and its importance in growth and other activities of fungi are discussed at some length.

The life processes of the fungi involve numerous chemical transformations. Living organisms make and use special organic catalysts, enzymes, which control these reactions. The actions of the enzymes in the living organism are coordinated and interrelated. A knowledge of the principles of enzyme action is essential to the study of fungus physiology.

The fungi are able to make a far greater contribution to the production of food and many other valuable products than they do at present. Both

the useful metabolic products, such as alcohols, organic acids, and antibiotics, and the harmful products (toxins) are discussed at some length.

Certain fungi cause diseases of plants and animals. The action of fungicides used to control these pathogens will be discussed from a theoretical viewpoint, since there is an enormous amount of literature dealing with the practical application of fungicides. Too little attention has been devoted to the mechanism of fungicidal action.

The production of spores, which is of fundamental importance to the fungus in the perpetuation of the species, affords many interesting problems in fungus physiology. Environmental and nutritional factors play important roles in determining whether a fungus will sporulate under a given set of conditions. These factors are discussed in some detail.

The latter portion of the text emphasizes the activities of fungi in nature. These topics include the discharge, dissemination, and germination of spores and the physiological aspects of parasitism, variation, and inheritance. The physiology of parasitism and resistance is of special interest to plant pathologists and medical mycologists. Most of the examples are taken from the field of plant pathology. Perhaps the discussion of these problems will stimulate the interest and curiosity of the student. A better understanding of parasitism will surely lead to a wiser choice of control methods for certain fungi.

No study of fungus physiology is complete without experimental work in the laboratory. The judgment necessary to evaluate one's own work is founded upon experience. Suggested laboratory exercises and demonstrations, with brief instructions, are given at the end of the text. These are selected to illustrate important principles, many of which can be illustrated clearly only by direct observation of the varied reactions of fungi to their environment.

HISTORICAL DEVELOPMENT

The development of fungus physiology is far from complete. While some of the main outlines are clearly visible, much remains to be done. Although space and time do not permit a complete review of the history of this science, it is important to realize that its development was the work of many minds and hands. The influence of the early investigators continues, not only in their published work but also in the students they trained.

Some of the outstanding leaders in the development of fungus physiology are worthy of special mention. Their names and their contributions are encountered frequently by all students of this subject. Brief mention of some of these men and their fields of interest and investigation is made below.

Louis Pasteur (1822–1895), *France.* Pasteur was a chemist who, as a result of his interest in microorganisms which cause disease and fermentations, became a biologist. No other scientist has opened up so many fields of fruitful study. Early in his career he discovered that fungi are able to discriminate between the optical isomers of tartaric acid. His student Raulin devised the first synthetic medium for the cultivation of fungi and published the first thorough study of the nutritional requirements of a fungus. Pasteur discovered that some organisms are inhibited by free oxygen and that some fungi change both their morphology and physiology when cultivated anaerobically. Pasteur's complete works have been collected and edited (1933–1939) by his grandson, Professor Pasteur Vallery-Radot. Dubos (1950) has published an evaluation of Pasteur's work.

Heinrich Anton de Bary (1831–1888), *Germany.* His principal contributions to mycology dealt with life histories and parasitism of fungi. His interests were primarily with biological adaptations and were more physiological than taxonomic. De Bary's influence as a teacher attracted many students who later were responsible for much of the development of plant pathology and mycology. Among his writings was "Morphologie und Physiologie der Pilze" (first edition 1866, second edition, English translation, 1887), which may be considered as the first book containing discussions of the physiology of the fungi.

Oscar Brefeld (1839–1925), *Germany.* We owe a great debt to this patient investigator, who developed methods of ensuring sterile media and apparatus for pure culture work. He was equally insistent with regard to the purity of his cultures. His chief interest in mycology was the study of life histories and development of fungi. This meant to him observation of a fungus from "Spore zu Spore." He was the first to use the single-spore technique. Besides his occasional papers, he published his monumental work (1872–1912) in 15 parts. This beautifully illustrated work is still of great value.

Georg Klebs (1857–1918), *Germany.* His important contributions to the study of fungus physiology concerned problems related to sporulation. In 1900 he summarized his conclusions in four statements or laws (Chap. 14). No better generalizations on this subject have appeared in the 50 years which have elapsed since they were published. For an evaluation of the significance of Klebs' work, see Kauffman (1929).

A. H. Reginald Buller (1874–1944), *England and Canada.* Many of his studies involved the activities of fungi in relation to structure. His chief interests lay in production of fruit bodies and spores, in spore discharge and dissemination, and in the effects of the environment on these activities. His keen observations are recorded in detail in seven volumes, "Researches on Fungi." These volumes are written in an interesting,

readable style and should be frequently consulted by all students of mycology.

Leon H. Leonian (1888–1945), *United States.* Trained as a mycologist under Kauffman, he was always interested in discovering the potentialities of living fungi. His principal contributions were made in the study of fungus nutrition with emphasis on the factors which are required by fungi for growth and reproduction. For a bibliography of his papers see Orton (1946).

The number of living investigators who have made and are continuing to make important contributions to fungus physiology is far too great to list here, and for this reason they have been omitted. An idea of the scope of their interests and activities may be gained from the references in the following chapters.

REFERENCES

Brefeld, O.: Botanische Untersuchungen über Schimmelpilze, Hefte 1–4, 1872–1881. Title changed to Botanische Untersuchungen über Hefenpilze Fortsetzung der Schimmelpilze for Heft 5, 1883; thereafter Untersuchungen aus dem Gesamtgebiet der Mykologie, Hefte 6–15, 1884–1912. Hefte 1–8, Arthur Felix, Leipzig. Hefte 9–15, Heinrich Schöningh, Muenster.

Buller, A. H. R.: Researches on Fungi, Longmans, Roberts and Green, London. Vol. I, 1909; Vol. II, 1922; Vol. III, 1924; Vol. IV, 1931; Vol. V, 1933; Vol. VI, 1934; Vol. VII, The University of Toronto Press, Toronto, 1950.

★De Bary, A.: Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria (trans. H. E. F. Garnsey), Oxford University Press, New York, 1887.

Dodge, B. O.: Some problems in the genetics of the fungi, *Science* **90**: 379–385, 1939.

★Dubos, R. J.: Louis Pasteur, Free Lance of Science, Little, Brown & Company, Boston, 1950.

Kauffman, C. H.: Klebs' theory of the control of developmental processes in organisms, and its application to fungi, *Proc. Intern. Congr. Plant Sci.* **2**: 1603–1611, 1929.

Klebs, G.: Zur Physiologie der Fortpflanzung einiger Pilze. III. Allgemeine Betrachtungen. *Jahrb. wiss. Botan.* **35**: 80–203, 1900.

Orton, C. R.: Leon Hatchig Leonian. *Phytopathology* **36**: 241–244, 1946.

Pasteur, L.: Oeuvres de Pasteur, réunies par Pasteur Vallery-Radot, 7 vols., Masson et Cie, Paris, 1933–1939.

Schopfer, W. H.: Plants and Vitamins, Chronica Botanica Co., Waltham, 1943.

CHAPTER 2

CULTURE MEDIA

Before discussing the nutrition of the fungi in detail, it will be helpful to consider the basic problems involved. For many purposes a knowledge of the nutrition of the fungi is necessary for culturing them in the laboratory or in industry. Like all living organisms the fungi must obtain from their environment the materials needed for the synthesis of protoplasm and other cellular constituents. Directly or indirectly, the fungi as well as animals and most bacteria are dependent upon green plants for "food" and energy.

Not all natural substrates are equally suitable for all fungi. In nature, the saprophytes are more widely distributed than the parasites, which are usually restricted to the range of their hosts. Many of the substances upon which the fungi grow in nature are chemically complex, and some, such as cellulose, starch, and proteins are insoluble or are only colloidally soluble. Before such compounds can be utilized, they must be changed into low-molecular-weight compounds which are soluble in water. This "digestion" is accomplished by means of enzymes which are excreted by the fungi. This is analogous to digestion in animals, which is also an enzymatic process. The complete utilization of a natural substrate is frequently due to the combined action of a succession of microorganisms. More than one organism may act at the same time, and often this simultaneous action is more effective than that of a single organism.

One may ask, Do the fungi simply incorporate within their own protoplasm the suitable elements and compounds found in the medium, or do they transform the compounds of the medium before building their own structures? Apparently the fungi do both. The essential elements such as potassium and magnesium are taken up as ions, although these elements may be in the state of chemical combination in the substrate and also in the fungus cells. Certain organic compounds, such as the vitamins, are undoubtedly absorbed as such from the medium by vitamin-deficient fungi; otherwise, these fungi would derive no benefit from them. The same statement is true for other necessary compounds which the various fungi are unable to synthesize.

By far the greater part of the compounds utilized by the fungi are modified or changed either before or after they are taken into the cells. Outside the fungus cells, these changes are largely in the direction of simplifying the molecular structure of compounds used. Within the fungus cells some of the metabolite molecules are oxidized to carbon

dioxide and water or to intermediate products. By this process the fungus obtains the chemical energy which it requires for the processes of synthesis.

KINDS OF MEDIA

No one knows when man began to cultivate fungi, but certainly it was many thousands of years ago. This cultivation was no doubt unintentional at first and was later developed into an art, in connection with the preparation of foodstuffs and beverages. The use of leaven (yeast) extends back to the beginning of agriculture. The yeast culture was preserved in a piece of dough which in turn was added to the next batch, much as buckwheat batter is prepared today. In the Orient, species of *Mucor* and *Aspergillus* have been used from the dawn of civilization in preparing food from rice and soybeans. Brewers used yeast many centuries before it was learned that yeast is a living organism. On the other hand, the science of growing fungi in pure culture is fairly recent.

Natural media. It was quite natural that, when mycologists and others began to cultivate fungi in the laboratory, they should turn to natural materials as media. A *natural medium* is one which is composed entirely of complex natural materials of unknown composition. Among the natural substances so used are the following: plant parts, malt, yeast, peptone, manure, bread, wort, fruit, and vegetables. Many of these substances are used in the form of extracts, infusions, or decoctions. The very diversity of these natural media is strong testimony to the fact that different species have different nutritional requirements. Brefeld (1881) was among the first to grow fungi in pure culture, and many of his techniques are in use today. Since his interest in cultivating the fungi was largely for the purpose of observing their development, it was necessary for him to select suitable media. He found two natural media to be of great utility: a decoction prepared from dried plums or raisins and a manure extract. This latter medium he considered "als Universalnährlösung für Pilzculturen." This medium is still used in some laboratories. Natural media have many advantages. They are cheap and easy to prepare. In many instances it is necessary only to add water to the base material and autoclave. More important yet is the fact that many fungi grow well upon a wide variety of natural media.

Certain of the more fastidious fungi have never been cultivated in the laboratory. These obligate parasites live only upon or within the living tissues of their hosts. *Puccinia graminis tritici* lives only on wheat, some species of grasses, and some species of barberry. These host plants when killed will no longer support growth of this fungus. However, many species, which in the past were considered to be obligate parasites, have since been cultured on nonliving media.

Semisynthetic media. A semisynthetic medium is one which is composed in part of natural materials of unknown composition. Such media are made by adding compounds of known composition to one or more natural materials. The widely used potato-glucose (dextrose) medium is an example of this type. The addition of agar to an otherwise synthetic medium introduces a natural material of unknown composition. Media which contain agar cannot be classed strictly as synthetic media. Semisynthetic media may be used for many types of physiological investigations.

The composition of a given natural or semisynthetic medium is not constant. Potato-glucose medium may vary greatly in composition depending upon whether or not the potatoes were peeled and upon the variety and age of the potatoes used. Neuberger and Sanger (1942) found a twofold difference in the amide nitrogen (asparagine and glutamine) among varieties. In this laboratory we have found that the amount of potato pulp which is allowed to enter this medium exerts a marked influence upon growth and reproduction of certain fungi. These differences, which may seem minor, are great enough to make comparisons between work done in different laboratories difficult.

Synthetic media. As the term is used in this book, a synthetic medium is one of *known composition and concentration.* It does not mean that every compound used is a product of the chemist's art. Some of the constituents, such as the sugars, may be of natural origin. The important condition is that the compounds used be pure, and this is difficult to attain in practice. "Chemically pure" compounds are usually far from being pure, as a glance at the labels will show. The ideal of using pure compounds is seldom realized, but the closer it is approached, the more we shall learn about the nutrition of the fungi.

Natural media and most semisynthetic media are of limited usefulness in studying nutrition of the fungi. The chief value of synthetic media is for nutritional studies. However, growth and reproduction are frequently poorer on a synthetic medium than on one containing some natural material. For example, *Aspergillus niger* grew well in a synthetic medium composed of sucrose, ammonium nitrate, magnesium sulfate, and dipotassium hydrogen phosphate (Steinberg, 1939). In addition to these major constituents, iron, zinc, copper, manganese, molybdenum, and gallium salts were present. Extraordinary care was taken in preparing the medium. The concentration of every constituent was so balanced that a decrease in concentration of any constituent resulted in diminished growth. Growth and sporulation were excellent upon this medium. When 20 mg. per liter of either peptone or yeast extract was added, the rate of growth was greatly increased and the time required for sporulation was decreased. The small amount of yeast extract or peptone used could have added only an insignificant amount of material from which

the fungus could synthesize protoplasm or derive energy. Steinberg's synthetic medium was adequate but not optimum for most rapid growth and sporulation. We may suppose that the yeast extract and peptone contained compounds the synthesis of which constituted a limiting effect upon the rate of growth and sporulation.

Synthetic media may be simple or complex but must contain the essential elements in utilizable form. Brefeld (1881) gave the following directions for preparing a synthetic medium: Add cigar ashes dissolved in nitric or citric acid to a solution containing a soluble carbohydrate, such as glucose, and an ammonium salt. The amount of ashes was not specified. The first synthetic medium was devised by Raulin (1869).

TABLE 1. COMPOSITION OF THE FIRST SYNTHETIC MEDIUM FOR CULTIVATING FUNGI (Raulin, *Ann. sci. nat.*, Ser. V, **11**, 1869.)

Ammonium nitrate	4.0	g.
Ammonium phosphate	0.6	g.
Magnesium carbonate	0.4	g.
Potassium carbonate	0.6	g.
Ammonium sulfate	0.25	g.
Zinc sulfate	0.07	g.
Iron sulfate	0.07	g.
Potassium silicate	0.07	g.
Sucrose	70	g.
Tartaric acid	4	g.
Water	1,500	ml.

However, not enough information is given in Table 1 for the duplication of this medium. Which ammonium phosphate, $(NH_4)H_2PO_4$ or $(NH_4)_2HPO_4$, was used by Raulin in the original work? Which zinc sulfate, $ZnSO_4 \cdot 7H_2O$ or $ZnSO_4 \cdot H_2O$, was used? Was the iron sulfate $FeSO_4$, $FeSO_4 \cdot 7H_2O$, or $Fe_2(SO_4)_3$? Did he use D-tartaric, L-tartaric, DL-tartaric, or *meso*-tartaric acid? These questions are asked for the purpose of emphasizing the need for exactness in reporting the composition of media used in experimental work. These uncertainties creep into the literature through ignorance or carelessness, or both. Nor are these ambiguities to be found only in the older literature, for they are present in papers published only yesterday. Either the specific name or the formula, or both, should be stated. If it is stated that dipotassium phosphate, K_2HPO_4, was used, the reader is certain of the identity of the compound. Potassium phosphate may designate at least five distinct chemical compounds.

NATURAL VERSUS SYNTHETIC MEDIA

In addition to the fact already noted that the composition of natural media is unknown, natural and synthetic media differ in two further respects. Natural media are more complex; *i.e.*, they contain more

chemical compounds than synthetic media. They also contain compounds ordinarily not present in synthetic media.

Specific metabolites. Only certain chemical compounds are utilized by fungi, but not all fungi are able to utilize the same compounds. Any compound utilized by a fungus is called a *metabolite*. Some fungi are unable to synthesize certain essential metabolites and are said to be "deficient" for the specific metabolites they are unable to synthesize. In order to cultivate such deficient fungi, these metabolites must be present in the medium. Natural media usually contain these metabolites. If a fungus grows upon a natural medium and fails to grow upon a variety of simple synthetic media, it may be suspected that specific metabolites are involved in its nutrition.

The following example will illustrate the role of specific metabolites in fungus nutrition. Fellows (1936) investigated the ability of *Ophiobolus graminis* to utilize different nitrogen compounds for growth. A sucrose–mineral salts solution was used as the basal medium to which various nitrogen sources were added. Only complex nitrogen sources such as egg albumen, peptone, casein, and nucleic acid allowed growth. Under the conditions used no growth resulted when ammonium compounds, nitrates, nitrites, and amino acids were tested. *O. graminis* in the presence of egg albumen utilized glucose, maltose, lactose, fructose, xylose, starch, and dextrin, in addition to sucrose. From these experimental results it was concluded that *O. graminis* requires a complex source of nitrogen for growth.

Later, White (1941) found that this fungus requires two specific metabolites, thiamine and biotin. When these vitamins were added to synthetic media containing simple nitrogen sources (sodium nitrate, ammonium nitrate, asparagine, or glycine), good growth was obtained. Thus, it appears obvious that *O. graminis* does not require a complex nitrogen source, but that it is unable to synthesize two specific chemical compounds. These papers illustrate the fact that fungus physiology is a young and developing science. Much of the early work needs reevaluation in the light of recent discoveries. A student should strive to develop a critical attitude toward the work of others, but he should be no less critical with regard to his own work. The evaluation of experimental results depends upon the conditions under which the work was done, and among these conditions the medium used is of first importance.

Complexity of media. It is a common experience to find that a trace of some crude natural product stimulates the rate of growth and sporulation of a fungus. This stimulation frequently occurs with fungi which grow well on synthetic media and which are not deficient for vitamins or amino acids. It appears that the complexity of natural media offers a clue to understanding this stimulatory effect. If a fungus is grown upon a simple

synthetic medium which has only one source of carbon and one source of nitrogen, it must synthesize many complex chemical compounds from constituents present in the medium. It may be suspected that these biochemical syntheses are slowed up under these conditions. When a mixture of many carbon and nitrogen sources is present, the fungus may function more efficiently, because the biochemical syntheses are easier since some of the intermediates are furnished. These speculations receive some support from evidence to be presented in Chaps. 6 and 7.

CHOICE AND PREPARATION OF MEDIA

Considerable care is needed in the selection of a suitable medium. A medium may be excellent for growth and unsuitable for reproduction or the production of an antibiotic. The method of preparation may influence the composition of a medium in unsuspected ways.

Choice of media. In selecting a medium the purpose for which it is to be used should be kept in view. For many purposes a natural medium is the one of choice. This is especially true for routine maintenance of cultures, for isolations, and for preliminary investigations. The composition of natural media may be varied by choosing different substrates. Frequently, a combination of natural products may be used to advantage, *e.g.*, malt and yeast extracts. In addition, these natural substrates may be fortified with one or more pure chemical compounds. The constituents of natural media are fixed by the substances used, but the amounts used may be changed at will.

More judgment enters into the selection of synthetic media. The essentials of a synthetic medium may be stated as follows: sources of carbon and nitrogen in utilizable forms; phosphate and sulfate ions; the metallic ions potassium, magnesium, iron, zinc, manganese, and others which are usually present as impurities in the chemicals used. These are the essential elements and will be considered at length in later chapters. Most fungi utilize glucose, so this sugar is frequently used as the carbon source. More fungi utilize nitrogen in organic combinations than in inorganic compounds. The question of specificity enters into the choice of the carbon and nitrogen sources, and this can be determined only by experiment. In order to cultivate deficient fungi on synthetic media, the specific metabolites for which the fungi are deficient must be added. Since synthetic media are used to study nutrition, the development of a suitable synthetic medium for a specific fungus may require considerable investigation. In our laboratory we commonly first use a glucose–casein hydrolysate medium containing the essential inorganic elements. This medium has been very useful in vitamin studies. Its composition is given in Chap. 10.

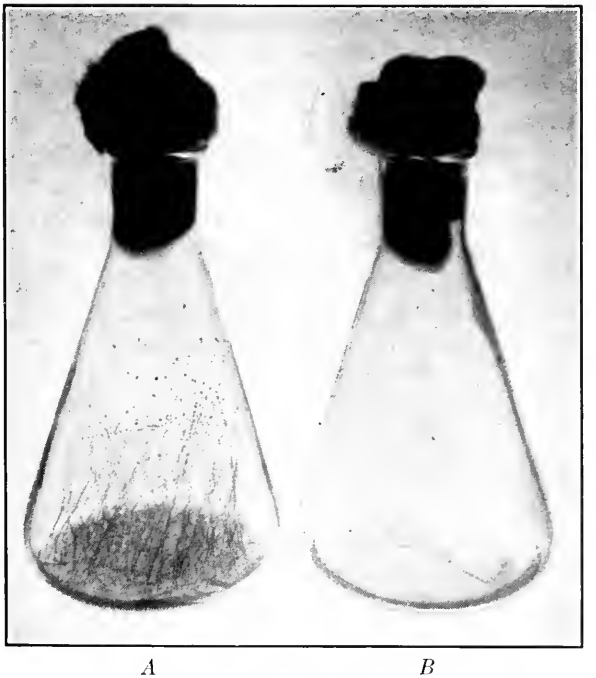
Solid versus liquid media. Both solid and liquid media are used in cultivating fungi. Media solidified with agar, or semisolid substrates such as corn meal, offer many advantages in that the culture vessels can be freely handled without disturbing the fungus. This feature is particularly valuable when one wishes to follow the development of a fungus. Microscopic examination is facilitated, and contaminants are more easily detected. Single-spore isolations can be made more easily from solid media. Agar media are used to maintain stock cultures and are recommended for many preliminary experiments.

Frau Hesse (Hitchens and Leikind, 1939) introduced the use of agar into microbiological procedures in 1881. Agar, which is obtained from various marine red algae, is a complex polysaccharide sulfate ester (Pigman and Goepp, 1948). It forms colloidal solutions at elevated temperatures and sets to a gel at temperatures around 45°C. On acid hydrolysis both D-galactose and its enantiomorph, L-galactose, as well as sulfuric acid, are formed. Agar must exist in the form of a salt (Ca, Mg, Na, K, etc.) to form a gel. Agar introduces physiologically active elements into media. It may contain significant amounts of zinc (Leonian and Lilly, 1940) and other micro essential elements. Mulder (1940) found that magnesium could be efficiently removed from agar by repeated soakings in 10 per cent sodium chloride solution, followed by washing with distilled water until the filtrate was free from chloride ion. Agar also contains growth factors such as thiamine (Day, 1942) (see Fig. 1). Many fungi make some growth on water agar, which indicates that agar or the "impurities" contained in it are utilized by fungi. Robbins (1939) found that leaching agar with 5 per cent aqueous pyridine removed many of the physiologically active compounds.

Liquid media should be used for precise investigations where it is desired to control as many variables as possible. The composition of the medium may be controlled and the amounts used measured accurately. Cultures may be aerated by shaking or by blowing sterile air through the media. Weighing the mycelium is facilitated. When it is desired to study the metabolic by-products of fungus metabolism (except gaseous products), it is almost necessary to use liquid media. Isolation of by-products is less complicated when liquid media are used. Studies of various metabolite deficiencies and many microbiological assays (Chap. 10) almost always require the use of liquid media. The choice between the use of solid or liquid media should be made on the basis of the known advantages and disadvantages of both and with regard to the purpose of the problem under investigation.

Designating media. It is common to find references to a medium by the name of the investigator who first used it. These names have served as convenient abbreviations and commemorate the pioneers in the art of

cultivating fungi. Some of these names are Blakeslee, Uschinsky, Coons, Czapek, Leonian, Sabouraud, Richard, Thaxter, Shear, Raulin. From a historical standpoint this practice has much to recommend it. However, this usage has many disadvantages. These distinguished names give no clue to the composition of these media. The original formulas have in many instances been changed. Some of these modifications have received



A B

FIG. 1. Growth of *Phycomyces blakesleeanus* on vitamin-free liquid medium solidified with two different brands of agar. Growth in *A* indicates relatively high content of thiamine of this agar. The trace of growth in *B* shows that this agar is relatively free of thiamine.

hyphenated names: *e.g.*, Czapek-Dox. Frequently the originator of a medium modified it from time to time. This introduces a further uncertainty as to its composition. In our opinion the use of personal names to designate media should be abandoned. It is much more helpful to designate media by descriptive titles than by names which tell nothing of the composition. The carbon and nitrogen sources are important constituents of every medium. Thus, sucrose-nitrate medium, glucose-asparagine medium, or malt extract–yeast extract medium are preferred to Czapek's medium, Schopfer's medium, or Leonian's medium. These descriptive terms afford valuable information that personal names do not. Even when the reader is familiar with the composition of a named medium,

there is a tendency to fail to associate experimental results with the composition.

Effect of autoclaving. Media are commonly and effectively sterilized by autoclaving. It should be noted, however, that such high temperatures may cause destruction or alteration of some constituents in the media. These changes are not serious for many uses; at least media prepared in this way are satisfactory. Sugars are among the substances most easily altered by autoclaving. The extent of decomposition depends upon the specific sugar used, the other constituents of the medium, and the time of autoclaving. It is desirable to adopt a uniform schedule for autoclaving media. An increase in the amount of caramelization occurs as the time of heating is increased. Maillard (1912) showed that a brown color results when reducing sugars (glucose, fructose, etc.) are autoclaved with amino acids. Hill and Patton (1947) have shown that growth of *Streptococcus faecalis* is reduced when tryptophane is autoclaved with sugars. Margolin (1942) found that no one method of sterilization resulted in best growth for all of the 14 species tested. *Phythophthora erythroseptica* made three times the amount of growth on glucose sterilized by filtration as when the entire medium was autoclaved. *Syncephalastrum racemosum*, however, made more growth on autoclaved than on sterile-filtered glucose (Table 2). The organisms most sensitive to heated glucose appear to be various species of *Cytophaga*, which failed to grow on glucose which had been heated to 50°C. (Stanier, 1942). These organisms utilized glucose which had been sterilized by filtration. Phosphates, a universal constituent of media, are active in converting glucose into ketoses and other products (Englis and Hanahan, 1945) during autoclaving.

Complex sugars and polysaccharides undergo some hydrolysis during autoclaving. The amount of hydrolysis is dependent upon the carbohydrate, the time and temperature of autoclaving, and the pH of the medium. Sucrose, when autoclaved in acidic media, may undergo sufficient hydrolysis to support some growth of species unable to utilize sucrose. This possibility must be guarded against in experiments on the availability of complex sugars.

Other substances used in media may be destroyed during autoclaving. To minimize or avoid such effects, heat-sensitive substances may be autoclaved separately, or they may be sterilized using special bacteriological filters. The Berkefeld and Chamberland filters are less used than formerly, while at present Seitz and fritted-glass filters are widely used. Fritted-glass filters are best for most purposes, inasmuch as the asbestos pad used in the Seitz filter may adsorb active compounds. All methods of sterilization which depend upon filtration are slow and can be used only with liquid media. Various volatile chemical sterilization agents such as

alcohol and acetone have been used. Hansen and Snyder (1947) have recommended the use of propylene oxide for the sterilization of plant parts used for culture media. Frequently a seemingly insignificant change in the method of preparing a medium may result in significant changes in the composition of the medium, which in turn may be reflected in the behavior of the organisms grown upon it. Even the volume of medium in culture vessels affects the amount of decomposition during autoclaving. Cotton plugs may introduce lint into the medium. Less refined grades of cotton release a volatile substance which affects the

TABLE 2. THE EFFECT OF DIFFERENT METHODS OF STERILIZING GLUCOSE UPON THE GROWTH OF SIX FUNGI, AT 25°C.

Growth reported as milligrams of dry mycelium.

The entire medium, containing a mixture of amino acids, was autoclaved in the control experiment. In the other experiments the glucose was sterilized by either Seitz filtration or treatment with acetone and added aseptically to the remainder of the sterile medium. (Margolin, thesis, West Virginia University, 1942.)

Species	Days of incubation	Control, entire medium autoclaved	Glucose sterilized by filtration	Glucose sterilized by treating with acetone
Phycomyces blakesleeanus	7	130	140	132
Rhizopus suinus	6	122	123	115
Syncephalastrum racemosum	5	103	68	80
Phytophthora erythroseptica	12	79	241	192
Diplodia macrospora	15	84	88	85
Phytophthora cactorum	15	142	147	103

germination of some spores (*Phycomyces blakesleeanus*, Robbins and Schmitt, 1945). Paper or aluminium caps may be used to replace cotton plugs. Residual soap films on improperly rinsed glassware may cause trouble in some cases.

Preparation of media. Directions for the preparation of specific media are given at the end of the text in the section Suggested Laboratory Exercises. Additional details concerning various media are to be found in Riker and Riker (1936) and Rawlins (1933).

WAYS OF EXPRESSING CONCENTRATION

Concentrations are frequently expressed in the literature as percentages. Unless the basis for calculating these values is given, percentage is an ambiguous way of reporting concentration. Buchanan and Fulmer (1928) have pointed out that there are six ways of calculating the percentage composition of a solution. A 10 per cent sulfuric acid solution may represent six different concentrations. For any precise work it is best to

avoid the use of percentages, but for routine work, where the composition of media is of less importance, the use of percentages may be allowed. Before the same medium can be prepared repeatedly, it is necessary to know what constituents are used and the amount of each.

Two general methods are used for reporting the composition of media. Either the weights of the constituents and the volume of water used are given, or the weights of the constituents are given and the medium made up to a definite volume. The first method is in common use; its simplicity conceals its disadvantages. The volume of a medium prepared by this method is never the same as the volume of water used. It is necessary to measure the volume of the medium *after* preparation in order to calculate the amount of any constituent in an aliquot.

The method of choice in accurate work is to weigh the constituents and make the medium up to a given volume. The amount of any constituent in any volume of medium may then be calculated. If a liter of medium contains 25 g. of sucrose, and 25-ml lots are dispensed, each lot contains $^{25}/_{1000} \times 25$, or 0.625 g. of sucrose.

Direct units. The units of volume most used are the liter (l.) and the milliliter (ml.). A cubic centimeter (cc.) is nearly, but not exactly, equivalent to a milliliter. Its use should be discouraged. The formulas for media are usually given on the basis of a liter. This practice is to be encouraged, as the liter is a convenient volume in preparing media. The weights of solid constituents should be reported as grams (g.) or decimal divisions thereof. The most commonly used decimal fractions of the gram are the milligram (mg.), the microgram (μg), and the millimicrogram (mμg), each of which is one-thousandth of the preceding weight. Since it is easy to make mistakes in reading small decimals, it is recommended that no decimals smaller than 0.1 be used. The use of 12 mg. is preferable to 0.012 g., although both mean exactly the same. It is easier to read 5 μg than 0.000005 g. One milligram of a substance in a liter of solution equals one part per million (p.p.m.). Each milliliter of such a solution will contain 1 μg of the substance. Similarly, a microgram of a substance in a liter of solution is present as one part per billion. The microgram has also been called the *gamma* (γ), but this usage should be abandoned inasmuch as *gamma* is not a regular prefix used in the metric system. The necessity of using such small units of weight arises from the physiological activity of certain compounds and elements. For example, a concentration of 1 mg. of biotin in a liter of medium is a relatively enormous concentration.

Derived units. Derived units *must* be used in comparing the effect of compounds which have different molecular weights. Among these derived units the mole is the most useful. A *mole* is the molecular weight of a chemical compound expressed in grams. A mole of glucose is 180 g.,

while a mole of sucrose is 342 g. A liter of solution containing one mole of a compound is said to be one molar (M). Equimolar solutions contain the same number of molecules. In problems in physiology, such as osmotic pressure, which have to do with the numbers of molecules it is necessary to use this way of expressing concentration. If it is desired to compare the effect of the osmotic pressure due to glucose and sucrose, the concentration must be expressed in terms of molar strengths, for the osmotic pressure is a function of the number of molecules of solute in a solution. If it is the purpose to compare the effect of glucose and sucrose on the amount of growth of a fungus, this method of expressing concentrations should not be used. Media of equal molarity with respect to sucrose and glucose do not contain the same amount of carbon. The first contains twice as much carbon as the second. Just as a milligram is one-thousandth of a gram, a millimole is one-thousandth of a mole. The meaning of micromole and millimicromole should be obvious.

If the weight of a compound is given in grams, this datum may be converted into moles. If a medium contains 50 g. of glucose per liter, the glucose concentration may be expressed as 50/180 or $5/18M$. Conversely, if the concentration of sucrose in a medium is stated to be $0.15M$, the weight of sucrose is 0.15×342 or 51.3 g. per liter. These conversions imply that the molecular weight is known or can be calculated. In preparatory work compounds are weighed on a balance as grams, not as moles, and unless the interpretation of the results demands conversion to moles, it is better to record the weights than to convert these data to derived units. The mole and molar solutions are particularly useful in dealing with non-ionizing compounds.

Another derived unit, the equivalent, is frequently used to express the concentration of ionized compounds. An equivalent is the atomic weight of an ion expressed in grams divided by the valence of the ion. If an ion is composed of more than one atom, the ion weight is computed by adding together the atomic weights. It is important to remember that, if an element has more than one valence, the equivalent weight depends upon the valence. An equivalent of ferrous (Fe^{++}) ion is 55.8/2 or 27.9 g., while an equivalent of ferric (Fe^{+++}) ion is 55.8/3 or 18.6 g. A normal solution (N) is one which contains one equivalent in a liter of solution. In dealing with small amounts it is convenient to use milliequivalents or microequivalents.

In preparing a series of media for the purpose of comparing the growth of a fungus on different nitrogen sources, the nitrogen content of the media should be equal. If urea, $CO(NH_2)_2$, and aspartic acid, $HOOC—CH_2—CH(NH_2)—COOH$, are used, it is obvious that different weights of these nitrogen sources must be used if the media are to contain equal amounts of nitrogen. Whenever media are modified by replacing one compound by

another, it should be done in such a way that the same amount of the essential element is present in all the media. If this is not done, the basis upon which the replacement was made should be stated. If 25 g. of glucose, $C_6H_{12}O_6$ is replaced by 25 g. of sucrose, $C_{12}H_{22}O_{11}$, it should be realized that the carbon contents of the two media are different. It is frequently difficult or impossible to find out from some papers in the literature how substitutions in the media were made.

TABLE 3. A COMPARISON OF TWO SYNTHETIC MEDIA UPON THE BASIS OF AMOUNTS OF ESSENTIAL ELEMENTS AND COMPOUNDS PRESENT IN ONE LITER
Both media were made with double-distilled water.

Element or compound	Glucose-asparagine*		Sucrose–ammonium nitrate†	
	Unit of meas.	Source	Unit of meas.	Source
	G.		G.	
C	4.0	D-Glucose, 10 g.	21.4	Sucrose, 50 g.
N	0.427	L-Asparagine, 2 g.	0.720	NH_4NO_3, 2.06 g.
Mg	0.049	$MgSO_4 \cdot 7H_2O$, 0.5 g.	0.025	$MgSO_4 \cdot 7H_2O$, 0.25 g.
S	0.065	$MgSO_4 \cdot 7H_2O$, 0.5 g.	0.032	$MgSO_4 \cdot 7H_2O$, 0.25 g.
K	0.287	KH_2PO_4, 1.0 g.	0.125	K_2HPO_4, 0.35 g.
P	0.228	KH_2PO_4, 1.0 g.	0.062	K_2HPO_4, 0.35 g.
	Mg.		Mg.	
Fe	0.2	As sulfate	0.3	As chloride
Zn	0.2	As sulfate	0.3	As chloride
Mn	0.1	As sulfate	0.075	As chloride
Cu	—		0.075	As chloride
Mo	—		0.02	As chloride
Ga	—		0.02	As chloride
	μg			
Thiamine hydrochloride	100			
Biotin	5			

* Medium 5, Suggested Laboratory Exercises.
† Steinberg, 1941.

Finally, it should be noted that the common practice of using one compound as the source of two essential elements does not permit perfect freedom in adjusting the composition of a medium. If magnesium sulfate heptahydrate is used to supply both magnesium and sulfur, it is obvious that the ratio Mg/S is fixed. If it is desired to vary the amounts of magnesium and sulfur independently, it is necessary to use different compounds of magnesium and sulfur; *e.g.*, magnesium chloride and sodium sulfate. This practice introduces other elements into the medium.

COMPARISON OF MEDIA

Media differ only in *constituents* and *amounts* used. It is desirable to be able to compare media in some uniform way. To do this, it is necessary to know not only the amounts of the elements present, but also the compounds in which these elements occur. A comparison of two synthetic media is given in Table 3.

From Table 3 it will be noted that these media contain the same essential elements. Copper, molybdenum, and gallium do not appear in the composition of the glucose-asparagine medium, but it should not be concluded that these elements were not present, since only c.p. chemicals were used to prepare this medium. Stout and Arnon (1939) note that a distinction must be made between ordinary chemical purity and biological purity. This will be considered in detail in Chap. 5. The two features which make these media quite distinct are the different sources of carbon and nitrogen used and the addition of two vitamins to the glucose-asparagine medium. The latter medium is suitable for the growth of more species of fungi than is the sucrose–ammonium nitrate medium.

SUMMARY

Fungi secure food and energy from the substrates upon which they live in nature. In order to culture fungi in the laboratory, it is necessary to furnish in the medium those essential elements and compounds they require for the synthesis of their cell constituents and for the operation of their life processes. The synthetic abilities of fungi differ. Some fungi are unable to synthesize certain key compounds that they require and must obtain them from the medium upon which they grow. All the fungi require much the same essential elements but differ widely in their ability to utilize compounds in which these elements occur. There is no universal natural substrate or artificial medium upon which all fungi will grow.

On the basis of composition there are three general types of media: natural media, which are composed entirely of natural products; semisynthetic media, which are composed in part of natural substances; and synthetic media, which are of known composition. Natural media are most useful for routine work, while synthetic media and, to a limited extent, semisynthetic media are used to investigate the nutritional requirements of the fungi. Media differ only with respect to constituents and concentrations.

The compounds and the amounts used in preparing a medium must be specified exactly. Media should be designated by naming the carbon and nitrogen sources used, *e.g.*, glucose-asparagine medium. The use

of proper names to designate the composition of a medium should be avoided.

The selection of a suitable medium depends upon the fungus under study and the purpose of the experiment. Not all media are equally suitable for all fungi, nor is one medium suitable for a complete physiological study of one fungus.

REFERENCES

Brefeld, O.: Botanische Untersuchungen über Schimmelpilze, Heft, IV Verlag Arthur Felix, Leipzig, 1881.

Buchanan, R. E., and E. I. Fulmer: Physiology and Biochemistry of Bacteria, Vol. I, The Williams & Wilkins Company, Baltimore, 1928.

Day, D.: Thiamin content of agar, *Bull. Torrey Botan. Club* **69**: 11–20, 1942.

Englis, D. T., and D. Hanahan: Changes in autoclaved glucose, *Jour. Am. Chem. Soc.* **67**: 51–54, 1945.

Fellows, H.: Nitrogen utilization by *Ophiobolus graminis*, *Jour. Agr. Research* **53**: 765–769, 1936.

★Hansen, H. N., and W. C. Snyder: Gaseous sterilization of biological materials for use as culture media, *Phytopathology* **37**: 369–371, 1947.

Hill, E. G., and A. R. Patton: The Maillard reaction in microbiological assay, *Science* **105**: 481–482, 1947.

Hitchens, A. P., and M. C. Leikind: The introduction of agar-agar into bacteriology, *Jour. Bact.* **37**: 485–493, 1939.

Leonian, L. H., and V. G. Lilly: Studies on the nutrition of fungi. IV. Factors influencing the growth of some thiamin-requiring fungi, *Am. Jour. Botany* **27**: 18–26, 1940.

Maillard, L. C.: Action des acides aminés sur les sucres; formation des mélanoïdines par voie méthodique, *Compt. rend. acad. sci.* **154**: 66–68, 1912.

Margolin, A. S.: The effect of various carbohydrates upon the growth of some fungi, thesis, West Virginia University, 1942.

Mulder, E. G.: On the use of micro-organisms in measuring a deficiency of copper, magnesium and molybdenum in soils, *Antonie van Leeuwenhoek* **6**: 99–109, 1939–1940.

Neuberger, A., and F. Sanger: The nitrogen of the potato, *Biochem. Jour.* **36**: 662–671, 1942.

Pigman, W. W., and R. M. Goepp, Jr.: Chemistry of the Carbohydrates, Academic Press, Inc., New York, 1948.

Raulin, J.: Études chimiques sur la végétation, *Ann. sci. nat.*, Ser. V, **11**: 93–229, 1869.

Rawlins, T. E.: Phytopathological and Botanical Research Methods, John Wiley & Sons, Inc., New York, 1933.

Riker, A. J., and R. S. Riker: Introduction to Research on Plant Diseases, John S. Swift Co., St. Louis, 1936.

★Robbins, W. J.: Growth substances in agar, *Am. Jour. Botany* **26**: 772–778, 1939.

Robbins, W. J., and M. B. Schmitt: Effect of cotton on the germination of *Phycomyces* spores, *Bull. Torrey Botan. Club* **72**: 76–85, 1945.

Stanier, R. Y.: The *Cytophaga* group: a contribution to the biology of myxobacteria, *Bact. Revs.* **6**: 143–196, 1942.

Steinberg, R. A.: Relation of carbon nutrition to trace-element and accessory requirements of *Aspergillus niger*, *Jour. Agr. Research* **59**: 749–763, 1939.

STEINBERG, R. A.: Sulfur and trace element nutrition of *Aspergillus niger*, *Jour. Agr. Research* **63**: 109–127, 1941.

STOUT, P. R., and D. I. ARNON: Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants, *Am. Jour. Botany* **26**: 144–149, 1939.

*WHITE, N. H.: Physiological studies of the fungus *Ophiobolus graminis* Sacc., *Jour. Council Sci. Ind. Research* **14**: 137–146, 1941.

CHAPTER 3

GROWTH

Growth may be considered either as an increase in cell *number* or as an increase in *mass*. Usually both these processes are concurrent in the phenomenon called growth. To a limited degree, fungus cells may divide and form new cells without an increase in mass. A spore may germinate in distilled water and give rise to a germ tube, but in the absence of nutrients this process soon stops. A few cell divisions exhaust the reserve material originally present in the spore, and growth soon ceases unless these new cells obtain nutrients from the external environment. Under certain conditions fungus cells may increase their store of reserve materials, and thus their mass, without an increase in cell number, but this process is also limited. Growth, excluding the limited meanings given above, involves an increase in *both the number and the mass of cells*.

This definition of growth neither "explains" the processes involved nor indicates their complexity. Rahn (1932) has expressed doubt that we will ever fully understand the process of growing. A yeast cell which buds and produces a daughter cell illustrates one of the striking features of growth: *growth involves duplication*. From a dozen or so simple chemical substances present in the medium the parent cell synthesizes at least a portion of the protoplasm of the daughter cell. The daughter cell has the same genetic constitution as the parent cell, and thus a duplication of genes is a feature of cell multiplication. The compounds which comprise protoplasm, enzymes, genes, and other substances are extraordinarily complex. Our meager knowledge concerning the chemical architecture of these substances only confirms this view. In the synthesis of such compounds we may assume that the chemical reactions which produce them are perfectly timed and coordinated, for no series of uncorrelated reactions could produce such compounds.

The growth processes of the filamentous fungi are still more complex than those of yeast, because of greater differentiation in structure. In those species of fungi which produce aerial mycelium these parts are nourished through the mycelium in contact with the medium. This involves translocation of nutrients over considerable distances. This is especially true of sporangiophores and aerial fruit bodies. The development of fruiting structures and spores is growth, in that the formation of new cells is involved. The formation of fruit bodies in many species

takes place at the expense of reserve materials and protoplasm formed by and stored in the vegetative mycelium.

PHASES OF GROWTH

Growth in the fungi, as in other organisms, follows a definite pattern. The way this development takes place depends upon the species and the environmental and nutritional conditions. In the present discussion, it will be assumed that the external conditions are favorable and that growth takes place in a limited volume of medium.

Unicellular organisms. The bacteriologists have long been interested in the mathematical analysis of the phenomenon of growth. The student is referred to Buchanan and Fulmer (1928) and to Rahn (1932, 1939) for further information on this subject. Among the fungi, the yeasts have somewhat the same type of development as the bacteria. Since bacteria multiply by fission and the yeasts (except *Schizosaccharomyces*) by budding, we cannot expect the growth pattern of yeasts to fit exactly the same formulas which have been developed for bacteria. But, in a general way, yeasts follow closely the phases of growth shown by bacteria. These phases of growth are as follows: (1) *Stationary phase.* When cells are inoculated into a medium, there is a period of time following inoculation when there appears to be no change in number. The stationary phase may be long or short depending upon the age and vigor of the inoculum, the medium, and other factors. (2) *Phase of accelerated growth.* Not until cell division is established and new protoplasm is being formed from the constituents of the medium may growth be considered as begun. This phase is characterized by an increase in the *rate* of cell division, *i.e.*, the generation time is decreasing. (3) *Exponential or logarithmic phase.* This phase is clearly defined for bacteria and approached by yeasts. It is characterized by a constant generation time. If the logarithms of the cell numbers are plotted against time, the curve is a straight line. (4) *Phase of declining acceleration.* As the nutrients become exhausted, or as toxic by-products accumulate, the average generation time increases. A combination of these and other factors results in a lessened rate of growth. If fresh medium were continuously supplied and toxic by-products removed, it is possible that this phase would never be attained. (5) *Maximum stationary phase.* This marks the attainment of maximum weight, or numbers of living cells. It is quite likely that the death of old cells is balanced by new growth. The duration of this phase is dependent upon the organism and upon the composition of the medium at this time. (6) *Phase of decline or autolysis.* Sooner or later, following attainment of maximum development, autolysis sets in. As the cells die, the cellular enzymes begin to digest the various cell constituents. Only the more resistant portions of the cell remain. Microscopic examination at this

time reveals that many cells are devoid of protoplasm. It is quite possible that some of the materials released by autolysis are used by the remaining living cells.

Filamentous fungi. With exception of the third phase of growth discussed above, the filamentous fungi follow the same order of development as the yeasts. The most obvious difference between the filamentous fungi and unicellular organisms is the failure to attain an exponential rate of growth. Usually, the exponential phase is replaced by a more or less linear phase of growth. Emerson (1950) found a straight-line relation between the cube root of the weight of mycelium produced by *Neurospora crassa* grown in nonagitated liquid medium and the time of incubation. This relation held for three surface-volume ratios. A comparison of the linear, logarithmic, and cube-root growth curves indicates that this fungus has a cube-root phase of growth during the interval when the linear graph is concave upward. Growth in the filamentous fungi is limited to the tips of the hyphae. The influence of neighboring cells which compete for nutrients is a much more important factor in the growth of filamentous fungi than in submerged unicellular organisms. In unagitaged cultures a portion of the mycelium is usually aerial at some stage of growth. The aerial mycelium derives its nutrients from the submerged cells, which involves the transport of these substances over some distance.

RATE OF GROWTH

To study growth, it is necessary to consider both the *rate* and *amount* of production of cells formed during incubation. The average rate of growth is obtained by measuring the amount of growth at two intervals of incubation and dividing the difference by the time interval. If the weight of a fungus colony increased from 50 to 98 mg. between the fourth and sixth days of incubation, the average rate of growth is 24 mg. per day, or 1 mg. per hr. In experimental work, measurements of growth should be made sufficiently often during the period of incubation so that a smooth graph (growth curve) can be plotted from the data. The intervals between measurements of growth may be as short as 1 day for a rapidly growing fungus and as long as a week for species which grow slowly. The rate of growth at any time may be determined by finding the slope (tangent) of the curve. The growth rates of fungi differ, as is illustrated in Fig. 2.

Since growth is a process which takes place in time, it can be studied only by making many growth measurements during the period of incubation. Such a study is not complete until the phase of autolysis is attained. Much of the information in the literature is incomplete because growth was measured only at one time. Many of the potentialities of the fungi can be discovered only by prolonged observation.

WAYS OF MEASURING GROWTH

The discussion of phases of growth presupposes methods of measuring growth. In choosing a method of measuring growth, or any other physiological process, the accuracy and type of information desired must be kept in mind. For some purposes the simplest methods are satisfactory; for others the most accurate methods should be chosen.

Visual inspection. The simplest way to measure growth is by inspection and comparison. The value of this method lies in the speed with which growth measurements are made. Elaborate equipment is not

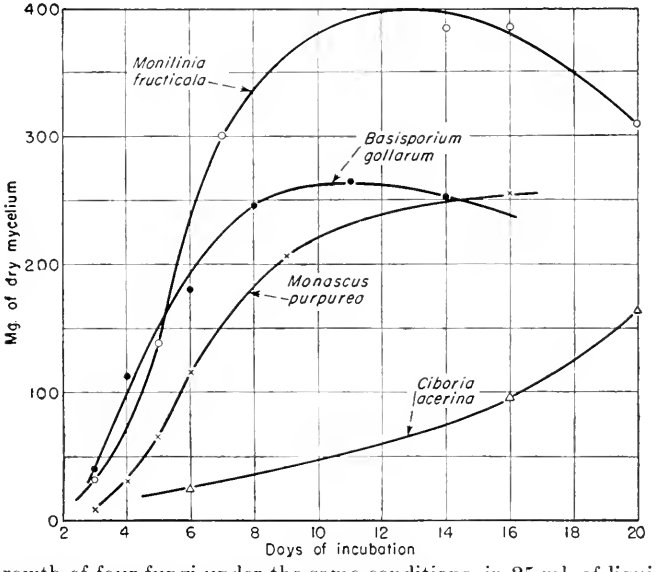


FIG. 2. Growth of four fungi under the same conditions, in 25 ml. of liquid glucose–casein hydrolysate medium at 25°C.

needed, as test tubes and Petri dishes are satisfactory culture vessels. This method has the further advantage that the *same* cultures may be kept under observation. It is frequently the method of choice for preliminary experiments, for the very appearance of the mycelium is a clue to the amount of growth. Growth under varying conditions may be compared if some condition is used as a standard for comparison (see Suggested Laboratory Exercises). It is obvious that a great deal of subjective judgment enters into this method of estimating growth, but it is very useful where fine distinctions are not required.

Linear growth. A second widely used method of measuring growth consists in growing fungi in Petri dishes and measuring either the diameter or the area of the colony. This is a useful method in some instances but

almost useless in others. At least these measurements can be made in an objective way. In this method, the diameter, radius, or area of a colony is used to express the amount of growth, while the daily increase represents the rate of growth. It is obvious that this method neglects the thickness of the colony. Worley (1939) has proposed to take the thickness of the mycelium into account when growth is measured by this method. Such measurements are difficult and neglect the mycelium buried in the agar. The rate of linear growth of some fungi has little relation to the composition of the medium. The rapid extension of mycelium on water-agar medium may serve as a familiar example.

It has been frequently assumed that fungi grow at a constant rate when maintained under constant environmental conditions. This assumption is not necessarily true, for the growth of *Aspergillus rugulosus* and many other fungi is self-limited under cultural conditions. Two factors may contribute to cause nonuniform rates of growth: (1) the change in concentration of nutrients due to diffusion and utilization; (2) the excretion of inhibitory metabolic products into the medium.

The same fungus may have a constant rate of growth at one temperature and not at another. The rate of growth is frequently not constant when fungi are cultured at temperatures higher than optimum. Fawcett (1921) found the rate of growth of *Phytiacystis citrophthora*, *Phytophthora terrestris*, *Phomopsis citri*, and *Diplodia natalensis* to decrease with time when these fungi were cultivated above the optimum temperature. Some of Fawcett's data which illustrate this phenomenon are given in Table 4.

TABLE 4. THE EFFECT OF TEMPERATURE UPON THE RATE OF GROWTH OF THREE FUNGI

The daily increase in the average radius of the colonies is given in millimeters. (From the data of Fawcett, *Univ. Calif. (Berkeley) Pubs. Agr. Sci.* **4**, 1921.)

Days of incubation	*Phytiacystis citrophthora*		*Phytophthora terrestris*		*Phomopsis citri*	
	23.5°C.	31.0°C.	30.0°C.	35.5°C.	27.5°C.	32.0°C.
1	5.4	6.3	5.5	4.8	4.6	0.9
2	10.0	5.5	13.8	4.2	8.0	0.3
3	10.2	3.5	13.3	2.6	8.0	0.2
4	10.5	1.5	13.2	2.5	8.5	0
5	10.5	0.5	10.9	0	8.5	0

If the rate of growth under a given condition does not change with time, this method is useful and simple. It permits observation of the same culture for the duration of the experiment. Ryan *et al.* (1943) have proposed the use of an ingenious growth tube in which linear growth can

be measured with ease and accuracy. This growth tube is illustrated in Fig. 3.

These authors (Ryan *et al.*, 1943) found the rate of linear growth of *Neurospora sitophila* in such a growth tube to be constant for 200 hr. The growth-tube method has been used to study the effect of temperature, pH, vitamin content, and other variables upon *Neurospora*. These special tubes have another advantage over Petri dishes in that cultures are well protected from contamination. The same culture may be exposed to a variety of environmental conditions such as light and temperature. These tubes have the disadvantage that it is more difficult to remove mycelium or fruit bodies for examination. In addition, aeration may be poor and become a limiting factor for some fungi.

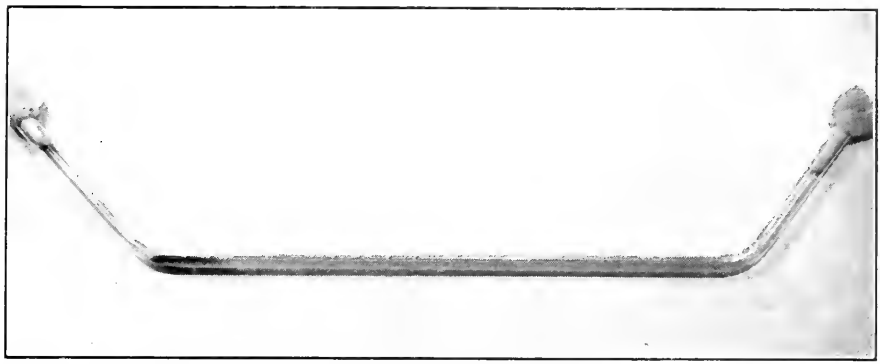


FIG. 3. Growth tube patterned after those described by Ryan, Beadle, and Tatum (*Am. Jour. Botany* **30**: 784–799, 1943) for measuring linear growth.

Dry weight. By weighing the mycelium and spores produced, an accurate and objective measure of growth is obtained. *For precise work it is the method of choice.* Where any significant weight of spores is produced, either Gooch or Alundum crucibles may be used to collect both mycelium and spores. For most purposes the mycelium may be filtered from the culture medium by use of a finely woven cloth and then transferred to weighing bottles or small aluminum cups. The excess medium should be removed by washing and pressing the mycelium, which is then dried to constant weight at 80 to 100°C. After the mycelium is dry, it is weighed on an analytical balance. It is usually sufficient to record the weight to the nearest milligram.

Some fungi make better growth and sporulate more readily on agar than in liquid medium. It is desirable to have an objective measure of growth of agar cultures. Fries (1943) and Day and Hervey (1946) have obtained the dry weight of cultures grown on agar. This technique should be more widely used. The mycelium is freed from agar by briefly autoclaving the cultures, filtering off the mycelial mats, and washing with

hot water. Frequently the mat can be removed from the melted agar with a pair of forceps instead of by filtering. Autoclaving removes some soluble constituents from the mycelium, but if a uniform procedure is adopted, the results are comparable.

Measuring yeast growth. The growth of yeasts may be measured by four methods. (1) Yeast cells may be counted in an aliquot of the medium by the use of a hemocytometer or other counting chamber. The method is tedious. (2) The volume of yeast cells in a given volume of medium may be measured in special graduated centrifuge tubes. Yeast

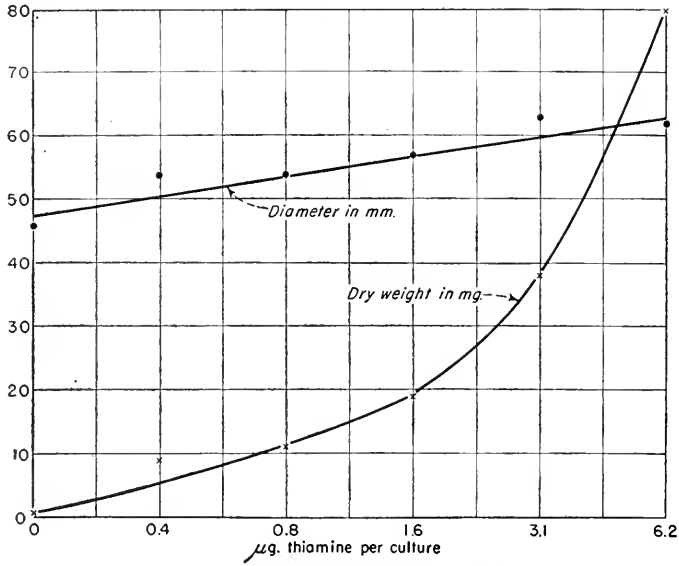


FIG. 4. Direct comparison between diameters and dry weights of the same 10-day-old cultures of *Ceratostomella fimbriata* in the presence of varying amounts of thiamine. Cultures were grown in Petri dishes on 25 ml. of glucose–casein hydrolysate agar at 25°C.

cells are large and easily separated from the medium by centrifuging. This method is less tedious than counting. (3) Turbidity may be used to measure the amount of yeast growth. Accurate determinations by this method require the use of a photoelectric photometer. This method is rapid and sufficiently accurate for many purposes. Lindegren and Raut (1947) have cultivated yeasts in colorimeter tubes and have followed the rate and amount of growth for as long as desired. (4) Yeast cells may be filtered under vacuum, washed, dried, and weighed. Selas porcelain crucibles with fritted bottoms are suitable. This method is accurate but somewhat time-consuming.

Comparison of methods. It should be clearly recognized that one method of measuring growth may not agree with another. This is illus-

trated by Fig. 4, where two methods of measuring the amount of growth of *Ceratostomella fimbriata* were used. This figure demonstrates that the diameter of a colony may be a very poor measure of the amount of growth. Fries (1943) grew *Ophiostoma* (*Ceratostomella*) *ulmi* on agar medium and measured the radii of the colonies and also weighed the mycelium after removing the agar. After 5 days the average radius of cultures without pyridoxine was 16.3 mm., while the average radius of cultures receiving pyridoxine was 12.3 mm; the weights of mycelium produced under these two conditions were 5.2 and 18.1 mg., respectively. It is clear from these examples that different methods of measuring growth do not always give comparable results. Before valid conclusions can be reached, it is necessary to use valid methods of measuring the quantities involved.

METHODS OF PRESENTING RESULTS

The data obtained in a well-planned and carefully executed experiment have value in themselves, but more frequently data are a means to an end. Experimental data form the basis upon which conclusions are reached and serve as a guide to further investigation. A conclusion is sound only if the data are sound. To be of greatest value, data must be presented in an understandable manner. Extensive data may be presented either as tables or graphs; each method has certain advantages.

Tables. The utility and conciseness of tables make them desirable for many purposes. Tables are especially suitable in comparing the amount of growth (or any other function under study) of a number of fungi under standard conditions or under a number of conditions. They give the reader the same basic and fundamental information available to the original investigator. The utility of such information can be appreciated only when one attempts to assess the reports in the literature.

Derived data, such as ratios or percentages, may be needed for the purposes of interpretation and study, and as such they are entirely proper. However, the original data from which the derived data were calculated should always be published. The original data frequently have values which are not perceived or considered by the original investigator. Derived data as such afford no clue as to the original magnitudes. Without the original data no comparison can be made with other experiments, whether in the same or other laboratories. The usefulness of many publications is severely limited because the author presented only ratios or percentages instead of the original data. If a datum represents an average value, the number of determinations upon which it is based should be stated. It is desirable to indicate the range of variation among replicates, or the standard deviation should be given if the number of observations is large.

Graphs. The significance of data is frequently best appreciated when presented in graphical form. A graph reminds one that growth is a continuous function in time, whereas a table may suggest a discontinuous process. Growth curves are especially suited to illustrate the *rate* and *amount* of growth as a function of time. In Fig. 2 the growth curves of four fungi illustrate differences among species. Growth curves are equally applicable to the study of a single species under different conditions. The points representing the data should be given, so that the reader may see how closely the curve fits the data.

Three-dimensional graphs may be used to represent the relations among three variables. Three-dimensional graphs take the form of a surface. Rahn (1939) has given concise directions for constructing such graphs and models. Schopfer (1943) has used such graphs to represent the growth of *Phycomyces blakesleeanus* with respect to the amount of thiamine and asparagine in the medium as a function of time of incubation (Fig. 33).

Another way of showing the relations among the variables involves the use of a triangular graph. Such a presentation is effective if one desires, for example, to show the effect of the concentrations of three constituents of a medium upon growth. For examples of the use of triangular graphs see Haenseler (1921) and Pratt and Hok (1946).

Photographs. The presentation of experimental results is frequently improved by the judicious use of photographs. Photographs are particularly useful in comparing the behavior of fungi under different experimental conditions. The behavior of different species under identical conditions may be effectively compared by the use of photographs. Well-labeled photographs also make excellent permanent records of certain types of experimental results.

FACTORS AFFECTING GROWTH

All the separate factors comprising the internal and external environment may affect either the *rate* or the *amount* of growth, or both. Among the internal factors are the genetic constitution and the internal modifications due to age and to the previous external environment. While more is known about the external factors which affect growth than about the internal factors, it should always be remembered that the external environment acts by modifying the internal environment.

Internal factors. One species differs from another, and even one isolate of a species may differ from another in genetic composition. Many mutations have been produced in the laboratory by the action of X rays, ultraviolet rays, and certain chemicals (see Chaps. 10 and 18). These mutants of a single species produced in the laboratory differ from the parent type in one or more biochemical or morphological characteristics and thus correspond to the different isolates of a species found in nature.

There is no reason to suppose that mutants produced in the laboratory differ fundamentally from those isolated in nature.

The potentialities of a fungus are limited by its genetic constitution. The realization of these potentialities may be denied or favored by the external environment, and only as the environment is suitable do these inherent factors find expression. Diversity, rather than uniformity, in behavior among species and isolates is the rule.

Only a small amount of inoculum is used in most studies. It is important to learn if the *age, history,* or *kind of inoculum* has any effect on the subsequent development of the fungus. All these factors may influence the rate and amount of growth and other functions of the fungi. Young and vigorously growing inoculum is most suitable, since old cells as a general rule are slow to start growth. Apparently one of the first functions a cell loses is the power of division. From this standpoint such cells are "dead," although they may be still capable of performing many vital functions, such as respiration. Difficulty is frequently experienced in making subcultures from old cultures. Certain species are difficult to maintain in culture unless they are frequently subcultured. In general, these species do not readily form resting cells. Among these are various species of *Pythium* and *Phytophthora,* test-tube cultures of *Choanephora cucurbitarum,* and others.

In experimental work of the highest precision neither the temperature nor the medium upon which the inoculum is grown may be neglected. Zikes (1919) investigated the generation time of six strains of yeast and found that the storage temperature of the inoculum affected the time required for cell division. These original cultures were grown at 8°C. and 25°C., and subcultures were incubated over a range of temperatures. When the inoculum which was grown and stored at 8°C. was subcultured at low temperatures, the generation time was less than that of the culture grown and stored at 25°C. At temperatures above 25°C. the generation time of the high-temperature yeast was less than that of the low-temperature yeast. In some way, yeast cells cultured over long periods of time at a certain temperature become adapted to this temperature, and when such cells are transferred to other temperatures, the influence of the original temperature of incubation persists for a time. It is evident that some change in the internal environment has occurred.

Comparable studies on the filamentous fungi are rare. From Fawcett's data on the rate of linear growth of four citrus pathogens it appears that the same phenomenon takes place with some filamentous fungi. Fawcett grew the inoculum at 20°C., and on subculturing at 7.5°C. the linear rate of growth increased with time, as is shown in Table 5.

Many fungi have latent abilities to synthesize various essential metabolites. In the virtual absence of these compounds in the medium and

after a shorter or longer period of incubation, a fungus may begin to synthesize these essential metabolites, and growth then takes place in a normal way. This is especially true of the yeasts with respect to vitamins. Many fungi lose their pathogenicity when cultured for a long time on laboratory medium. Host passage frequently restores pathogenicity. The indiscriminate use of inoculum from a variety of substrates and of different ages may introduce unexpected variation in experimental work and should be guarded against.

TABLE 5. DAILY INCREASE (IN MILLIMETERS) IN DIAMETER OF COLONIES OF FOUR FUNGI

Inoculum grown at 20°C.; subcultures incubated at 7.5°C. (From the data of Fawcett, *Univ. Calif. (Berkeley) Pubs. Agr. Sci.* 4, 1921.)

Species	1st day	2d day	3d day	4th day	5th day
Phythiacystis citrophthora	0.04	0.4	0.6	0.8	1.2
Phytophthora terrestris	0.02	0.14	0.21	0.7	0.8
Phomopsis citri	0.01	0.16	0.83	0.9	1.0
Diplodia natalensis	0.05	1.9	2.1	—	—

External factors. Among the external factors which influence the growth of fungi, *temperature* plays an extremely important role. Temperature affects almost every function of the fungi. For each fungus there is a temperature below which it will not grow, the minimum temperature. Likewise there is a temperature above which growth ceases, the maximum temperature. These two temperatures indicate the *temperature range* of an organism. A few fungi are capable of growing below 0°C., but for most species the minimum temperature is 0 to 5°C. The maximum temperature varies from 27°C. for *Phacidium infestans* (Pehrson, 1948) and *Sclerotinia camelliae* (Barnett and Lilly, 1948) to 45 or 50°C. for *Aspergillus fumigatus* (Thom and Raper, 1945). The maximum tempera-

TABLE 6. CARDINAL TEMPERATURES FOR VARIOUS FUNGI

Species	Minimum, °C.	Optimum, °C.	Maximum, °C.	Citation
Neurospora sitophila	4	36	44	Ryan *et al.*, 1943
Ceratostomella pilifera	5	25–30	35	Lindgren, 1942
C. ips	5	30	40	Lindgren, 1942
Phacidium infestans	−3	15	27	Pehrson, 1948
Phytophthora infestans	2	18–21	26	Crosier, 1933
P. terrestris	12.0	31.5	36.1	Fawcett, 1921
Various yeasts	0.5	25–30	40	Zikes, 1919

ture is sometimes an important factor limiting the attack of plant pathogens.

The *cardinal temperatures* of a few fungi are given in Table 6. A more extensive compilation is given by Wolf and Wolf (1947). The characteristic effect of different temperatures on the rate of growth of two fungi is shown in Fig. 5. Further examples may be found in the work of Lindgren (1942).

Most reports on the effect of *light* on the fungi have been concerned with reproduction rather than vegetative growth. However, Elfving (1890) found strong diffuse daylight to depress the growth of *Penicillium glaucum* and a species of *Briarea*. The amount of inhibition was least when the culture medium contained complex nutrients such as peptone. Greater inhibition resulted when the media contained glucose, mannitol, and malic acid. Scattered observations indicate that the depressing effect of strong light may be rather common. In the old literature some mention is made of the favorable effect of light on red yeasts. The sporangiophores of *Phycomyces blakesleeanus* attain a greater length in darkness than in intense light. The role of light in the sporulation of some fungi is discussed in Chap. 14.

Conclusive evidence that light affects the amount of growth of *Karlingia* (*Rhizophylctis*) *rosea*, one of the lower Chytridiales, was presented by Haskins and Weston (1950). This fungus when grown in liquid glucose-nitrate medium produced twice the amount of dry weight of cells when cultured in light than when the cultures were kept in total darkness. With the exception of the factor of illumination, the experimental conditions were the same. Approximately twice as much glucose was utilized by cultures exposed to light as those kept in darkness. On the other hand, when *K. rosea* was grown in a liquid cellobiose-nitrate medium, more growth resulted in total darkness than in light. The explanation for this behavior of *K. rosea* is not known.

The *moisture* requirements of fungi differ. Most species in nature live on substrates which are not saturated with water. The low moisture content of a substrate is often a factor which limits the growth of fungi. Particularly is this true of the species which live on wood or in soil. As a general rule, wood which contains less than 20 per cent moisture is immune to fungus decay. A difference of a few per cent in the moisture content may determine whether a species will be able to grow or not. Lindgren (1942) has reported that *Ceratostomella pilifera*, a wood-staining fungus, does not grow in pine wood having a moisture content of 23 per cent but develops in wood containing 24.5 per cent moisture. The maximum rate of penetration was attained on wood having a moisture content of 29 per cent or more. Jute sacking is subject to fungus attack only if the moisture content exceeds 17 per cent.

In physiological studies dealing with high concentrations of nutrients, it is important to distinguish between osmosis and osmotic pressure. *Osmosis* is the transfer of water through a membrane permeable to water

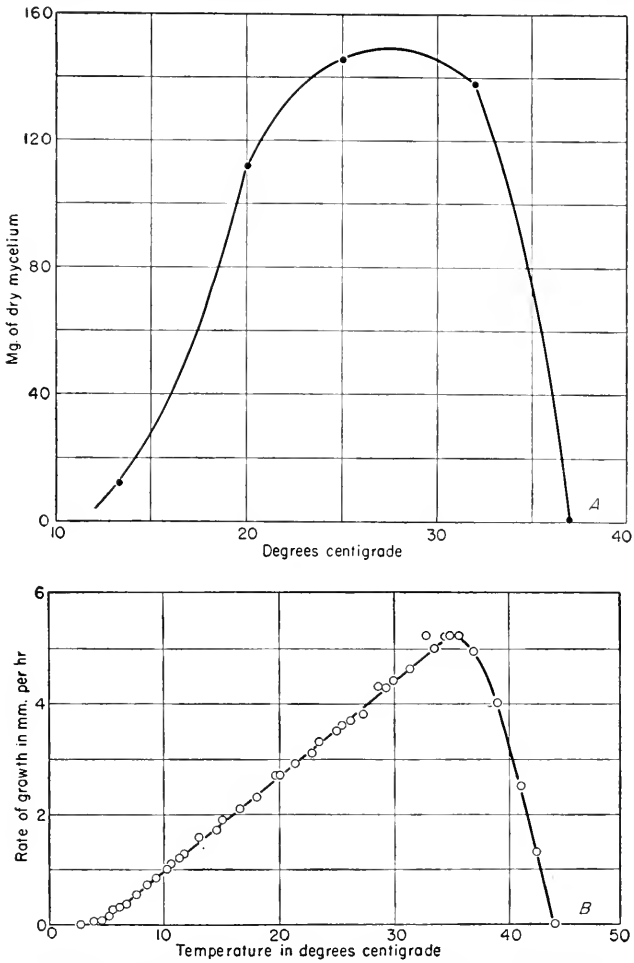


FIG. 5. *A*, the effect of temperature on the dry weight of mycelium produced by *Glomerella cingulata* after 5 days in 25 ml. of liquid glucose-asparagine medium. (Drawn from the data of I. G. Bennett, 1951.) *B*, the effect of temperature on the rate of linear growth of *Neurospora crassa*. (Courtesy of Ryan, Beadle, and Tatum, *Am. Jour. Botany* **30**: 785, 1943.)

but not to the solute molecules. In simple systems water passes from a dilute to a more concentrated solution. *Osmotic pressure* is the force necessary to restrain the movement of water from a dilute to a concentrated solution through a semipermeable membrane. The osmotic pressure which a solution is capable of developing is a function of the number

of ions and molecules of solute contained in a unit volume of solution. A mole of a non-ionized compound in 1,000 g. of water at 0°C. has an osmotic pressure of 22.4 atm. if separated from pure water by a semipermeable membrane. For a fuller discussion of osmosis and osmotic pressure the student is referred to Gortner (1949), Seifriz (1936), and Meyer and Anderson (1948).

If concentration were the sole factor which determines whether growth is possible, all solutions having the same osmotic pressure would be equally inhibitory. Table 7 indicates that this is not true.

TABLE 7. HIGHEST OSMOTIC PRESSURES (ATMOSPHERES) OF SOLUTIONS OF FOUR COMPOUNDS IN WHICH VARIOUS FUNGI GREW
(Hawkins, *Jour. Agr. Research* **7**, 1916.)

Species	Glucose*	Sucrose	Potassium nitrate	Calcium nitrate
Plenodomus destruens	58.3	47.4	54.5	33.6
Diplodia tubericola	63.2	42.1	58.8	33.6
Rhizopus nigricans	63.2	42.1	27.5	15.9
Botrytis cinerea	63.2	47.4	54.5	27.7
Ceratostomella fimbriata	63.2	47.4	54.5	19.5

* Limiting concentrations not used.

These data and others show that the limiting osmotic pressure depends upon the fungus and the compounds used. It is difficult to evaluate the effects of osmotic pressure upon the fungi, for the cell membrane is permeable to other compounds in addition to water. Calculations of osmotic pressure are made by assuming that an indifferent semipermeable membrane separates solutions of different concentrations. The effect of osmotic pressure upon the fungi cannot be considered as a simple physiochemical process. However, the ability of many fungi to grow in solutions having high osmotic pressures is advantageous. Parasitic fungi characteristically have a higher osmotic pressure than the cell sap of the plants they parasitize (Thatcher, 1939). For further references to the effect of osmotic pressure on fungi, see Kroemer and Krumbholz (1931).

Another process involved in the entrance of water into fungus cells is *imbibition*. Gortner (1949) has defined imbibition as the process whereby colloidal substances such as protoplasm take up water, and *imbibition pressure* as the pressure against which a colloid will imbibe liquid. Raciborski (1905) grew a species of *Torula* in saturated lithium chloride (1,000 atm.) and *Aspergillus glaucus* in a saturated sodium chloride solution.

Aside from osmotic effects, the *concentration of the medium* has a great effect on the rate and amount of growth of fungi. The concentration of

nutrients which is most favorable for growth may be poor in other respects, *e.g.*, for reproduction. The concentration may be varied in two ways: (1) by diluting the entire medium, whereby the ratios among the constituents remain unchanged, and (2) by varying the concentration of one constituent. These methods are not equivalent and yield different results.

When an entire medium is diluted, it might be expected that the decrease in amount of mycelium produced would be directly proportional to the amount of dilution. Such is not always the case. When *Chaetomium convolutum* was grown in full-strength medium and in medium diluted to one-fourth and one-sixteenth full strength, the maximum weights of mycelium produced were 220, 75, and 22 mg., respectively (Lilly and Barnett, 1949). *C. convolutum* grew most efficiently in the most dilute medium. This principle appears to be generally valid and is also illustrated by *Ceratostomella fimbriata* (Table 57).

Table 8. The Effect of Different Volumes of Medium upon the Rate and Maximum Amount of Growth of *Sordaria fimicola*
Dry weight of mycelium in milligrams.

Days of incubation	Ml. medium per 250-ml. Erlenmeyer flask			
	6.25	12.5	25.0	50.0
3	47	80	63	22
4	75	99	129	99
5	71	113	166	160
6	65	100	156	238
9	57	107	168	269

When the concentration of *one* constituent in the medium is changed, over a certain range, the amount of growth will be proportional to the concentration. Above a certain concentration there will be no further increase in the amount of growth. This is due to the limiting concentration of some other constituent in the medium. This is the principle upon which fungi are used in vitamin and other assays (Chap. 10).

The maximum weight of mycelium which is obtained from a given volume of medium depends upon the *type and size of the culture vessels* used. The rate of growth is also affected. These results appear to be due mainly to differences in aeration, and perhaps to a lesser degree to diffusion. The effect of *depth* of medium on rate and amount of growth in nonagitated cultures may be demonstrated by using a constant volume of medium in different-sized flasks, or by varying the volume of medium in flasks of the same size. Data illustrating this latter condition are presented in Table 8. The slow initial rate of growth when the mycelium is

entirely submerged is due to lack of an adequate supply of oxygen. The efficiency of *Sordaria fimicola* in converting the constituents of the medium into mycelium decreased as the depth of the medium increased. This fungus was less than half as efficient when grown in 50 ml. of medium as when grown in 6.25 ml.

EFFECT OF EXTERNAL FACTORS ON MORPHOLOGY

While the study of morphology, as such, is not within the province of physiology, there is a close connection between these two aspects of mycology. Form and function are the two ways in which the potentialities of organisms come to expression. The morphology of a fungus may be modified by environmental factors to such a degree as to be unrecognizable. These changes in morphology may be microscopic as well as grossly visible.

Pasteur (1879) noted that species of *Mucor*, when grown submerged in liquid and in the absence of air, assumed a yeast-like form. Not only did they resemble yeasts, but under these conditions they fermented sugar to alcohol. Under aerobic conditions no detectable amounts of alcohol were formed. Reproductions of Pasteur's drawings have been published by Foster (1949).

When yeasts are cultured in liquid media and allowed to age undisturbed, a film or membrane frequently covers the surface of the liquid. Film formation frequently starts as a ring of cells on the wall of the flask at the air-liquid interface. The morphology of the yeast cells in such films is unusual in that the cells are joined together in filaments. The supply of oxygen must play an important role in the formation of filaments. The temperature range within which film formation occurs varies with the species of yeast and is usually considerably less than the temperature range for growth. Most species of yeasts forms films only between 6 and 30°C., although Zikes (1919) found *Monilia candida* and *Mycoderma cerevisiae* to form films at 37°C. The early literature on this subject has been summarized by La Far (1911).

Nickerson and Van Rij (1949) have reviewed the mechanisms of filament formation in yeast and conclude that the processes of cell elongation and cell division are controlled by different enzyme systems. Apparently, the sulfhydryl enzymes which regulate the process of cell division may be inhibited without greatly interfering with cell elongation. Among the agents which inhibit cell division are cobalt, iodoacetate, and penicillin. The effect of penicillin on *Saccharomyces cerevisiae* is shown in Fig. 6. Camphor and other narcotizing agents produce somewhat the same changes in morphology of yeast cells (Levan, 1947).

Many pathogenic fungi which cause disease in man are dimorphic. These fungi are usually yeast-like in the host but frequently form myce-

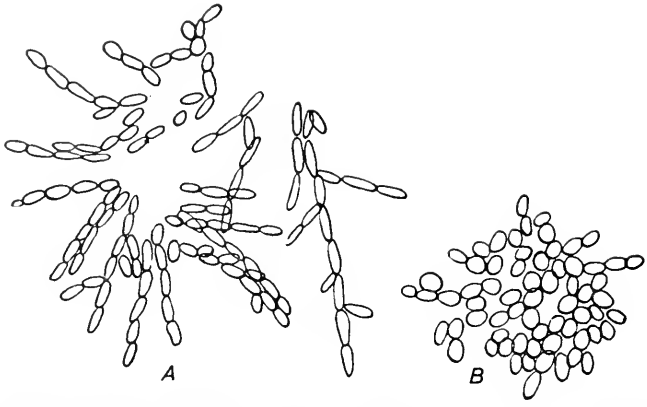
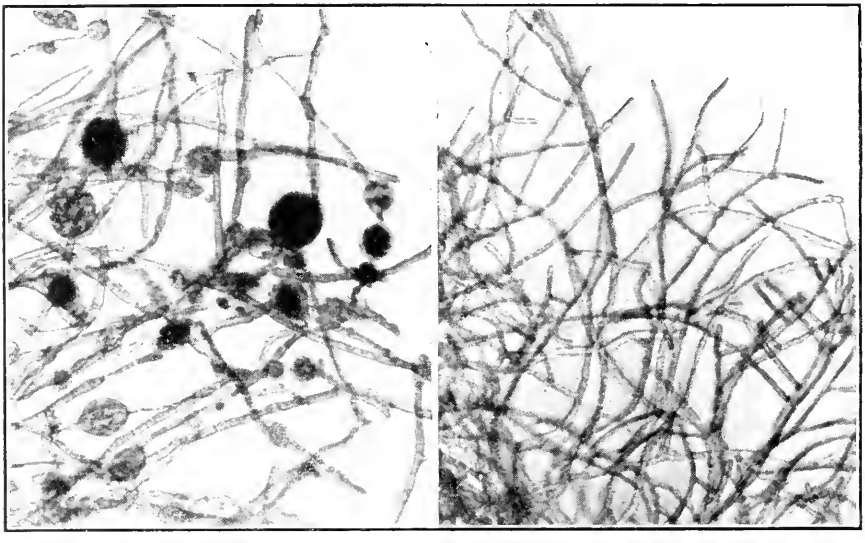


FIG. 6. *Saccharomyces cerevisiae*, camera lucida drawings of cells from agar cultures. *A*, culture treated with penicillin; *B*, culture treated with penicillin plus cysteine. (Courtesy of Nickerson and Van Rij, *Biochim. et Biophys. Acta* **3**: 461–475, 1949. Published by permission of Elsevier Book Company, Inc.)



A *B*

FIG. 7. The effect of hydrogen-ion concentration on the morphology of cells of *Sordaria fimicola*. *A*, rounded swollen cells produced in glucose–casein hydrolysate medium at initial pH 3.6. *B*, normal mycelium from the same culture a few days after a drop of NaOH was added.

lium in culture. *Blastomyces dermatitidis* and *B. brasiliensis* exhibit thermal dimorphism (Nickerson and Edwards, 1949). When these fungi are cultured on certain media at 37°C., they are yeast-like, while at lower temperatures of incubation they form mycelium. This change in morphology is accompanied by changes in the rate of respiration and type of

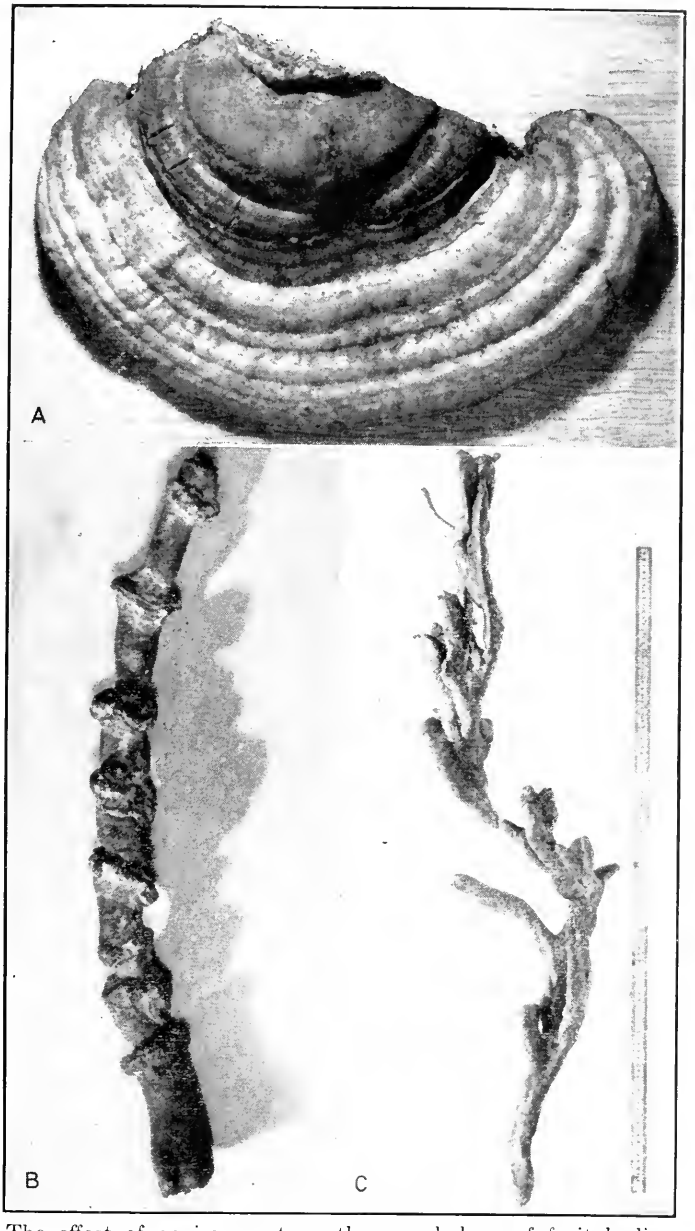


FIG. 8. The effect of environment on the morphology of fruit bodies of *Fomes applanatus*. *A*, normal fruit body developed in nature; *B*, *C*, malformed fruit bodies of the same (?) fungus developed under water in abandoned coal mines. The "nodes" in *B* are believed to be caused by different water levels.

metabolism. Chemical agents may favor or prevent similar morphological changes. *Trichophyton rubrum* produces two metabolic products of unknown constitution which inhibit the transformation of *Candida albicans* to the mycelial form (Jillson and Nickerson, 1948). The addition of excessive amounts of inositol to the culture medium causes *Ophiostoma* (*Ceratostomella*) *multiannulatum* to grow almost entirely in the form of conidia (Fries, 1949). The morphology of the vegetative mycelium and sporangia of various species of *Phytophthora* was found to depend upon the medium used (Leonian, 1925).

The form of mycelial growth of many species, when grown on agar media, is an aid in identification. The colony form may be altered beyond recognition when cultures are grown in agitated liquid medium. In general, spherical colonies or balls form in agitated medium. Burkholder and Sinnott (1945) investigated colony form of a large number of species when subjected to agitation.

The acidity of the medium affects the size and shape of the vegetative cells of some fungi. In a medium so acid as to allow only very slow growth the cells often become swollen or nearly spherical in shape, much like chlamydospores, but the wall remains thin (Fig. 7). This may be accompanied by excessive branching.

Unusual environmental conditions often affect the morphology of both vegetative and reproductive structures. The environment which exists in coal mines is unnaturally uniform with respect to temperature, moisture, and absence of light. Basidiomycetes growing on old mine timbers either fail to fruit or produce odd-shaped sterile fruit bodies (Fig. 8).

SUMMARY

Normal growth results in an increase in cell number and mass. Limited growth may result from either of these two processes alone. Growth is a phenomenon which requires time for its various manifestations. Growth follows a pattern which differs from species to species, but the general sequence of phases is much the same for all fungi. Growth studies are based upon measuring both the *amount* and the *rate* of growth. The rate and amount of growth are controlled by the internal and external environment. The potentialities of a fungus are limited by its genetic constitution, but the expression of these potentialities is controlled by external factors such as temperature, light, composition, and concentration of the medium. Even the size and shape of the culture vessels used affect the rate and amount of growth.

The amount of growth can be estimated by visual comparison or measured by determining the diameter of a colony or by harvesting the mycelium and weighing it after drying to constant weight. The amount of yeast growth may be measured by counting the numbers of cells produced,

by centrifuging and measuring the volume of cells, by turbidity, or by weighing. The most direct way of measuring growth of either yeast or filamentous fungi is by weighing the crop produced. The various methods of measuring growth are not strictly comparable.

The morphology of a fungus may be changed by environmental factors so that it becomes unrecognizable. The processes of cell elongation and cell division are controlled by different enzyme systems. In some instances it has been possible to inhibit cell division without interrupting cell elongation. Frequently a change in physiology accompanies a change in morphology.

REFERENCES

★Barnett, H. L., and V. G. Lilly: The interrelated effects of vitamins, temperature, and pH upon vegetative growth of *Sclerotinia camelliae*, *Am. Jour. Botany* **35**: 297–302, 1948.

Bennett, I. G.: Thesis, West Virginia University, 1951.

Buchanan, R. E., and E. I. Fulmer: Physiology and Biochemistry of Bacteria, Vol. I, The Williams & Wilkins Company, Baltimore, 1928.

Burkholder, P. R., and E. W. Sinnott: Morphogenesis of fungus colonies in submerged shaken culture, *Am. Jour. Botany* **32**: 424–431, 1945.

Crosier, W.: Studies in the biology of *Phytophthora infestans* (Mont.) de Bary, *Cornell Univ. Agr. Expt. Sta. Mem.* 155, 1933.

Day, D., and A. Hervey: *Phycomyces* in the assay of thiamine in agar, *Plant Physiol.* **21**: 233–236, 1946.

Elfving, F.: Studien über die Einwirkung des Lichtes auf die Pilze, Helsingfors Central-Druckerei, Helsingfors, 1890.

Emerson, S.: The growth phase in *Neurospora* corresponding to the logarithmic phase in unicellular organisms, *Jour. Bact.* **60**: 221–223, 1950.

★Fawcett, H. S.: The temperature relations of growth in certain parasitic fungi, *Univ. Calif. (Berkeley) Pubs. Agr. Sci.* **4**: 183–232, 1921.

Foster, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

Fries, N.: Die Einwirkung von Adermin, Aneurin und Biotin auf das Wachstum einiger Ascomyceten. *Symbolae Botan. Upsalienses* **7**(2): 1–73, 1943.

Fries, N.: *Ophiostoma multiannulatum* (Hedgc. and Davids) as a test object for the determination of pyridoxin and various nucleotide constituents. *Arkiv für Botanik* **1**: 271–287, 1949.

Gortner, R. A.: Outlines of Biochemistry, 3d ed., John Wiley & Sons, Inc., New York, 1949.

Haenseler, C. M.: The effect of salt proportions and concentrations on the growth of *Aspergillus niger*, *Am. Jour. Botany* **8**: 147–163, 1921.

★Haskins, R. H., and W. H. Weston, Jr.: Studies in the lower Chytridiales. I. Factors affecting pigmentation, growth, and metabolism of a strain of *Karlingia* (*Rhizophlyctis*) *rosea*, *Am. Jour. Botany* 37: 739–750, 1950.

Hawkins, L. A.: Growth of parasitic fungi in concentrated solutions, *Jour. Agr. Research* **7**: 255–260, 1916.

Jillson, O. F., and W. J. Nickerson: Mutual antagonism between pathogenic fungi. Inhibition of dimorphism in *Candida albicans*, *Mycologia* **40**: 369–385, 1948.

Kroemer, K., and G. Krumbholz: Untersuchung über Osmophile Sprosspilze. I. Mitteilung, Beiträge zur Kenntniss der Gärungsvorgänge und der Gärungserreger der Trockenbeerenauslesen, *Arch. Mikrobiol.* **2**: 352–410, 1931.

La Far, F.: Technical Mycology. Vol. II, Eumycetic Fermentation (trans. C. T. C. Salter), Chas. Griffin & Co., Ltd., London, 1911.

Leonian, L. H.: Physiological studies on the genus *Phytophthora*, *Am. Jour. Botany* **12**: 444–498, 1925.

Levan, A.: Studies on the camphor reaction of yeast, *Hereditas* **33**: 457–514, 1947.

Lilly, V. G., and H. L. Barnett: The influence of concentrations of nutrients, thiamin, and biotin upon growth and formation of perithecia and ascospores by *Chaetomium convolutum*, *Mycologia* **41**: 186–196, 1949.

Lindegren, C. C., and C. Raut: The effect of the medium on apparent vitamin-synthesizing deficiencies of microorganisms. A direct relationship between pantothenate concentration and the time required to induce the production of pantothenate-synthesizing "mutants" in yeasts, *Ann. Missouri Botan. Garden* **34**: 75–90, 1947.

Lindgren, R. M.: Temperature, moisture, and penetration studies of wood-staining Ceratostomellae in relation to their control, *U.S. Dept. Agr. Tech. Bull.* 807, 1942.

Meyer, B. S., and D. B. Anderson: Plant Physiology, D. Van Nostrand Company, Inc., New York, 1948.

Nickerson, W. J., and G. A. Edwards: Studies on the physiological bases of morphogenesis. I. The respiratory metabolism of dimorphic pathogenic fungi, *Jour. Gen. Physiol.* **33**: 41–55, 1949.

Nickerson, W. J., and N. J. W. van Rij: The effect of sulfhydryl compounds, penicillin, and cobalt on the cell division mechanism of yeasts, *Biochim. et Biophys. Acta* **3**: 461–475, 1949.

★Pasteur, L.: Studies on Fermentation. The Diseases of Beer, Their Causes, and the Means of Preventing Them (trans. from Etudes sur la bière by F. Faulkner and D. C. Robb.), Macmillan & Co., Ltd., London, 1879.

Pehrson, S. O.: Studies of the growth physiology of *Phacidium infestans* Karst., *Physiologia Plantarum.* **1**: 38–56, 1948.

Pratt, R., and K. A. Hok: Influence of the proportions of KH_2PO_4, $MgSO_4$, and $NaNO_3$ in the nutrient solution on the production of penicillin in submerged cultures, *Am. Jour. Botany* **33**: 149–156, 1946.

Raciborski, M.: Ueber die obere Grenze des osmotischen Druckes der lebenden Zelle, *Bull. internat. acad. sci. Cracovie, Cl. sci. math. et nat.* **7**: 461–471, 1905.

Rahn, O.: Physiology of Bacteria, The Blakiston Company, Philadelphia, 1932.

Rahn, O.: Mathematics in Bacteriology, Burgess Publishing Co., Minneapolis, 1939.

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

Schopfer, W. H.: Plants and Vitamins, Chronica Botanica Co., Waltham, 1943.

Seifriz, W.: Protoplasm, McGraw-Hill Book Company, Inc., New York, 1936.

★Thatcher, F. S.: Osmotic and permeability relations in the nutrition of fungus parasites, *Am. Jour. Botany* **26**: 449–458, 1939.

Thom, C., and K. B. Raper: A Manual of the Aspergilli, The Williams & Wilkins Company, Baltimore, 1945.

Wolf, F. A., and F. T. Wolf: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

Worley, C. L.: Interpretation of comparative growths of fungal colonies on different solid substrata, *Plant Physiol.* **14**: 589–593, 1939.

Zikes, H.: Ueber den Einfluss der Temperature auf verschiedene Funktionen der Hefe, *Cent. Bakt.*, Abt. II, **49**: 353–373, 1919.

CHAPTER 4

ENZYMES AND ENZYME ACTION

The fungi, in common with other living organisms, possess tools or reagents far more specific, more delicate, and more powerful than those available in the laboratory. The most complex natural substances such as proteins, polysaccharides, and lipoids are degraded into simpler compounds which are soluble in water. Fungi also synthesize similar complex compounds from relatively simple molecules. These transformations are carried out under such mild conditions of temperature and pressure and in such low concentrations of acid and alkali that it is certain the means used are of a peculiar kind. For in the absence of these special agents formed by the living organisms, these reactions do not take place or do so at a very slow rate. These organic catalysts produced by living organisms are called *enzymes*. The life processes of organisms are controlled and directed by a complicated and interrelated series of enzymes or enzyme systems (Dixon, 1949).

Some enzymes formed by fungi are excreted and normally perform their functions outside the cells that produce them. These are termed *exoenzymes* (extracellular enzymes), such as cellulase, amylase, and pectinase. Exoenzymes perform the functions of digestion; *i.e.*, the degradation of complex food materials into low-molecular-weight compounds which are able to enter the cell. After entering the cell, these metabolites are acted upon by the enzymes within the cell. These enzymes are called *endoenzymes* (intracellular enzymes).

Naturally enough, exoenzymes were recognized and studied first. In the early literature, these exoenzymes were called unorganized ferments because of their solubility. In contrast to these unorganized ferments it was recognized that other ferments (enzymes) occurred in an insoluble organized form. These were called organized ferments. Pasteur (1875) still spoke of yeast as "ferment alcoolique ordinaire du vin." Thus, the name organized ferment took on a dual meaning, that of a living organism and the various chemical reactions caused by these organisms. In 1878 Kühne suggested that the word enzyme be used to replace the terms organized and unorganized ferments. Enzyme is derived from the Greek phrase, *en zymē*, which means in yeast or leaven. For excellent summaries of the historical development of the relation between fermentation

and the action of microorganisms, see Stephenson (1939) and Harden (1932).

It was not until late in the nineteenth century that Buchner (1897) succeeded in releasing certain enzymes from yeast cells and demonstrating that the endoenzyme(s) in yeast causing fermentation was also active entirely apart from the living yeast cells. While yeast juice prepared according to the method of Buchner contained a variety of enzymes, it contained fermentative enzymes never before obtained apart from the living cell. These enzymes cleaved sugar into alcohol and carbon dioxide. This was truly a monumental step in the science of enzymes, for it afforded a way of studying "life" processes apart from the terrible complexity of the living organism. The study of isolated enzyme systems has led to important advances in our knowledge and understanding of life processes; yet the student should be reminded that life is more complex than its parts. Leibowitz and Hestrin (1945) say:

> . . . it has become clear that the risk involved in translating results from lifeless to living systems is a two-way one: not only may mechanisms which operate *in vivo* be absent *in vitro;* mechanisms may be present *in vitro* and yet not necessarily function *in vivo.* In fermentative physiology, as in biology generally, *selective* and *restrictive activity* by the living organism must always be taken into account.

The rate of many chemical reactions is changed by the presence of traces of substances which do not appear to enter into permanent chemical combination with the reactants and which appear unchanged when the reaction has come to equilibrium. Substances which alter the rates of chemical reactions are called *catalysts,* and the process *catalysis.* Enzymes are catalysts of a very special kind, and many of them catalyze but a single reaction. For example, lactose reacts with water to form glucose and galactose. Unless a catalyst is present, this reaction occurs at a very slow rate. Even at 100°C. a long time is required for an appreciable amount of lactose to react with water. If, however, some acid is added to the lactose solution, the rate of the reaction is greatly increased, varying in degree with the amount and kind of acid used. This same reaction is catalyzed when the enzyme, lactase, produced by some yeasts and certain other fungi, is added to a solution of lactose. In general, enzymes are *specific* catalysts. There is no stoichometric relation between the amount of catalyst (acid or enzyme) and the amount of substrate decomposed. Within limits, the amount of substrate decomposed per unit of time is dependent upon the amount of catalyst present.

For a given set of conditions there is a position of equilibrium where the rate of reaction of the reactants is equal and opposite to the rate of combination of the products. The position of equilibrium is not changed by

the presence of a catalyst. The same catalyst will effect synthesis as well as decomposition; the position of equilibrium as well as the relative concentrations of reactants and products determines which reaction predominates. It is possible to choose conditions, in some instances, so the equilibrium conditions favor synthesis. Bourquelot (1915) demonstrated α-methylglucoside was readily formed from methyl alcohol and glucose in the presence of yeast juice.

CLASSIFICATION OF ENZYMES

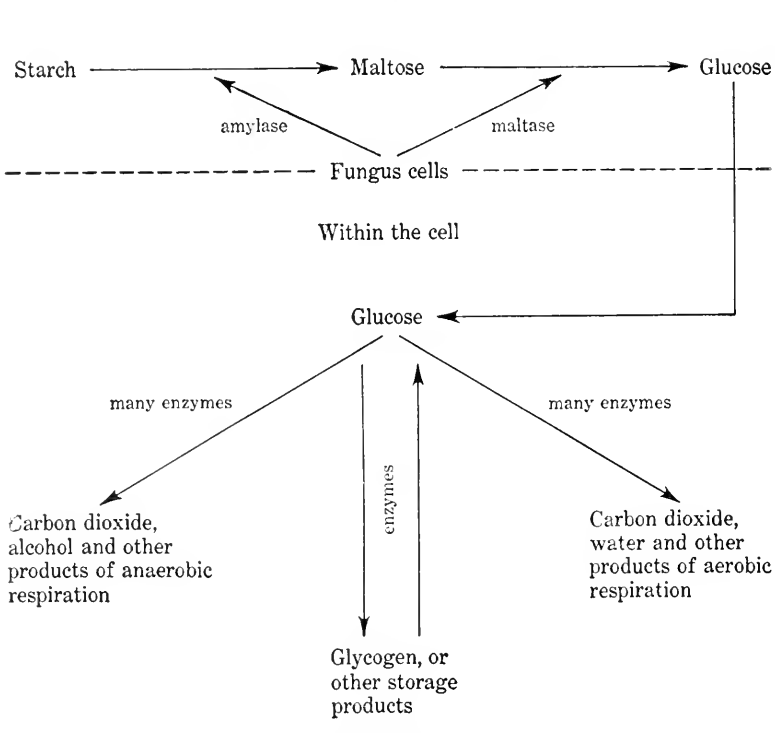
It is more important to classify enzymes upon the basis of function rather than the site of action (endo- and exoenzymes). Many enzymes catalyze reactions in which water is either a product (synthesis) or a reactant (degradation). These enzymes are called *hydrolases.* These reactions usually involve only moderate energy changes. Another class of enzymes, usually intracellular, catalyze oxidation and reduction reactions and reactions involving the scission (or formation) of carbon-to-carbon linkages. These enzymes are known as *desmolyzing* enzymes and include *oxidases, dehydrogenases,* and *desmolases.* Energy changes involved in these reactions are usually large. For more detailed classifications of enzymes see Gortner (1949) and Sumner and Somers (1947).

Since an enzyme acts upon a restricted number of compounds, it is convenient to name enzymes with reference to the substrate acted upon. In general, enzymes are named either by adding the suffix *-ase* to the name of the substrate or by replacing the final syllable of the name of the substrate by this suffix. The following examples give the substrate followed by the name of the enzyme: maltose, maltase; lactose, lactase; cellulose, cellulase; starch (amylum), amylase; protein, proteinase; pectin, pectinase. The suffix *-ase* is also used to designate classes of enzymes. Thus, esterases are members of that group of enzymes which catalyze the hydrolysis and synthesis of esters; oxidases are enzymes which activate oxygen, and dehydrogenases are enzymes which activate the hydrogen of various metabolites. An enzyme may have several names. The enzyme which catalyzes the hydrolysis of sucrose is known also as saccharase and invertase. Amylase is also called diastase.

Hydrolases. The hydrolases catalyze a wide variety of reactions in which water is either a reactant or a product. Hydrolysis is generally thought of as a process whereby complex molecules react with water to form simpler substances. Many hydrolases are exoenzymes which function by preparing the substrate for assimilation. Among these the following should be noted: cellulase, amylase, pectinase, various disaccharidases, proteinases, and peptidases. Others are endoenzymes (the same enzymes in some instances), which catalyze the same or similar reactions within the cells. It would be expected that the process of synthesis within the cells

would be of much more common occurrence than outside the cells. In the medium the process of degradation may be expected to go more or less to completion, since the soluble products of the reaction are assimilated by the organism and hence equilibrium is not reached. Within the cell, however, the reverse may be true. Here, the products of hydrolysis may accumulate, a situation which would tend to favor the reverse reaction, or synthesis. Therefore, synthesis within the cell would be expected to occur when a plentiful supply of simple metabolite molecules continue to reach the cell. When few, if any, metabolite molecules are entering the cell, the hydrolysis of reserve materials would take place. These products of hydrolysis within the cell are then used in other metabolic processes until the store of reserve material is exhausted. Some of these functions are illustrated in scheme I.

SCHEME I. GENERAL SCHEME OF STARCH UTILIZATION



Esterases. These enzymes catalyze the hydrolysis of esters, an acid and an alcohol being formed. The most important natural esters are the fats, which are the glycerol esters of the long-chain fatty acids. Enzymes which catalyze the hydrolysis of fats are called lipases. Both exo- and

endolipases are known. Many fungi store fat as reserve material, and presumably the first step in utilization is hydrolysis.

Phosphatases are classified as esterases because of the fact that they catalyze the hydrolysis of esters of phosphoric acid. Phosphorus is an essential element which enters into many metabolic processes and is a constituent of many physiologically important compounds. Many coenzymes are esters of pyrophosphoric acid (thiamine pyrophosphate, and diphosphopyridine nucleotide, DPN), while triphosphoric acid is a constituent of triphosphopyridine nucleotide, TPN. The synthetic capacity of the phosphatases has been rarely demonstrated. Other enzymes, *phosphorylases*, are apparently the catalytic agents active in forming many phosphate esters. In many instances the substrates from which these esters are formed are different from the products of phosphatase hydrolysis.

Carbohydrases. The enzymes which catalyze the hydrolysis of complex carbohydrates, or polysaccharides, are called *carbohydrases*. These enzymes appear to be highly specific; thus each of the common disaccharides requires a different enzyme for hydrolysis. Sucrase is found in many fungi, including the common strains of *Saccharomyces cerevisiae*, although it is apparently absent in *Schizosaccharomyces octosporus*. The enzyme which hydrolyzes maltose to glucose is called maltase. Maltase is very widely distributed among the fungi. The enzyme which catalyzes the hydrolysis of lactose to glucose and galactose is called lactase. While this enzyme is less widely distributed among the fungi than sucrase and maltase, it is produced by many species.

While it is doubtless correct to assume that the more complex and insoluble carbohydrates must be hydrolyzed before utilization, this assumption may, in some instances, be false with regard to the disaccharides. It is possible that some fungi may employ a phosphorylative degradation of the disaccharides rather than hydrolysis. For a critical review of carbohydrate utilization without preliminary hydrolysis, see Hestrin (1948).

In addition to the water-soluble polysaccharides there is a wide variety of water-insoluble high-molecular-weight carbohydrates which are utilized by many fungi as carbon sources. Only two of these complex polysaccharides will be considered here. The empirical formula for cellulose is $(C_6H_{10}O_5)_n$. On complete hydrolysis by acids, glucose is the only product. Less complete hydrolysis produces a disaccharide known as cellobiose. The majority of fungi, according to Norman and Fuller (1942), are able to attack cellulose. The early work is reviewed by Thaysen and Bunker (1927). With respect to the fungi which attack cellulose, a great deal of variation in cellulolytic ability is found (see White *et al.*, 1948). The enzyme which catalyzes the hydrolysis of cellulose is called cellulase.

While starch has the same empirical formula as cellulose, it is more

easily hydrolyzed. Glucose is likewise the end product of hydrolysis. The enzyme (or enzymes) which catalyzes the hydrolysis of starch is called amylase. In general, the end product of enzymatic hydrolysis of starch is maltose and glucose. The various intermediate degradation products are called dextrins.

Starch appears to be composed of two main types of compounds: amylose (20 to 25 per cent) and amylopectin. Amylose appears to consist of long, unbranched molecules containing some 300 glucose residues, whereas amylopectin has a branched structure. There are two types of amylase: β-amylase, which hydrolyzes off two glucose residues at a time to form maltose, and α-amylase, which attacks the 1,4-glucosidic linkages in such a way as to produce starch fragments (dextrins) as the primary products. The dextrins are further hydrolyzed to form maltose and some glucose. The primary function of α-amylase is thus liquefaction; that of the β-amylase is saccharification. The Aspergillus amylases are of the *alpha* type. The student is referred to the excellent reviews of Hopkins (1946) and Myrbäck (1948) for critical summaries of amylase activity. Amylase is widely distributed among the fungi but is not universal.

Pectinase. The pectins are colloidal carbohydrate-like compounds found in fruits and in the middle lamellae of plants. Many fungi produce pectinase, which catalyzes the hydrolysis of pectin. When the pectin is hydrolyzed, the cells fall apart. Harter and Weimer (1921) tested the ability of nine species of *Rhizopus* to produce pectinase in culture but were unable to correlate the pathenogenicity of these species with the amount of pectinase secreted. In fact, some of the pathogenic species (*R. nigricans* and *R. autocarpi*) secreted less pectinase than did two nonpathogenic species (*R. chinensis* and *R. microsporus*).

Pectins were formerly believed to yield a considerable variety of hydrolytic products, including acetic acid, galactose, and arabinose in addition to methyl alcohol and D-galacturonic acid. More recent work indicates that pectins are methylated polymers of D-galacturonic acid (Schneider and Bock, 1937). The chemistry and physiology of the pectins have been reviewed by Bonner (1936).

Proteinases and peptidases. These enzymes, also called proteolytic enzymes, catalyze the hydrolysis (and synthesis) of proteins and peptides. These enzymes have been separated into two groups upon the basis of ability to attack native protein. Those enzymes which act upon intact proteins are called proteinases, while those which attack peptides are called peptidases. It seems that the fundamental difference between these two classes of enzymes lies in the point of attack. The proteinases attack the protein molecule in such a way as to produce various peptides as well as amino acids, while the peptidases act only on the ends of the peptide chains. This is analogous to the action of the two amylases.

The proteolytic enzymes are a very complex group of hydrolases. In view of the complexity of protein structure this is not unexpected. The question of specificity of the proteolytic enzymes has been considered by Bergmann (1942), who emphasizes that the specificity of a given enzyme for a certain substrate may be modified by the presence of a second substrate. Johnson and Berger (1942) have reviewed the enzymatic properties of the peptidases, including those produced by the fungi.

Oxidases, hydrogenases, and desmolases. One of the central problems in metabolic processes is how and by what means oxidation of metabolites to carbon dioxide and water is brought about. Some organisms (bacteria) are inhibited or killed by free oxygen (anaerobes). Others may live either in the presence or absence of free oxygen (facultative anaerobes), while others require free oxygen (aerobes) to carry on their metabolic processes and to maintain life. Thus, one organism may degrade a substrate only partially, and these intermediate oxidation products become substrates for other organisms. In the end complete oxidation takes place. In other instances an organism may first carry out a partial degradation and complete it later. Thus, yeast produces alcohol by fermentation. In the presence of oxygen, alcohol is utilized for the synthesis of cellular constituents and as a source of energy. Many fungi possess two ways of obtaining energy by the degradation of metabolites: an anaerobic (fermentative) and an aerobic (oxidative) pathway. Both may function in the same organism at the same time, although external conditions may favor one process at the expense of the other, or a substance may inhibit one without affecting the other.

Biological oxidations are carried out in two ways: by the removal of hydrogen from, or by the addition of oxygen to, substrates. The name of Wieland is associated with the process of dehydrogenation, and that of Warburg with the second process.

The theory of Wieland stressed the importance of the enzyme systems which activated hydrogen or removed hydrogen from substrate molecules, while Warburg's theory focused attention upon the enzyme systems which activated oxygen and which carried oxygen to the substrates. These two theories might seem irreconcilable, but today they are considered as mutually complementary. Both types of enzymatic oxidation are known for the same organism. For further discussion of this problem the student is referred to Elvehjem and Wilson (1944) and Meyerhof *et al.* (1942). For a classification of the respiratory enzymes see Gortner (1949) and Sumner and Somers (1947). For the electronic mechanism involved in biological oxidation-reduction see Michaelis (1946).

Some representative dehydrogenases and oxidases are aerobic dehydrogenases (xanthine oxidase, and uricase); anaerobic dehydrogenases, (succinic dehydrogenase, glucose dehydrogenase, triose phosphate dehy-

drogenase); oxidases (cytochrome oxidase, tyrosinase, polyphenol oxidase). Succinic acid dehydrogenase oxidizes succinic acid to fumaric acid by the removal of two hydrogens; but this reaction takes place only in the presence of another system (cytochromes) which "carries" the hydrogen to an oxidizing enzyme, which converts the hydrogen to water and regenerates the cytochrome system so that it can transport more hydrogen. In the cell, succinic acid dehydrogenase is said to be cytochrome-linked. In the laboratory, hydrogen carriers other than cytochrome may be used. Various other dehydrogenases are linked to the cytochrome system.

Another oxidase, tyrosinase, is found in many fungi. It is well established that copper is an essential constituent of this enzyme system (Kubowitz, 1937) and may be removed by dialyzing the enzyme against cyanide solutions. The activity which is lost by this treatment is restored by cupric ion, Cu^{++}, but other divalent metals do not replace copper. Various reagents which react with copper, such as cyanide, diethyl dithiocarbamate, salicylaldoxine, and carbon monoxide, inhibit the action of tyrosinase. Among the fungi which produce tyrosinase are the following species (Nelson and Dawson, 1944): *Boletus luridis*, *Russula foetens*, *R. niger*, *Lactarius piperatus*, and *Psalliota campestris*. It is probable that the darkening and coloration of the fruit bodies of these fungi depend upon the activity of tyrosinase.

Pyruvic acid, $CH_3—CO—COOH$, is a key compound in carbohydrate utilization, and perhaps in other metabolic processes as well. The enzyme, carboxylase, catalyzes the decomposition of pyruvic acid in the following way:

$$\underset{\text{Pyruvic acid}}{CH_3—CO—COOH} \xrightarrow{\text{carboxylase}} \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Acetaldehyde}}{CH_3—CHO}$$

The carbon dioxide formed escapes, while the acetaldehyde formed may be either oxidized to acetic acid or reduced to ethyl alcohol. The enzyme which catalyzes the decarboxylation of pyruvic acid to carbon dioxide and acetaldehyde is abundant in yeast and other fungi. This enzyme consists of three moieties, a specific protein, a magnesium ion, and thiamine pyrophosphate.

CHEMICAL NATURE OF ENZYMES

In the past there has been a great deal of controversy over the chemical nature of enzymes. Sumner (1926) was the first to isolate an enzyme (urease) in pure crystalline condition. Since then a dozen or more enzymes have been prepared in pure crystalline form. All the enzymes which have been isolated in pure crystalline condition have proved to be proteins.

Some enzymes are specific proteins requiring neither coenzymes nor metals for activity. These enzymes must contain as an integral part of their structure the specific groups whereby they react with the substrate. Other enzymes consist of two moieties, a specific protein and a specific nonprotein compound which can be detached from the protein. In the process of purifying an enzyme by dialysis the activity may be lost and later restored by adding to the dialyzed material some boiled juice from the tissue under investigation. These specific nonprotein compounds are known as *coenzymes*. Neither the specific protein nor the coenzyme alone functions as the enzyme; both are required for activity. The specific protein is called the *apoenzyme*, while the combination of apoenzyme and coenzyme is called the *holoenzyme*. Still other holoenzymes consist of an apoenzyme, a coenzyme, and a metallic ion. Coenzymes, being nonprotein in nature, have proved to be more easily isolated and studied than the specific protein moieties of enzymes. Coenzymes are a varied group of compounds, some relatively simple in structure and others more complex. The vitamins are known to enter into the structure of some coenzymes, and it is supposed it is through such coenzyme molecules that the vitamins exert their specific effects. The same coenzyme may combine with many specific proteins to form different enzymes.

FACTORS AFFECTING ENZYME ACTIVITY

Some of the factors influencing enzyme activity affect the intact organism as well as isolated enzyme systems. While the situation within the intact organism is more complex, a knowledge of the behavior of isolated systems will be useful in interpreting the behavior of living fungi. The factors which will be discussed are temperature, hydrogen-ion concentration (pH), chemical reagents (activators and inhibitors), and radiation.

Temperature. The rate of many reactions is approximately doubled for each 10°C. increase in temperature. The rate of reactions catalyzed by enzymes also increases with temperature. This increase is not maintained indefinitely, for enzymes are destroyed by temperatures of less than 100°C. Although there are some reports in the literature of the rate of enzymatic reactions being increased as much as fivefold by a 10°C. increase in temperature, for most enzymatic reactions the increase in rate is less than twofold. This increase between two temperatures 10°C. apart is called the *temperature coefficient*, or Q_{10}. Since the increase in rate is not exactly constant, it is desirable to specify the temperatures involved; *e.g.*, Q_{20-30}.

A reaction with a Q_{10} of 2 proceeds sixteen times faster at 40°C. than at 0°C. Or, the transformation of a given amount of substrate which requires 16 hr. at 0°C. will occur within 1 hr. at 40°C. Figure 9 shows the theoretical effect of temperature upon the amount of substrate trans-

formed when Q_{10} is 2, 3, and 4. It was assumed that one unit of substrate was transformed per unit of time at 0°C. For a reaction with a Q_{10} of 2 an increase in temperature from 28 to 30°C. causes as great an increase in the amount of substrate transformed as does the increase from 0 to 10°C. A small increase in temperature in the range 25 to 35°C. has a greater effect on the rate of reaction than a much greater increase in temperature in the lower temperature range.

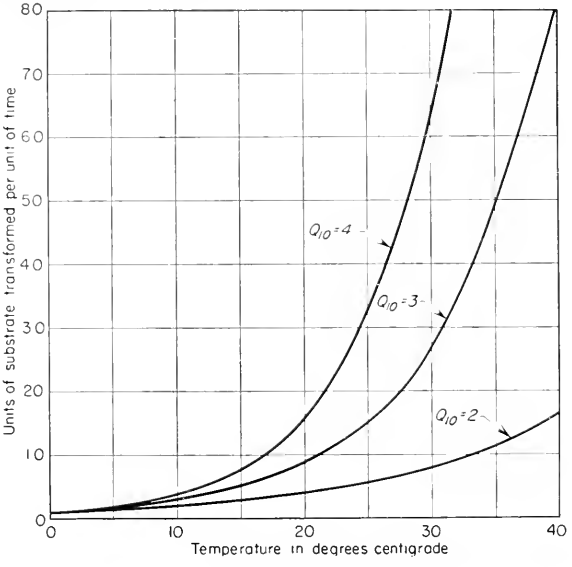


FIG. 9. The theoretical effect of temperature on the rate of enzymatic reactions for different assumed values of Q_{10}.

Enzymes are inactivated by heat. The inactivation may be reversible or irreversible depending upon the enzyme involved, the duration of heating, and other factors. The temperature at which the increased rate of reaction is balanced by destruction of an isolated enzyme is the so-called *optimum temperature* (Bayliss, 1925).

The life processes of a fungus are mediated by a large number of enzymes, which differ in their sensitivity to heat. Fungi cease to grow or reproduce at temperatures lower than that required to kill them. It may be assumed that the enzymes most sensitive to heat are gradually inactivated as the temperature increases. This situation in the living fungus is different from that of an isolated system in that the enzyme is in its natural surroundings and the fungus is able to synthesize or repair the vital enzymes in question. At some temperature we may suppose that the rate of synthesis or repair of the enzyme system is exceeded by the rate of inactivation. When this temperature is reached, or exceeded, the activity of these enzyme systems decrease. This decreased activity is

reflected in a lowered rate of growth or may be seen in other behavior of the fungus. With further increases in temperature, the enzyme systems become less and less operative. So long as the temperature does not exceed the point which produces irreversible inactivation, lowering the temperature will enable the fungus to resume growth or other activity. The temperature of inactivation is not fixed unless the length of exposure is also considered.

The effect of temperature upon growth is shown in Figs. 5 and 39. The portions of the curves in the optimum temperature range represent a balance between inactivation and increased rate of reaction. Above optimum temperature, the rate of growth falls off abruptly. In a general way the rate of growth parallels that expected of enzymatic processes.

Hydrogen-ion concentration. Long ago it was recognized that strong acids and alkalies were destructive to enzymes. A second effect was also recognized: some enzymes exhibited maximum activity only in the presence of weakly acidic or alkaline solutions (see Chap. 8 for a discussion of pH). The effect of pH on the activity of urease is shown in Fig. 25. It should be noted that the pH optimum is dependent upon the concentration of urea.

Haldane (1930) compiled the pH optima of 105 enzymes and found that the range extended from pH 2 to 10. However, all but nine of these enzymes had pH optima between 4 and 8. Most fungi grow between these limits. The effect of the pH of the medium upon the pH of the cell contents is unknown in most instances. Bünning (1936) has reported that the internal pH of the cells of *Aspergillus niger* is influenced by the pH of the medium. The activities of the exoenzymes are affected by the pH of the medium.

Chemical reagents. Some enzymes are inactive or nearly so until they have been treated with certain reagents. A group of the plant proteinases which includes papain and bromelin are activated by hydrogen sulfide and hydrogen cyanide (inhibitors for many enzymes), glutathione, and other thiol compounds. These various activators do not act by removing heavy metals (inactivators for many enzymes) but by reducing the disulfide linkage, —S—S—, to thiol (sulfhydril), —SH. Neutral salts activate some enzymes (emulsin, pancreatic amylase). The mode of activation by neutral salts is unknown. Many of the metallic ions (Mg^{++}, Ca^{++}, Fe^{++}, Cu^{++}, Mn^{++}) are required for enzyme activity, but it seems better to consider them as essential parts of some enzymes rather than activators.

Inhibitors are substances which reduce or destroy enzyme activity. Inhibition may be reversible or irreversible. A few enzyme inhibitors are cyanides, monoiodoacetate, fluoride, and the heavy metals (lead, copper, mercury, silver, etc.). An inhibitor is active against certain enzymes and

not others. There appears to be a close relation between the chemical constitution of the prosthetic group of the enzyme and the inhibitors which inactivate it. We may postulate that inactivation results from a chemical reaction between the inhibitor and the prosthetic group of an enzyme.

One characteristic of an oxidase is inhibition by cyanide and hydrogen sulfide. This points to some common moiety in these enzymes which is

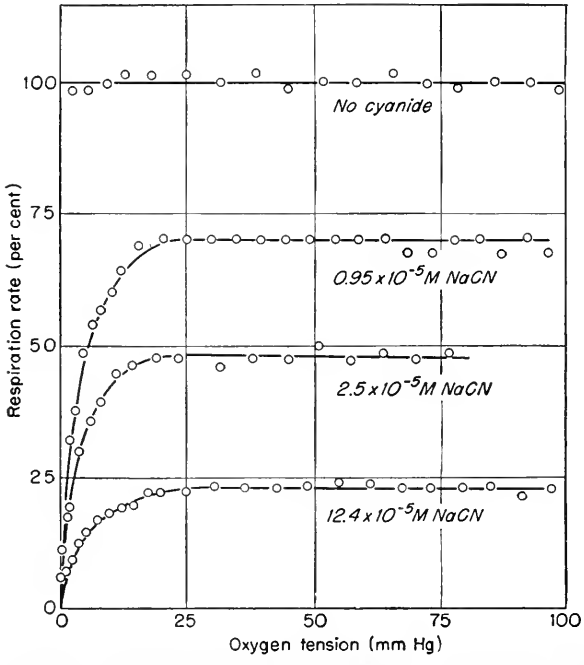


FIG. 10. The effect of cyanide on yeast respiration. (Courtesy of Winzler, *Jour. Cellular Comp. Physiol.* **21**: 238, 1943. Published by permission of Wistar Institute of Anatomy and Biology.)

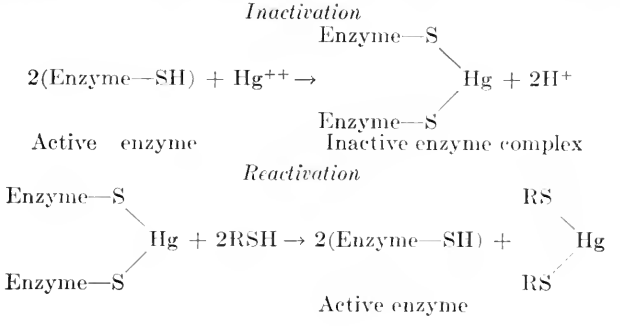
able to react with cyanide. The oxidases are metalloproteins, and in view of the property of cyanides of reacting with metals to form complexes, it would appear likely that cyanide reacts with the metal to form inactive little-ionized compounds. The typical properties of ferrous and ferric ions are masked by cyanide. Tyrosinase, a copper-containing enzyme, is inactivated by cyanide. Hydrogen sulfide acts on many of the same enzymes which are inhibited by cyanide; the action may be assumed to be due to the formation of insoluble metal compounds rather than the formation of non-ionized complexes.

Winzler (1943) studied the effect of different concentrations of cyanide upon the respiration of yeast maintained under different oxygen tensions. The effect of cyanide on yeast respiration is shown in Fig. 10. It may be

noted that the percentages of inhibition of respiration (oxygen uptake) depend upon two conditions, the amount of oxygen available and the concentration of cyanide present. We may assume that the cyanide inhibited one or more respiratory enzymes and that, as the concentration of cyanide increased, more and more of these enzymes were inactivated. When the oxygen tension was reduced, these effects were increased.

While it is known that salts of the heavy metals may denature proteins, and this explanation has been advanced to account for enzyme inactivation by them, recent opinion inclines to the view that the heavy metals inactivate enzymes either by combining with —SH groups, or, under alkaline conditions, by oxidizing thiol sulfur to disulfide. Mercuric ions, especially, may combine with specific metabolites which contain —SH groups (glutathione, thioamino acids), as found by Fildes (1940). Certain metals may inactivate enzymes by replacing the normal metal, rendering the enzyme inoperative. It is noteworthy that many enzymes which are inactivated by heavy metals may be either "protected" or restored to activity by the addition of thiol compounds. We may assume for the purpose of illustration that, when a heavy metal combines with an enzyme, an inactive complex or compound is formed as shown in scheme II. Two factors would influence the effectiveness of thiol compounds in preventing or reversing enzyme inactivation, the relative affinity of the enzyme —SH groups and the thiol compound for mercury, and the relative concentration of enzyme and thiol compound.

SCHEME II. A SCHEME ILLUSTRATING A POSSIBLE MECHANISM OF INACTIVATION OF A SULFHYDRIL ENZYME BY MERCURIC ION AND REACTIVATION OF THE INACTIVE ENZYME-MERCURY COMPLEX BY THE ADDITION OF A THIOL COMPOUND



Radiation. Many reports are to be found in the literature that radiation affects enzymes adversely (see the review of Schomer, 1936). Radiation may affect not only the enzymes of an organism but also the substrates. Ionizing short-wave radiations may cause the formation of hydrogen peroxide from water. Barron *et al.* (1947) were able, by adding

glutathione, to reactivate phosphoglyceral dehydrogenase which had been inactivated by X rays.

Whether radiation is absorbed or not depends upon the chemical constitution of the absorbing molecule and the wave length of the radiation. The energy thus obtained may disrupt the molecule or may merely increase its ability to react. These generalizations are not very helpful in either predicting the effect of light upon living fungi or interpreting the observed effects of light on growth and reproduction. It is probable that light acts on various enzyme systems. Light is known to affect one specific enzyme system (cytochrome–cytochrome oxidase). Warburg (1926) showed that the respiration of baker's yeast was inhibited to the extent of 70 per cent in the *dark* when exposed to carbon monoxide containing 5 per cent oxygen, while respiration was inhibited only 14 per cent in *light*. The same effect of light on carbon monoxide inhibition of respiration has been demonstrated with larvae of *Tenebris molitor* and the heart of embryo trout.

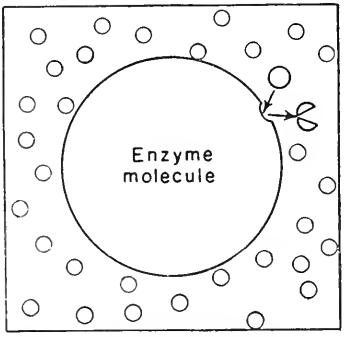


FIG. 11. Diagrammatic illustration of the mechanism of enzymatic hydrolysis. The substrate molecules are represented by small circles, the products of hydrolysis by semicircles. (Courtesy of Van Slyke, *Advances in Enzymol.* **2**: 38, 1942. Published by permission of Interscience Publishers, Inc.)

Ultraviolet radiation and X rays have a lethal effect on fungi. A small percentage of the spores which survive exposure to ultraviolet radiation may produce mutants. It has been noted recently (Kelner, 1949) that the lethal effect of ultraviolet radiation upon spores of *Streptomyces griseus* is overcome to a considerable extent by exposing irradiated spores to visible light. Whether this is due to reactivation of certain enzyme systems is not known.

MECHANISM OF ENZYME ACTION

The most generally accepted theory of enzyme action postulates that the enzyme and substrate unite to form a molecular compound or complex (enzyme-substrate complex). In favorable instances the existence of such enzyme-substrate complexes has been demonstrated (Stern, 1936). During this temporary union the substrate molecule is "strained" or activated so that it undergoes reaction. The products of the reaction have less affinity for the enzyme surface than the substrate molecules and hence diffuse away, and other substrate molecules unite temporarly with the enzyme and the process continues. If the product molecules are present in excess, they may compete more successfully for the enzyme surface than the substrate does. During synthesis, when the reactants

(products) are present in solution in greater than equilibrium concentrations, the reactants combine with the enzyme, unite, and diffuse away. Figure 11 gives a diagram which is helpful in visualizing these processes.

ADAPTIVE ENZYMES

Some fungi produce certain enzymes only in response to particular environmental conditions. Such enzymes are called *adaptive enzymes.* Whether they are produced under all cultural conditions, but in such small amounts as to be undetectable, or whether they are produced *de novo* is questionable. However, this phenomenon is of great importance. Two types of behavior may be noted when a fungus is placed upon unsuitable medium for the first time. Either the fungus may die, owing to lack of ability to synthesize the enzymes to cope with the new environment; or

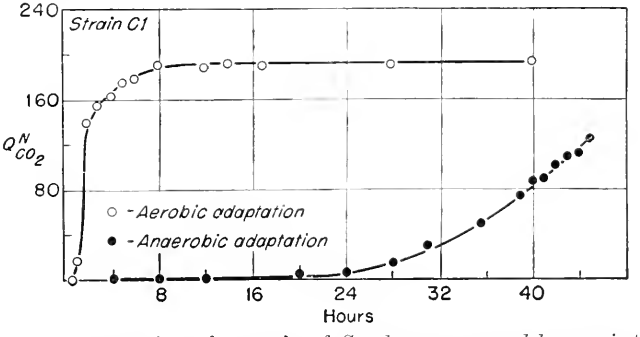


FIG. 12. Rate of adaptation of a strain of *Saccharomyces carlsbergensis* to galactose under aerobic and anaerobic conditions. (Courtesy of Spiegelman, *Jour. Cellular Comp. Physiol.* **25**: 128, 1945. Published by permission of Wistar Institute of Anatomy and Biology.)

after a time it may synthesize the necessary enzymes, and the fungus is then able to grow and function under the new surroundings. Whether or not the fungus is able to synthesize "new" enzymes depends upon its genetic constitution. The biochemical and physiological responses of an organism may change when it is placed on a different kind of medium. These changes ordinarily are called forth by deficiencies in the medium. The substrate upon which the inoculum grew may be very important in governing the various responses of the organism.

Spiegelman (1945) has shown that the adaptation of yeasts to galactose is affected by aerobic and anaerobic conditions. Adaptation is more rapid in air than in nitrogen, and some strains of yeast are unable to adapt to galactose in the absence of oxygen. Figure 12 shows that only some 30 min. is required for *Saccharomyces carlsbergensis* to begin to utilize galactose under aerobic conditions, while about 20 hr. are required under anaerobic conditions.

The effect of composition of the medium on the readaptation of panto-

thenate-dependent strains of yeast to the synthesis of pantothenate has been studied in some detail (Lindegren and Raut, 1947; Lindegren, 1949). Changes to pantothenate independence occurred by an adaptation, which was transmitted vegetatively, and by a gene mutation. The adaptation occurred only in the media of low pantothenate content, while the mutations were apparently not affected by the concentration of pantothenate.

Leonian and Lilly (1943) studied the induced ability of eight strains of *Saccharomyces cerevisiae* to synthesize various vitamins for which they were normally deficient. This was accomplished by long "training" in media "free" from various vitamins. The ability of various yeast strains to synthesize a given vitamin varied. These yeasts which had been trained "reverted" to their deficient status when cultured for 6 months on media containing vitamins and yeast extract.

ENERGY AND ENERGY UTILIZATION BY FUNGI

Fungi need energy, as well as certain elements and chemical compounds, for life, growth, and reproduction. Since the life processes of the fungi are controlled by interlocking systems of enzymes, the utilization of energy is also an enzymatic process. The chemical reactions which accompany or underlie life processes may be divided into those which yield energy (exergonic) and those which require energy (endergonic) (Coryell, 1940). The oxidation reactions whereby such substrate molecules as glucose are converted into carbon dioxide and alcohol or carbon dioxide and water yield energy, while the reactions involved in the synthesis of protoplasm and reserve materials require energy. Let us consider an analogy first. When water falls from a higher to a lower level, there is a decrease in energy content, and this decrease in energy content is the same whether the water has passed through a turbine or not. The water that passes over a spillway does no useful work, while the water that turns a turbine makes part of the energy available (as mechanical or electrical power) for doing useful work. The energy given up by the falling water is the same in both cases, but only where the proper mechanism is available is any useful work obtained.

A similar situation occurs when a fungus oxidizes glucose to water and carbon dioxide. If energy-requiring synthetic reactions are coupled with the degradation reactions, a portion of the available energy becomes useful to the fungus. The remainder of the energy liberated appears as heat, which is unavailable to the fungus for lack of suitable mechanisms to utilize it.

Winzler and Baumberger (1938) have investigated the liberation of energy by yeast cells during metabolism. Washed yeast cells were suspended in a phosphate buffer containing glucose but no nitrogen. The reaction vessel was placed in an adiabatic calorimeter, and the heat

evolved and the amount of oxygen absorbed and of carbon dioxide evolved were measured. In the absence of a nitrogen source the synthesis of protoplasm was avoided. The rate at which heat was evolved was constant until all the glucose was consumed (exogenous respiration), after which the rate of heat formation decreased (endogenous respiration) (see Fig. 13).

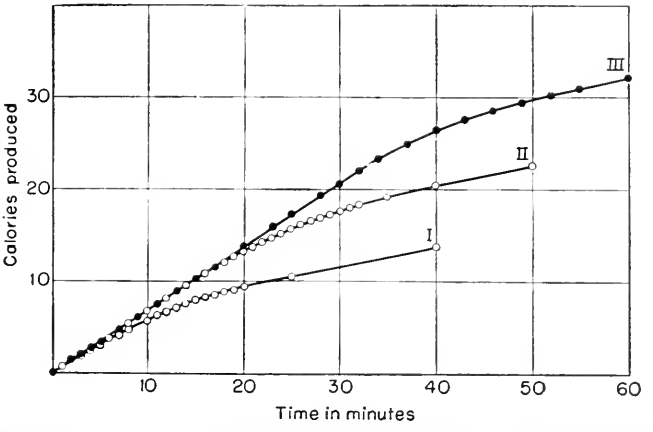


FIG. 13. Heat produced from glucose oxidation by yeast in the absence of a nitrogen source; 10, 20, and 30 mg. of glucose were added at zero time in curves I, II, and III, respectively. In all cases, only 26 per cent of the expected amount of heat was evolved before the endogenous respiration rate was resumed. (Courtesy of Winzler and Baumberger, *Jour. Cellular Comp. Physiol.* **12**: 199, 1938. Published by permission of Wistar Institute of Anatomy and Biology.)

In this experiment the theoretical amount of heat could be calculated for the amounts of glucose used. Only 26 per cent of the theoretical heat was produced before endogenous respiration set in. The volume of oxygen used was equal to the volume of carbon dioxide evolved, *i.e.*, the R.Q. was 1. These data may be interpreted as follows: For every molecule of glucose oxidized to carbon dioxide and water, three molecules were synthesized into a carbohydrate, presumably glycogen. When sodium acetate was the substrate, about 59 per cent of the theoretical heat was evolved, but in the presence of dinitrophenol the theoretical amount of heat was evolved. This inhibitor, therefore, blocked the assimilative mechanism but not the oxidative processes.

Within recent years it has been discovered that certain phosphate esters may play a very important role in energy transfer. The student is referred to the review of Lipmann (1941) for further information on this subject.

The utilization of energy derived from degradation reactions depends upon such energy-yielding reactions being coupled with energy-requiring reactions. Degradation reactions which are not so coupled (blocked)

waste energy in the form of heat which is not utilized by the fungi. The efficiency of utilization depends upon the substrate utilized and upon the nature of the coupled reactions. In any case only a part of the energy available in the substrate does useful chemical work for the fungus utilizing it. The application of these ideas with any rigor requires a sound knowledge of thermodynamics.

SUMMARY

The chemical reactions which underlie the life processes of fungi and other organisms are initiated by organic catalysts, or enzymes. Enzymes catalyze synthetic as well as degradation reactions and are mediators of energy transfer as well.

Enzymes are specific proteins which in some instances require certain metallic ions or organic coenzymes, or both, before they are active. In general, an enzyme controls but a single type of reaction. In living organisms these enzyme-controlled reactions are correlated and integrated to a high degree.

Among the external factors which modify the action of enzymes the following are especially important: temperature, hydrogen-ion concentration, concentration of substrate and products, and inhibitors. The effects of these factors on isolated enzymes and intact organisms are much the same.

While the role of enzymes in maintaining life processes in fungi and other organisms is well established, the application of this information to living fungi must be made with due caution and the realization that a living organism is more complex than its parts.

REFERENCES

Barron, E. S. G., S. Dickman, and T. P. Singer: On the inhibition of enzymes by ionizing radiations, *Fed. Proc.* **6**: 236, 1947.

*Bayliss, W. M.: The Nature of Enzyme Action, 2d ed., Longmans, Roberts and Green, London, 1925.

Bergmann, M.: A classification of proteolytic enzymes, *Advances in Enzymol.* **2**: 49–68, 1942.

Bonner, J.: The chemistry and physiology of the pectins, *Botan. Rev.* **2**: 475–497, 1936.

Bourquelot, E.: La Synthèse biochimique des *d*-glucosides d'alcools monovalents. II. Alcool-*d*-glucosides α, *Ann. chim.*, Ser. IX, **3**: 287–337, 1915.

Buchner, E.: Alcoholische Gärung ohne Hefezellen, *Ber. d. deut. chem. Ges.* **30**: 117–124, 1897.

Bünning, E.: Ueber die Farbstoff- und Nitrataufnahme bei *Aspergillus niger*, *Flora* **131**: 87–112, 1936.

Coryell, C. D.: The proposed terms "exergonic" and "endergonic" for thermodynamics, *Science* **92**: 380, 1940.

Dixon, M.: Multi-enzyme Systems, Cambridge University Press, New York, 1949.

Elvehjem, C. A., and P. W. Wilson (Editors): Respiratory Enzymes, Burgess Publishing Co., Minneapolis, 1944.

Tildes, P.: The mechanism of the anti-bacterial action of mercury, *Brit. Jour. Exptl. Path.* **21**: 67–73, 1940.

Gortner, R. A.: Outlines of Biochemistry, 3d ed., John Wiley & Sons, Inc., New York, 1949.

Haldane, J. B. S.: Enzymes, Longmans, Roberts and Green, London, 1930.

Harden, A.: Alcoholic Fermentation, 4th ed., Longmans, Roberts and Green, London, 1932.

Harter, L. L., and J. L. Weimer: A comparison of the pectinase produced by different species of *Rhizopus*, *Jour. Agr. Research* **22**: 371–377, 1921.

Hestrin, S.: The fermentation of disaccharides. I. Reducing disaccharides and trehalose, *Wallerstein Labs. Communs.* **11**: 193–206, 1948.

Hopkins, R. H.: The action of the amylases, *Advances in Enzymol.* **6**: 389–414, 1946.

Johnson, M. J., and J. Berger: The enzymatic properties of peptidases, *Advances in Enzymol.* **2**: 69–92, 1942.

Kelner, A.: Effect of visible light on the recovery of *Streptomyces griseus* conidia from ultraviolet irradiation injury, *Proc. Natl. Acad. Sci. U.S.* **35**: 73–79, 1949.

Kubowitz, F.: Ueber die chemische Zusammensetzung der Kartoffeloxydase, *Biochem. Zeit.* **292**: 221–229, 1937.

Leibowitz, J., and S. Hestrin: Alcoholic fermentation of the oligosaccharides, *Advances in Enzymol.* **5**: 87–127, 1945.

Leonian, L. H., and V. G. Lilly: Induced autotrophism in yeast, *Jour. Bact.* **45**: 329–339, 1943.

Lindegren, C. C.: The Yeast Cell, Its Genetics and Cytology, Educational Publishers, St. Louis, 1949.

Lindegren, C. C., and C. Raut: A direct relationship between pantothenate concentration and the time required to induce the production of pantothenate-synthesizing "mutants" in yeasts. *Ann. Missouri Botan. Garden* **34**: 85–93, 1947.

Lipmann, F.: Metabolic generation and utilization of phosphate bond energy, *Advances in Enzymol.* **1**: 99–162, 1941.

★Meyerhof, O., *et al.*: Symposium on Respiratory Enzymes, University of Wisconsin Press, Madison, 1942.

Michaelis, L.: Fundamentals of oxidation and reduction *in* Currents in Biochemical Research (edited by D. E. Green), Interscience Publishers, Inc., New York, 1946.

Myrbäck, K.: The structure of starch, *Wallerstein Labs. Communs.* **11**: 209–218, 1948.

Nelson, J. M., and C. R. Dawson: Tyrosinase, *Advances in Enzymol.* **4**: 99–152, 1944.

Norman, A. G., and W. H. Fuller: Cellulose decomposition by microorganisms, *Advances in Enzymol.* **2**: 239–264, 1942.

Pasteur, L.: Etudes sur la vin, Librairie F. Savy, Paris, 1875.

Schneider, G. G., and H. Bock: Ueber die Konstitution der Pektinstoffe, *Ber. d. deut. chem. Ges.* **70**: 1617–1630, 1937.

Schomer, H. A.: The effects of radiation on enzymes *in* Biological Effects of Radiation (edited by B. M. Duggar), McGraw-Hill Book Company, Inc., New York, 1936.

★Spiegelman, S.: The effect of anaerobiosis on adaptation to galactose fermentation by yeast cells, *Jour. Cellular Comp. Physiol.* **25**: 121–131, 1945.

Stephenson, M.: Bacterial Metabolism, 2d ed., Longmans, Roberts and Green, London, 1939.

Stern, K. G.: On the mechanism of enzyme action. A study of the decomposition

of monoethyl hydrogen peroxide by catalase and of an intermediate enzyme-substrate compound, *Jour. Biol. Chem.* **114**: 473–494, 1936.

SUMNER, J. B.: The isolation and crystallization of the enzyme urease, *Jour. Biol. Chem.* **69**: 435–441, 1926.

★SUMNER, J. B., and G. F. SOMERS: Chemistry and Methods of Enzymes, Academic Press, Inc., New York, 1947.

THAYSEN, A. C., and H. J. BUNKER: The Microbiology of Cellulose, Hemicelluloses, Pectins and Gums, Oxford University Press, New York, 1927.

★VAN SLYKE, D. D.: The kinetics of hydrolytic enzymes and their bearing on methods for measuring enzyme activity, *Advances in Enzymol.* **2**: 33–47, 1942.

WARBURG, O.: Ueber die Wirkung des Kohlenoxyds auf den Stoffwechsel der Hefe, *Biochem. Zeit.* **177**: 471–486, 1926.

WHITE, W. L., R. T. DARBY, G. M. STECHERT, and K. SANDERSON: Assay of cellulolytic activity of molds isolated from fabrics and related items exposed in the tropics, *Mycologia* **40**: 34–84, 1948.

WINZLER, R. J.: A comparative study of the effects of cyanide, azide, and carbon monoxide on the respiration of bakers yeast, *Jour. Cellular Comp. Physiol.* **21**: 229–252, 1943.

★WINZLER, R. J., and J. P. BAUMBERGER: The degradation of energy in the metabolism of yeast cells, *Jour. Cellular Comp. Physiol.* **12**: 183–211, 1938.

CHAPTER 5

ESSENTIAL METALLIC ELEMENTS

The fungi need about 17 elements to supply their nutritional requirements. These elements are utilized in the form of specific compounds, as ions, and as free elements. Some of the essential elements are required by all fungi. Other elements are required only by certain species. In a general way, the elements required by the fungi are the same ones required by bacteria, green plants, and animals. There are, however, striking differences in the essential-element requirements of different groups of organisms (Table 15). Differences in ability to utilize specific compounds containing these essential elements are common in the fungi and bacteria.

BIOLOGICALLY ESSENTIAL ELEMENTS

Before seeking to determine which elements are essential, it is necessary to define what is meant by the term *biologically essential element*. An essential element is *indispensable* in that no other element may entirely replace it. Without these essential elements life is impossible. An element needed in extremely small amounts may be just as essential as carbon, which comprises almost half the weight of a fungus.

There are some 92 chemical elements (if we exclude the recently isolated transuranic elements), most of which are known to exist, or may exist, as a mixture of isotopes. So far as is known, all the isotopes of an element (with the possible exception of the isotopes of hydrogen) have the same chemical and biological properties. Even radioactive isotopes, before they decay, exhibit the same biological properties as the stable isotopes. The biological effects of radiation in inducing mutations are considered briefly in Chapter 18. In spite of the limited number of elements, the question of essentiality is not settled completely for all.

The problem of determining which elements are essential for the fungi has been approached from the standpoint of ultimate analysis of mycelium and spores. If certain elements, such as carbon, potassium, and magnesium, are always found in all samples analyzed, irrespective of the substrates upon which these fungi grew, it may be concluded with a high degree of probability that these elements are essential for the fungi. Some of the analytical results of ultimate analyses of mycelium and spores have been collected by Buchanan and Fulmer (1928) and Foster (1949).

Organic materials are dried before analysis. On the average about 75 per cent of the fresh weight of mycelium is water, while spores contain only about 40 per cent water. It is probable that the water driven off when fungus cells are dried to constant weight is in part free water and in part water bound to various colloidal cell constituents.

Ultimate analyses of mycelium and spores always reveal the presence of carbon and nitrogen. On the average about 45 per cent of dry mycelium is carbon. This high content of carbon makes it certain that carbon is an essential element. The percentage of nitrogen found is quite variable. Phosphorus, potassium, magnesium, calcium, sodium, sulfur, and iron are found in the ash that remains after burning mycelium and spores. More refined methods of analysis reveal that fungus ash contains still other elements. Richards and Troutman (1940) investigated the composition of yeast ash by spectrographic analysis and found the following elements: iron, sodium, boron, bismuth, barium, magnesium, manganese, copper, zinc, tin, lead, tellurium, silver, chromium, potassium, gold, and lanthanum. However, the mere presence of an element in fungus cells does not necessarily mean that it is essential.

Since many of these elements in fungus ash occur in minute traces only, it is desirable to approach the problem of essentiality in another way. This is done by omitting from the medium the element in question. Raulin (1869) was apparently the first to use this method. He found that the omission of phosphorus, sulfur, magnesium, zinc, or iron from the basal medium allowed very little growth of *Aspergillus niger*. These elements are thus shown to be essential by the two methods of investigation. In general, the experimental work in which specific elements have been omitted from the medium is more convincing than the method of ultimate analysis. This is the preferred method of testing the essentiality of elements required in small amounts.

Functions of the essential elements. Thatcher (1934) has attempted to classify the essential elements into groups: structural, functional, and those utilized in the transfer of energy. This classification has some validity and may serve to fix attention upon the more salient biological features of an element. However, most, if not all, of the essential elements play many roles in the life processes of the fungi. In general, the nonmetallic elements may be classified as structural elements. This means that the compounds which make up the structural units such as the protoplasm are largely composed of the nonmetallic essential elements: carbon, nitrogen, hydrogen, oxygen, sulfur, and phosphorus. The functional uses of these elements by the fungi are no less important. The essential metallic elements may be classified as functional elements, but this does not mean that these metallic elements have no structural functions.

The elements are in the form of chemical compounds, some of which are relatively simple, while others are complex. With the exception of oxygen the essential elements are usually utilized in the form of compounds or ions. An essential element may exist in a chemical compound and be unavailable. The properties of a chemical compound are determined by all the atoms that compose it and by the way in which atoms are joined together in the compound. It is convenient to consider the essential elements one by one, but this is done only to simplify the approach to a complex subject. These separate factors must be considered in relation to the organism as a whole.

A fungus is no more capable of growth on an iron-free medium than on a carbon- or nitrogen-free medium. Yet, in a balanced medium the ratio of iron to carbon is in the neighborhood of 1 to 50,000. The essential metallic elements function in conjunction with enzyme systems (Chap. 4). This accounts for the small amounts of these elements required. If a vital enzyme system lacks an essential metal ion, it will not function. It appears that in processes such as growth a suboptimal amount of an essential metal will stop growth because the apoenzymes or coenzymes synthesized will lack the necessary activating metal. The ratios as well as the amounts of the various essential metallic ions affect certain metabolic processes other than growth.

The absolute amounts of the essential metallic elements required differ widely. Raulin (1869) found that *Aspergillus niger* required 1 g. of potassium to produce 64 g. of mycelium, while 1 g. of magnesium sufficed for the synthesis of 200 g. of mycelium. Recent work of Steinberg (1946) with *A. niger* indicates still higher yields per gram of these two elements. The yield of mycelium per gram of iron and zinc was in the neighborhood of 55,000 g.

The list of metallic elements known to be essential to fungi has increased over the years. The list now includes potassium, magnesium, iron, zinc, copper, calcium, gallium, manganese, molybdenum, vanadium, and scandium. Others will probably be added as cultural methods become more refined and more species are studied. It is unfortunate that only a few fungi have been investigated thoroughly with respect to mineral nutrition. In stating that the above elements are essential, the reservation must be made that they are essential for some fungi under certain conditions. While it may be assumed that all fungi require the same essential elements, experimental evidence is lacking for most species.

For the purpose of discussion the essential metallic elements will be divided into two groups, macro and micro metallic elements. This grouping is made solely for convenience and on the basis of the amounts ordinarily employed in culturing fungi under laboratory conditions.

THE ESSENTIAL MACRO ELEMENTS

Potassium. This element is essential for all organisms, so far as is known. There is an immense amount of information on the specific effects of potassium on green plants and animals, but such data are not common for the fungi. The quantitative relation between the amount of potassium in the medium and the weight of mycelium produced by *Aspergillus niger* was studied by Steinberg (1946). This work was done with extraordinary care using a highly purified optimal medium (except potassium). The optimum amount of potassium was 150 mg. per liter. The relative amounts of mycelium formed increased as the potassium content of the medium decreased. The fungus produced almost three times as much mycelium per milligram of potassium when 15 instead of 150 mg. per liter were used. Jarvis and Johnson (1950) have reported that *Penicillium chrysogenum* Q176 requires 40 mg. of potassium and 8 mg. of magnesium per liter of medium for optimum growth.

The physiological effects of potassium on fungi have been studied but little. The enzymes in yeast maceration juice which ferment glucose are activated by either potassium or ammonium ions (Muntz, 1947). Molliard (1920) noted that a low potassium content of the medium resulted in increased synthesis of oxalic acid by *A. niger*. The chemical composition of *A. niger* mycelium varies, depending upon the amount of potassium in the medium (Rippel and Behr, 1934).

The problem of *biological substitution* arose early in the study of fungus nutrition. Biological substitution means that one element can replace another, in whole or in part. The possibility of biological substitution was investigated by Steinberg (1946) using *A. niger* as the test fungus. This investigation was made to determine whether the alkali metals (lithium, sodium, rubidium, or cesium) could replace potassium, and whether the alkaline-earth metals (calcium, beryllium, strontium, or barium) could replace magnesium. Under these conditions sodium and beryllium gave increased yield of mycelium in media containing suboptimal amounts of potassium and magnesium. These effects are illustrated in Table 9.

Some increases in weight of mycelium were noted under certain conditions with some of the other metallic ions tested, but the effects of these elements were ascribed to ion antagonism.

Studies of biological substitution require great care and a detailed and extensive knowledge of the composition of the media and of the behavior of the fungus under the experimental conditions used.

Magnesium. This element is one of the alkaline-earth group. It is essential for green plants and animals as well as for fungi and bacteria. *Aspergillus niger* has been more carefully investigated with respect to the

effects of magnesium than any other fungus. Within certain limits of concentration, the amount of growth of *A. niger* is proportional to the concentration of magnesium in the medium. This has been demonstrated by Steinberg (1946), Lavollay and Laborey (1938), and others. The application of this principle to the microbiological assay of magnesium is discussed in Chap. 10. *Penicillium glaucum*, *Botrytis cinerea*, and *Alternaria tenuis* failed to grow in the absence of magnesium (Rabinovitz-Sereni, 1933). Excess magnesium was not harmful to these three fungi until the concentration of magnesium sulfate in the medium reached about 40 per cent. These three species were able to grow in the presence of traces of magnesium but sporulated only when the concentration of magnesium was increased. Respiration also increased as the magnesium content of the medium increased. Failure to sporulate unless sufficient magnesium is available is probably to be expected with many fungi.

TABLE 9. THE EFFECT OF 50 MILLIGRAMS OF SODIUM ON THE AMOUNT OF MYCELIUM PRODUCED BY *Aspergillus niger* IN AN OPTIMAL MEDIUM CONTAINING TWICE THE NORMAL AMOUNTS OF MICRO ELEMENTS WHEN THE CONCENTRATION OF POTASSIUM WAS VARIED

(Steinberg, *Am. Jour. Botany* **33**, 1946.)

Potassium, mg. per liter	Control, mg. mycelium	Sodium added, 50 mg. per liter, mg. mycelium
15	256.3	401.3
30	446.1	783.1
45	641.2	896.7
60	823.4	1,042.0
75	955.2	1,089.0
90	988.0	1,070.0
105	1,065.2	1,093.1
120	1,059.2	1,095.5
135	1,113.9	1,084.9
150	1,145.9	1,146.5

Most of the magnesium in the mycelium of *Aspergillus niger* can be extracted by means of dilute acids (Ripple and Behr, 1930), which indicates that this element does not form stable organic compounds. A relation between the optimum concentrations of magnesium and phosphorus for *A. niger* was discovered by Laborey *et al.* (1941). Some 36 phosphate ions are required for every ion of magnesium. Many enzyme systems are activated by magnesium ion, and in view of the role of phosphate in enzymatic transformations it is not surprising that there should be a close relation between magnesium and phosphate concentrations. Magnesium is involved in many of the enzymatic reactions involved in fermentation

(Sumner and Somers, 1947). It is equally likely that magnesium is involved in aerobic oxidation of carbohydrate. Low concentrations of magnesium in the medium led to increased synthesis of riboflavin by *A. niger* (Lavollay and Laborey, 1938).

One ion may affect the physiological action of another. This is called *ion antagonism*. In nature and in the laboratory fungi come in contact with compounds of both essential and nonessential elements. Many of the nonessential elements are toxic, although toxicity is not limited to the nonessential elements. Copper is an essential element, but it is toxic to most fungi when the concentration exceeds certain limits (Chap. 12). The toxic effect of an ion may be overcome by the presence of one or more other ions in the medium. Gortner (1949) has reviewed this subject from the standpoint of colloidal chemistry and suggests that the relative concentrations of various metallic ions may regulate the process of adsorption.

As an example of ion antagonism Lohrmann (1940) described the toxic action of mercuric chloride and boric acid on *Aspergillus niger, A. flavus, Mucor pusillus, Penicillium glaucum, Fusarium coeruleum, Cunninghamella elegans, Absidia cylindrospora,* and *Rhizopus nigricans.* The inhibition caused by either of these toxic compounds was overcome in part by increasing the concentration of magnesium sulfate. Similarly, the toxic effects of high concentrations of magnesium sulfate were overcome by mercuric chloride. Either mercuric chloride or boric acid in certain concentrations "stimulated" growth in the nutrient solution used. This is not evidence that either boron or mercury is an essential element, but it does show that the nutrient solution used was unbalanced. The effect of sodium and calcium ions upon growth and respiration of *A. niger* depended upon the ratio of these nonessential ions present in the medium. A sodium-calcium ratio of 19 to 1 gave the highest rate of respiration, while a ratio of 4 to 1 was most favorable for growth (Gustafson, 1919). Aluminum inhibits the production of itaconic acid by *A. terreus.* This inhibition is overcome by magnesium sulfate (Lockwood and Reeves, 1945). Nickerson (1946) found the inhibitory effects of zinc ion on the rate of respiration of *Epidermophyton floccosum* to be reversed by calcium or magnesium ions.

The phenomenon of antagonism is not confined to ions. Organic compounds present in media may modify the activity of ions, and organic compounds may antagonize the physiological activity of other organic compounds (Chap. 11). All these possibilities exist. Whether a given ion or compound will be physiologically active depends upon the other constituents of the medium and the metabolic compounds excreted by the fungus under study.

ESSENTIAL MICRO ELEMENTS

These elements have been called heavy-metal nutrients, trace elements, micronutrients, and minor elements. The literature on this subject is extensive and often conflicting. Reviews of this subject are given by Perlman (1949), Foster (1939), and Steinberg (1939). A collection of 10,000 abstracts on the effects of the micro elements on green plants and animals has been published by the Chilean Nitrate Educational Bureau (1948).

In spite of Raulin's (1869) discovery that iron and zinc are essential for *Aspergillus niger*, there arose a school of investigators who considered the micro elements to be *stimulatory* rather than essential. This view is no longer held. There are a number of reasons for this misinterpretation: (1) The failure to realize that the "chemically pure" compounds used in preparing media are grossly contaminated from the biological standpoint and that rigorous purification of media is essential in work of this kind. (2) Distilled water is often a source of metallic ions unless it has been redistilled in Pyrex, or preferably quartz, stills. (3) Many kinds of chemical glassware are sufficiently soluble to furnish the fungi all or a part of the micro elements required. (4) The inoculum, whether mycelium or spores, may introduce sufficient micro elements to obscure the need for these elements. Serial transfer using media free from the element in question and the use of small inocula minimizes this source of error.

Steinberg (1936) has indicated that the optimum concentration of the essential micro metallic elements for *A. niger* ranges from 0.3 mg. of iron to 0.02 mg. of gallium per liter of medium. Lest the reader conclude that these concentrations are so small as to be meaningless, it is revealing to calculate the number of atoms of iron in 0.3 mg. From the atomic weight of iron and Avagadro's number it may be calculated that there are about 3×10^{18} atoms in 0.3 mg. of iron. If the number of cells produced by *A. niger* under these conditions were known, the number of iron atoms available for each cell could be calculated. In lieu of this information we may use data from experiments on the number of yeast cells produced in a liter of medium. Under favorable conditions there are roughly 500 billion yeast cells produced in a liter of medium (Stark *et al.*, 1941). If *A. niger* produces the same number of cells per liter as yeast, there would be available 6.4×10^6 atoms of iron per cell.

The prime essential in investigations dealing with the effects of the essential micro elements is a medium free from the element under study. This ideal is difficult to attain in practice. Equal care is necessary in the choice of culture vessels, for it is wasted effort to remove an element from the medium rigorously and then contaminate it by using glassware which

furnishes the metal. The culture vessels should be of quartz for work of the most exacting kind, although Pyrex or other suitable glassware may be used. It is desirable in any event to use a few quartz culture vessels as controls. In part, the long controversy over the effect of zinc on fungi was due to the liberation of sufficient amounts of this element from certain kinds of glassware used as culture vessels. Javillier (1914) showed that the addition of zinc to cultures of *A. niger* has little effect when Jena glass culture flasks were used. When quartz vessels were used, the crop increased from 291 mg. in the control without added zinc to 1,624 mg. when zinc was added. Steinberg (1919) found essentially the same results except that zinc deficiency could be demonstrated for *A. niger* when Pyrex vessels were used (Table 10).

TABLE 10. THE AVERAGE WEIGHT OF FIVE CULTURES OF *Aspergillus niger* CULTIVATED ON THE SAME BASAL MEDIUM IN THREE MAKES OF GLASSWARE
(Steinberg, *Am. Jour. Botany* **6**, 1919.)

Make of glassware	Mg. mycelium	
	No zinc	Zinc, 10 mg. per liter
Jena	950	987
Kavalier Bohemian	301	943
Pyrex	287	957

Purification of culture media. Progress in the study of essential micro elements depends upon methods of removing them from media. As long as these elements occur in the ingredients of the media, their need may be unnoticed and unsuspected. In 1919 Steinberg devised a useful method of reducing the concentration of heavy metals, especially iron and zinc, in media. In essentials, this method consists in autoclaving the complete medium with 15 g. per liter of calcium carbonate. The hot solution after autoclaving is filtered through paper or a fritted-glass filter, or allowed to cool and the supernatant liquid decanted off. The precipitate must be removed; otherwise the essential elements will be released by the fungi. Calcium oxide and magnesium carbonate may replace calcium carbonate in some applications (Steinberg, 1935*a*). The mode of purification appears to be as follows: During autoclaving, heavy-metal carbonates or their hydroxides are formed. The excess calcium carbonate serves to adsorb these insoluble compounds The composition of a medium is somewhat changed by this treatment, part of the phosphate being removed as calcium phosphate. In practice this is compensated by using an excess of phosphate. A medium which is treated by this process is essentially neutral in reaction, which may lead to some changes in the sugar during autoclaving.

Sugars are frequently highly contaminated with metallic compounds. Steinberg (1937) has reported a sample of glucose to contain the following elements: lithium, sodium, strontium, calcium, rubidium, potassium, manganese, aluminum, iron, rhodium, nickel, silver, copper, magnesium, tin, boron, and silicon. The metallic contamination of non-ionic compounds such as the sugars can be sharply reduced by a variety of mild procedures. Shu and Johnson (1948) give these details for an aluminum hydroxide coprecipitation method: To 140 g. of glucose contained in 500 ml. of solution, 1.25 g. of $Al_2(SO_4)_3 \cdot 18H_2O$ were added. Dilute ammonium hydroxide was added until the pH rose to 9, and the precipitate of $Al(OH)_3$ with the adsorbed impurities was filtered off. This treatment was repeated until the desired degree of purification was attained. Non-ionic substances such as glucose and urea may be purified by treatment with cation-exchange materials operating on the hydrogen cycle. Perlman (1945) used Zeo-karb H (Permutit Corporation) for this purpose. Various ion-exchange materials are used to purify beet juice in the manufacture of sugar. Mulder (1939–1940) found the combination of ammonium sulfide and Norit to be efficient in removing copper from media. The sulfide ion forms insoluble heavy-metal sulfides while the activated carbon serves as a "gatherer."

Complex-forming reagents such as diphenylthiocarbazone (dithizone) (Stout and Arnon, 1939), and 8-hydroxyquinoline (Waring and Werkman, 1943) are useful in removing heavy-metal ions from, or testing the purity of, salts used in preparing media. These reagents and the metal complexes they form are removed from solutions by extraction with chloroform or other organic solvents. The chemistry of complex formation between organic compounds and ions is treated by Yoe and Sarver (1941). Others have merely added such complex-forming reagents to the media, in which the various metallic ions combine with the reagent to form non-available compounds. The specificity of the reagent, concentrations of reagent and the metallic ion or ions, the pH of the medium, as well as the stability of the complex, enter into the success of this type of treatment. Hickey (1945) found that 2,2′-bipyridine inactivated ferrous iron in media treated with this reagent. It is better to remove metallic impurities from the media by extraction than to depend upon complexing compounds to hold these ions in non-ionic combination.

Certain compounds used in making media such as the amino acids and hydroxy acids form non-ionized complexes with various metallic ions. Media containing these types of compounds are difficult to free from metallic contamination. In addition, some fungi excrete hydroxy acids, such as citric acid, which may modify the availability of the essential micro elements.

Media may be freed of essential micro elements by a biological process.

If a fungus is grown on a medium, it will absorb and utilize the essential elements present in the medium. The success of this procedure depends upon having a low initial concentration of the essential micro elements, which soon become exhausted so that the culture liquid no longer supports growth. Removal of the mycelium will thus remove the elements which have been taken up. The culture filtrate may then be used as a medium relatively free of micro elements. However, fungi excrete various compounds which may affect the results. MacLeod and Snell (1947) have recently utilized this method in studying the mineral nutrition of some lactic acid bacteria.

Iron. Raulin's claim that iron was essential for fungi was questioned at first, but his findings were soon confirmed. So far as is known, iron is essential for all fungi. It may be noted that, in the absence of another essential element in the medium, iron alone may cause little or no response. If the zinc content of a medium is low, the addition of iron to an iron-free medium will have little effect. This situation is true of any essential nutrient. Only one element may be studied at a time, but all the other essential nutrients must be present before the effect of the nutrient under investigation can be studied. Some results of Steinberg (1919) with *Aspergillus niger* on media purified by the calcium carbonate method are given in Table 11. Neither iron nor zinc *alone* had much effect on the growth of *A. niger*, since both of these elements are essential for this fungus.

TABLE 11. THE EFFECT OF IRON AND ZINC, SINGLY AND IN COMBINATION, ON THE AMOUNT OF GROWTH OF *Aspergillus niger*
(Steinberg, *Am. Jour. Botany* **6**, 1919.)

Essential Micro Element Added	Mg. Mycelium
Control (none added)	18
Iron	44
Zinc	40
Iron plus Zinc	731

Little interest has been shown in recent years in proving iron to be an essential element for a large number of fungi. In view of the almost universal occurrence of a group of iron-containing enzymes (catalase, the cytochromes, cytochrome oxidase, etc.), the essential role of iron is taken for granted.

The most obvious effect of suboptimal iron concentrations upon fungi is decreased growth. This result is probably due to the decreased and limited amounts of iron-containing enzymes formed under these conditions. It was shown by Yoshimura (1939–1940) that the amount of catalase produced by *Aspergillus oryzae* increased as the amount of iron in the medium increased. Lilly and Leonian (1945) showed that a rela-

tion existed between the amount of iron supplied in the medium and the ability of *Rhizobium trifolii* to synthesize certain vitamins. In the presence of suboptimal concentrations of iron the addition of certain vitamins replaced iron to a certain degree. A quantitative study of the vitamins synthesized by *Torulopsis utilis* has shown the iron concentration to be important (Lewis, 1944). Increased amounts of thiamine, riboflavin, nicotinic acid, and pyridoxine were synthesized on media low in iron, while the amounts of biotin, inositol and *p*-aminobenzoic acid were decreased.

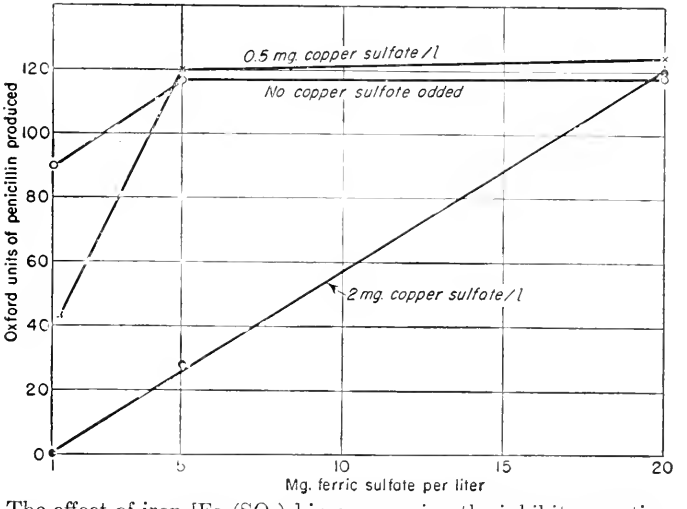


FIG. 14. The effect of iron [$Fe_2(SO_4)_3$] in overcoming the inhibitory action of copper ($CuSO_4 \cdot 5H_2O$) on the production of penicillin by *Penicillium chrysogenum* X-1612. An amount of copper sufficient to inhibit penicillin production entirely did not affect the amount of growth. The fungus was cultured submerged in a lactose–starch–dextrin–ammonium sulfate medium for 7 days. (Curves drawn from data of Koffler *et al.*, *Jour. Bact.* **53**: 120, 1947. Published by permission of The Williams & Wilkins Company.)

There has been a great deal of interest in the effects of iron and other metallic ions on various microbiological processes. Perlman *et al.* (1946) have shown that the iron concentration is an important factor in citric acid fermentation by *Aspergillus niger*. The optimum iron concentration for citric acid production varied over tenfold for different strains of *A. niger*. The effect of iron on penicillin production has been studied by Koffler *et al.* (1947), who concluded that the effect of the ash of corn steep is due to iron and phosphate. Chromium increased penicillin production above that obtained with iron and phosphate, presumably by neutralizing the effect of other ions. Similarly an antagonism was shown to exist between copper and iron. The antagonistic effect of copper and iron on the production of penicillin by *Penicillium chrysogenum* X-1612 is shown in Fig. 14.

The iron concentration of the medium has been shown to affect the amount of pigmentation of *Torulopsis pulcherrima* (Roberts, 1946).

Zinc. This element is essential for *Aspergillus niger* (Raulin, 1869; Steinberg, 1919). Foster (1939) lists *Trychophyton interdigitale*, *Rhizopus nigricans*, and *Saccharomyces cerevisiae* as requiring zinc, and Roberg (1928) found zinc to be essential for *A. fumigatus* and *A. oryzae*. Blank (1941) reported the amount of growth of *Phymatotrichum omnivorum* to be increased by the addition of zinc to a medium treated with calcium carbonate, and Perlman (1948) noted that the sclerotia of *Sclerotium delphinii* are more highly pigmented in the presence of added zinc.

Zinc ions activate (and inhibit) various enzymes such as enolase and dipeptidase. Zinc is contained in carbonic anhydrase, an enzyme which catalyzes the decomposition of carbonic acid to carbon dioxide and water. In addition to these specific uses the zinc concentration has a decided effect on a number of physiological or biochemical processes in fungi. Foster and Waksman (1939) found that the production of fumaric acid from glucose by *Rhizopus nigricans* varied according to the amount of zinc added to the medium. Fumaric acid was produced most efficiently when the concentration of zinc was low (1.2 mg. per liter). Higher concentrations of zinc resulted in increased growth and decreased production of fumaric acid. From these results it appears that zinc plays a role in the utilization of glucose, the completeness of oxidation and assimilation being favored by relatively high concentrations of zinc. A somewhat similar effect of zinc on the production of lactic acid by *Rhizopus* sp. has been noted (Waksman and Foster, 1938). Zinc was found to cause increased growth and a decrease in the production of lactic acid, while the effect of iron is to increase the yield of lactic acid. For a further discussion of the mechanism of zinc in fungus metabolism, see Foster (1949).

Copper. This element is essential for animals, green plants, and fungi. From the work of Steinberg (1936) it appears that 0.04 mg. of added copper per liter of purified medium is sufficient for the maximum growth of *Aspergillus niger*. Under these conditions omission of copper decreased the yield only from 984.8 to 774.3 mg. It is probable that purification of the medium by the calcium carbonate treatment is not very satisfactory for this element. The weight of metal needed to obtain maximum growth with *A. niger* is much less for copper than for iron or zinc. The experimental difficulties increase as the amount of a micro element needed becomes less. Apparently it is very difficult to prepare a copper-free medium. Roberg (1931) made use of Bortel's method of adding a trace of ammonium sulfide to convert heavy metals to sulfides and adsorbing these impurities with charcoal. This treatment is very efficient in removing iron and zinc but somewhat less satisfactory for removing copper. The essential nature of copper for *A. flavus* and *Rhizopus nigricans* was

shown by McHargue and Calfee (1931). The full effect of copper was dependent upon the presence of other essential elements. The coloration of conidia of *A. niger* has been shown to depend upon the copper content of the medium (Javillier, 1939).

Although copper is an essential element, it is a constituent of many fungicides (Chap. 12). The concentration, therefore, is a very important consideration in studying the effect of this element. The phenomenon of ion antagonism must also be considered, for the effect of a given amount of copper is dependent upon the other constituents of the medium. Marsh (1945) investigated the antagonistic effects of three salts upon copper as it affected germination of conidia of *Sclerotinia fructicola*

TABLE 12. THE ANTAGONISTIC EFFECT OF THREE SALTS ON COPPER AS SHOWN BY THE GERMINATION OF CONIDIA OF *Sclerotinia fructicola*
(Marsh, *Phytopathology* **35,** 1945.)

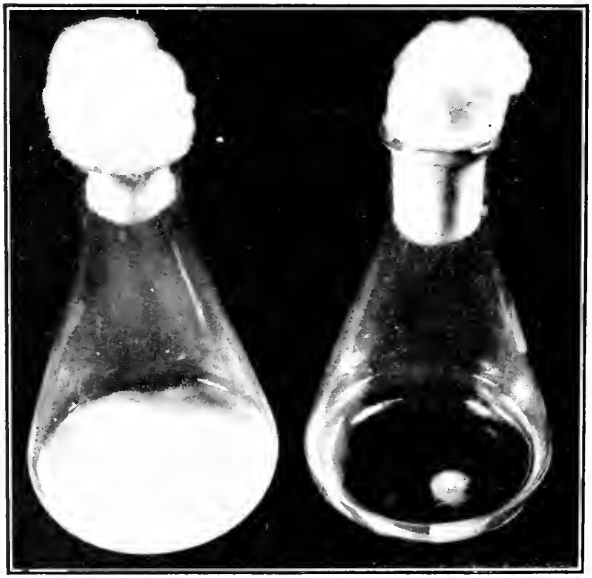
Salt concentration	Percentage Germination in $4 \times 10^{-7}M$ $CuSO_4$, plus 0.01% glucose		
	$MgSO_4$	$CaCl_2$	KCl
0.0	1.2	0.8	2.9
$10^{-5}M$	54.0	31.0	3.9
$10^{-4}M$	67.0	62.0	2.6
$10^{-3}M$	78.0	83.0	3.9
$10^{-2}M$	—	—	59.0

(Table 12). It was shown that the mechanism of the protective action of these salts was to decrease absorption of copper. There is no reason to assume that the absorption and utilization of copper from nutrient solutions would not be affected similarly. Thus, the amount of copper added to a nutrient solution may reflect only imperfectly the amount absorbed and used by a fungus.

It was noted in Chap. 4 that copper is an essential constituent of certain enzymes, including tyrosinase, which occurs in many fungi. Nelson and Dawson (1944) suggest that tyrosinase functions in the respiration chain as an oxygen shuttle.

Manganese. The classification of this element as essential rests upon the experimental findings that omission of this element from media results in decreased yields. The multiplication of examples strengthens the validity of this conclusion, although most investigators have confined their attention to a relatively few species. The results of Robbins and Hervey (1944) with *Pythiomorpha gonapodyoides* indicate that investigation of fungi other than *Aspergillus niger* with regard to micro-element

nutrition may be rewarding. It was unnecessary to resort to elaborate methods of medium purification to demonstrate that manganese is essential for *P. gonapodyoides*. This situation occurred only when reagent magnesium sulfate of a certain manufacture was used. Substitution of another brand of magnesium sulfate revealed heavy (biological) contamination by manganese (Fig. 15). The inoculum was found to carry sufficient manganese and other micro elements to influence the amount of growth in the first passage. No growth resulted in the third passage in



A *B*

FIG. 15. *Pythiomorpha gonapodyoides* growing in a basal solution with no added mineral supplements. *A*, medium prepared with Baker's Analyzed magnesium sulfate. *B*, medium prepared with Mallinckrodt's magnesium sulfate analytical reagent. Age, 5 days. Note the extensive white mycelium in *A* and the slight growth in *B*. (Courtesy of Robbins and Hervey, *Bull. Torrey Botan. Club* **71**: 263, 1944.)

the absence of added manganese. The range of manganese concentrations for optimum growth was narrow and appeared to depend upon the concentration of other micro elements present, particularly zinc. Steinberg (1935) found manganese to be essential for *A. niger*. McHargue and Calfee (1931, 1931*a*) noted that growth of *A. flavus*, *Rhizopus nigricans*, and *Saccharomyces cerevisiae* increased in the presence of added manganese. Steinberg (1945) showed that omission of manganese from a balanced medium resulted in a decrease in yield of *A. niger* from 1,084.8 to 356.6 mg. No spores formed when manganese was omitted. It is interesting to note that, as the numbers of spores used for inoculum

decreased, *A. niger* became more sensitive to micro-element deficiencies in the medium. The favorable effect of adding biotin to the medium when only a few spores were used as inoculum suggests an intimate connection between micro-element nutrition and the synthesis of this vitamin. Whether the decreased yield due to small inoculum was due to other deficiencies or to a decreased rate of growth is not entirely clear, as all harvests were made after 4 days.

Manganese (Mn^{++}) has been shown to be the natural activator of yeast arginase. Other enzymes are activated by this element (Sumner and Somers, 1947). In view of the small amounts of manganese required by fungi, it may be assumed that manganese functions as a constituent of various enzymes.

Molybdenum. The study of the role of this element emphasizes the similarity in certain physiological processes throughout the plant kingdom. The most striking feature of this essential element is its role in nitrogen metabolism. The utilization of nitrate nitrogen by green plants and fungi and the fixation of atmospheric nitrogen by bacteria (*Azotobacterchroococcum*, *Clostridium pasteurianum*) is dependent upon molybdenum (Bortels, 1930, 1936).

Our knowledge of the effect of molybdenum on fungi is largely confined to *Aspergillus niger*. Steinberg (1936, 1937) found that more molybdenum was required by *A. niger* for maximum growth in media containing nitrate nitrogen than in media with ammonium nitrogen. Steinberg expressed the opinion that molybdenum is essential for *A. niger* even when ammonium nitrogen is available. Additional studies on *A. niger* and other organisms (Mulder, 1948) indicated that an increased need for molybdenum is associated with nitrate utilization. It may be assumed that the enzymatic reduction of nitrate is carried out by enzymes which require molybdenum as an activator.

In view of the important role of molybdenum in the utilization of nitrate nitrogen, care should be used in comparing the value of different nitrates. Unless sufficient molybdenum is present, misinterpretations may result. Steinberg (1937) found the amount of molybdenum present as an impurity in various nitrates to vary. One sample of calcium nitrate contained enough molybdenum to support maximum growth of *A. niger*. Perhaps the report of Young and Bennett (1922) that many fungi made better growth on calcium than on potassium nitrate may be partially explained on the basis of the molybdenum content of these two salts. This explanation, of course, must allow for the effect of calcium, which is now known to be essential for certain fungi.

Calcium. This element was one of the first to be recognized as essential for green plants and animals. In 1922, Young and Bennett reported that *Rhizoctonia solani* made no growth in the absence of this element

This report apparently attracted little attention since most investigators working on this problem confined their attention to *Aspergillus niger*. The value of using more than one fungus to demonstrate the essential nature of calcium was strikingly shown by Steinberg (1948, 1950). These data are given in Table 13.

It is evident from the data in Table 13 that the essential nature of calcium for certain fungi is established. The concentrations of calcium required for maximum growth varied from 2 to 6 mg. per liter of medium. On the other hand, neither *A. niger* nor *Fusarium oxysporum* needs more

TABLE 13. EFFECT OF THE OMISSION OF CALCIUM FROM THE MEDIUM ON THE GROWTH OF SEVEN FUNGI
(Steinberg, *Science* **107**, 1948.)

Fungus	Calcium added, mg. mycelium	Calcium not added, percentage of yield
Aspergillus niger	1,250.0	100.0
Rhizoctonia solani	1,215.1	14.3
Selerotium rolfsii	1,082.3	49.5
Cercospora nicotianae	1,380.2	90.1*
Fusarium oxysporum var. *nicotianae*	823.3	100.0
Pythium irregulare	459.0	60.1*
Thielaviopsis basicola	364.2	82.0*

* Asparagine of unknown purity was used as a source of nitrogen.

than spectroscopic traces of calcium, if they require this element at all. Steinberg is of the opinion that further advances in purity of nutrient solutions will reveal more uniformity in the essential element requirements of organisms.

Lindeberg (1944) has demonstrated a synergistic effect between manganese and calcium upon the growth of various species of *Marasmius*.

TABLE 14. THE EFFECT OF INCREASING CONCENTRATIONS OF CALCIUM AND MANGANESE, ALONE AND IN COMBINATION, ON THE GROWTH OF *Marasmius epiphyllus*
(Lindeberg, *Symbolae Botan. Upsaliensis*, **8**: 2, 1944.)
(Dry weight mycelium in milligrams.)

Mn, millimoles per liter	Ca, millimoles per liter			
	0	0.005	0.05	0.5
0.0	10.1	19.8	33.0	73.5
0.0005	11.1	18.0	38.4	83.8
0.005	10.7	18.3	35.7	78.5
0.05	18.2	20.8	47.6	77.0
0.5	20.3	35.8	48.6	52.6

Within limits, the growth of *M. alliaceus* and *M. epiphyllus* was proportional to the concentration of either of these elements, and the response to each element was modified by the presence of the other. The data in Table 14 illustrate this effect.

In addition to the essential micro elements discussed above, there is some evidence which indicates the essentiality of other metallic elements for the fungi. Certain of these elements are essential for other organisms.

Gallium. Under certain conditions Steinberg (1938) was able to show that omission of this element from the medium led to decreased yield and sporulation of *Aspergillus niger*. Extraordinary care was needed to demonstrate gallium deficiency. The chemicals used were spectroscopically pure with the exception of traces of iron, calcium, and sodium. The sucrose, after 6-hr. extraction with alcohol, contained only 0.0014 per cent ash. The water used was triple-distilled, the last distillation being made in a quartz still. Spectroscopically pure calcium oxide was used to purify the sucrose further. Under these conditions the yield of *A. niger* increased from 814 mg. to 1,053 mg. when gallium (0.02 mg. per liter) was added to the medium. The salts of 76 other chemical elements were tested, and none was found to replace gallium. In view of the similar chemical behavior of gallium and aluminum, Steinberg considers it possible that the biologic activity sometimes attributed to aluminum may in reality be due to gallium.

Scandium. In the discussion of the role of manganese in nitrogen metabolism it was noted that the amount of manganese required was determined by the nitrogen source used. In a somewhat similar fashion, Steinberg (1939) found that scandium appeared to be essential when glycerol was used as a carbon source for *Aspergillus niger*. Growth was poor on this carbon source; omission of copper or manganese increased the yield somewhat. Omission of scandium decreased the yield from 269.4 to 107.4 mg. Interestingly enough, scandium appeared to have no effect on growth when sucrose was used as a source of carbon. Addition of lysine or proline (20 mg. per liter) to the glycerol medium increased growth and at the same time prevented the effect of scandium. These results suggest that the need for certain elements may be shown only under certain nutritional conditions.

Vanadium. Bertrand (1943) reported the presence of this element in all fungi examined. *Amanita muscaria* contained from 61 to 156 mg. of vanadium per kilogram. Bertrand (1941) considers vanadium as an essential element for *Aspergillus niger*.

Cobalt. Whether fungi require some, or all, of the other metallic elements required by other organisms is not known. Cobalt is required by animals. Lack of sufficient amounts of this element in the soil causes severe cobalt deficiency in animals which are pastured on such soils.

Recently, a cobalt-containing vitamin (B_{12}) was isolated. This vitamin is synthesized by *Streptomyces griseus* (Rickes *et al.*, 1948) and some bacteria. Whether *S. griseus* requires cobalt as an essential element for growth or reproduction is not known. The synthesis of this vitamin is necessarily dependent upon a supply of cobalt. Some bacteria are known to be deficient for vitamin B_{12}.

PERIODICITY OF BIOLOGICALLY ESSENTIAL ELEMENTS

Steinberg (1938*a*), Frey-Wyssling (1935), and others have considered the problem of biologically essential elements in relation to the structure

Table 15. A Portion of the Periodic Table of Elements Based on Atomic Number

The biologically essential elements are set in italics. Those elements essential for fungi are marked with an asterisk.

Group 0	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8		
	*H**									
	1									
He	Li	Be	*B*	*C**	*N**	*O**	*F*			
2	3	4	5	6	7	8	9			
Ne	*Na*	*Mg**	Al	*Si*	*P**	*S**	*Cl*			
10	11	12	13	14	15	16	17			
A	*K**	*Ca**	*Sc**	Ti	*V**	Cr	*Mn**	*Fe**	*Co*	Ni
18	19	20	21	22	23	24	25	26	27	28
	*Cu**	*Zn**	*Ga**	Ge	As	Se	Br			
	29	30	31	32	33	34	35			
Kr	Rb	Sr	Y	Zr	Cb	*Mo**	Tc	Ru	Rh	Pd
36	37	38	39	40	41	42	43	44	45	46
	Ag	Cd	In	Sn	Sb	Te	*I*			
	47	48	49	50	51	52	53			

and atomic number of the elements. The biologically essential elements are in italics in Table 15. It is noteworthy that the essential elements tend to occur in groups with consecutive atomic numbers. Atomic number is a fundamental property of atoms and denotes the number of excess positive charges on the nucleus. Only those elements which have certain configurations are required by organisms. Why some organisms require certain elements not required by others is not known.

SUMMARY

The role of the essential metallic elements is primarily functional rather than structural. Presumably these ions usually function in ionizable combinations, but some compounds containing metals in non-ionizable compounds have been isolated from fungi. It may be assumed that many of these metallic ions activate enzyme systems, while others are integral parts of enzymes and other essential organic compounds. An element is essential because some of its vital functions cannot be replaced by any other element. Some functions may be performed by other closely related elements.

The concentration of an essential element affects many life processes besides growth, which is the usual criterion of essentiality. The concentrations of various essential ions influence the formation of pigments, the synthesis of vitamins and other products, and the dissimilation of carbohydrates. While the essential elements may be supposed to participate uniquely in certain life processes, the concentrations of other ions, both of essential and nonessential elements, modify the action of a given element. The phenomenon of ion antagonism no doubt exists among all ions, and in evaluating the effects of any element it is necessary to consider the other constituents present in the medium. It is probable that the mechanism involved is one of modified adsorption rather than any direct chemical reaction in the medium.

The widespread use of *Aspergillus niger* as a test fungus in micro-element studies has had the advantage that the work in many laboratories may be compared. The careful and long-continued studies by Steinberg are especially valuable. The almost exclusive use of this fungus has also had its disadvantages. Comparatively little is known about the need of other species for micro elements. Other fungi may require some of these elements in amounts which make it comparatively easy to demonstrate deficiency. The evidence for the essentiality of iron, zinc, copper, manganese, molybdenum, and calcium is impressive in most instances, but the need for the elements on the part of all fungi under all cultural conditions has not been established. In a few instances the evidence is confined to a single fungus. The micro-element nutrition of a wide range of species needs further study.

REFERENCES

Bertrand, D.: Le Vanadium comme facteur de croissance pour l'*Aspergillus niger*, *Bull. soc. chim. biol.* **23**: 467–471, 1941.

Bertrand, D.: Le Vanadium chez les champignons et plus spécialement chez les Amanites, *Bull. soc. chim. biol.* **25**: 194–197, 1943.

Blank, L. M.: Response of *Phymatotrichum omnivorum* to certain trace elements, *Jour. Agr. Research* **62**: 129–159, 1941.

Bortels, H.: Molybdän als Katalysator bei der biologischen Stickstoffbindung, *Arch. Mikrobiol.* **1**: 333–342, 1930.

Bortels, H.: Weitere Untersuchungen über die Bedeutung von Molybdän Vanadium, Wolfram und anderen Erdaschenstoffen für stickstoffbindende und andere Mikroorganismen. *Cent. Bakt.*, Abt. II **95**: 193–218, 1936.

Buchanan, R. E., and E. I. Fulmer: Physiology and Biochemistry of Bacteria, Vol. I, The Williams & Wilkins Company, Baltimore, 1928.

Chilean Nitrate Educational Bureau: Bibliography of the Literature on the Minor Elements and Their Relation to Plant and Animal Nutrition, New York, 1948.

★Foster, J. W.: The heavy metal nutrition of fungi, *Botan. Rev.* **5**: 207–239, 1939.

Foster, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

Foster, J. W., and S. A. Waksman: The specific effect of zinc and other heavy metals on growth and fumaric acid production by *Rhizopus*, *Jour. Bact.* **37**: 599–617, 1939.

Frey-Wyssling, A.: Die unentbehrlichen Elemente der Pflanzennährung, *Naturwissenschaften* **23**: 767–769, 1935.

Gortner, R. H.: Outlines of Biochemistry, 3d ed., John Wiley & Sons, Inc., New York, 1949.

Gustafson, F. G.: Comparative studies on respiration. IX. The effects of antagonistic salts on the respiration of *Aspergillus niger*, *Jour. Gen. Physiol.* **2**: 17–24, 1919.

Hickey, R. J.: The inactivation of iron by 2,2′-bipyridine and its effects on riboflavin synthesis by *Clostridium acetobutylicum*, *Arch. Biochem.* **8**: 439–447, 1945.

Jarvis, F. G., and M. J. Johnson: The mineral nutrition of *Penicillium chrysogenum* Q176, *Jour. Bact.* **59**: 51–60, 1950.

Javillier, M.: Une cause d'erreur dans l'étude de l'action biologique des éléments chimiques: la présence de traces de zinc dans la verre, *Compt. rend. acad. sci.* **158**: 140–143, 1914.

Javillier, M.: Cuivre et *Aspergillus niger*. Rappel de quelques faits anciens, *Ann. fermentations* **5**: 371–381, 1939.

★Koffler, H., S. G. Knight, and W. C. Frazier: The effect of certain mineral elements on the production of penicillin in shake flasks, *Jour. Bact.* **53**: 115–123, 1947.

Laborey, F., J. Lavollay, and J. Neumann: Coefficient d'action du magnésium vis-à-vis d'*Aspergillus niger:* variation linéaire de ce coefficient avec la concentration en phosphore, *Compt. rend. acad. sci.* **212**: 624–626, 1941.

Lavollay, J., and F. Laborey: Sur les circonstances d'apparition de pigments jaunes dans le liquide de culture d'*Aspergillus niger*, *Compt. rend. acad. sci.* **206**: 1055–1056, 1938.

Lewis, J. C.: Relationship of iron nutrition to the synthesis of vitamins by *Torulopsis utilis*, *Arch. Biochem.* **4**: 217–228, 1944.

Lilly, V. G., and L. H. Leonian: The interrelationship of iron and certain factors in the growth of *Rhizobium trifolii*. strain 205, *Jour. Bact.* **50**: 383–395, 1945.

Lindeberg, G.: Ueber die Physiologie Ligninabbauender Boden Hymenomyzeten, *Symbolae Botan. Upsalienses* **8**(2): 1–183, 1944.

Lockwood, L. B., and M. D. Reeves: Some factors affecting the production of itaconic acid by *Aspergillus terreus*, *Arch. Biochem.* **6**: 455–469, 1945.

Lohrmann, W.: Untersuchungen über die antagonistische Wirkung von Magnesium gegenüber Bor und Quecksilber bei einigen Pilzen, *Arch. Mikrobiol.* **11**: 329–367, 1940.

McHargue, J. S., and R. K. Calfee: Effect of manganese, copper and zinc on growth and metabolism of *Aspergillus flavus* and *Rhizopus nigricans*, *Botan. Gaz.* **91**: 183–193, 1931.

McHargue, J. S., and R. K. Calfee: Effect of manganese, copper and zinc on the growth of yeast, *Plant Physiol.* **6**: 559–566, 1931*a*.

MacLeod, R. A., and E. E. Snell: Some mineral requirements of the lactic acid bacteria, *Jour. Biol. Chem.* **170**: 351–365, 1947.

Marsh, P. B.: Salts as antidotes to copper in its toxicity to the conidia of *Sclerotinia fructicola*, *Phytopathology* **35**: 54–61, 1945.

Molliard, M.: Influence d'une dose réduite de potassium sur les charactères physiologiques du *Sterigmatacystis nigra*, *Compt. rend. acad. sci.* **170**: 949–951, 1920.

Mulder, E. G.: On the use of microorganisms in measuring a deficiency of copper, magnesium and molybdenum in soils, *Antonie van Leeuwenhoek* **6**: 99–109, 1939–1940.

Mulder, E. G.: Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants, *Plant and Soil* **1**: 94–119, 1948.

Muntz, J. A.: The role of potassium and ammonium ions in alcoholic fermentation, *Jour. Biol. Chem.* **171**: 653–665, 1947.

Nelson, J. M., and C. R. Dawson: Tyrosinase, *Advances in Enzymol.* **4**: 99–152, 1944.

Nickerson, W. J.: Inhibition of fungus respiration: a metabolic bio-assay method, *Science* **103**: 484–486, 1946.

Perlman, D.: Some effects of metallic ions on the metabolism of *Aerobacter aerogenes*, *Jour. Bact.* **49**: 167–175, 1945.

Perlman, D.: On the nutrition of *Sclerotium delphinii*, *Am. Jour. Botany* **35**: 360–363, 1948.

★Perlman, D.: Effects of minor elements on the physiology of fungi, *Botan. Rev.* **15**: 195–220, 1949.

Perlman, D., D. A. Kita, and W. Peterson: Production of citric acid from cane molasses, *Arch. Biochem.* **11**: 123–129, 1946.

Rabinovitz-Sereni, D.: Influenza del magnesio sullo sviluppo di alcuni funghi, *Bol. R. Staz. Veg.* **13**: 203–226, 1933.

Raulin, J.: Etudes chimiques sur la végétation, *Ann. sci. nat.*, Ser. V, **11**: 93–299, 1869.

Richards, O. W., and M. C. Troutman: Spectroscopic analysis of the mineral content of yeast grown on synthetic and natural media, *Jour. Bact.* **39**: 739–746, 1940.

Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers: Crystalline vitamin B_{12}, *Science* **107**: 396–397, 1948.

Rippel, A., and G. Behr: Ueber die Verteilung des Magnesiums im Pilzmycel, *Arch. Mikrobiol.* **1**: 271–276, 1930.

Rippel, A., and G. Behr: Ueber die Bedeutung des Kaliums im Stoffwechsel von *Aspergillus niger*, *Arch. Mikrobiol.* **5**: 561–577, 1934.

Robbins, W. J., and A. Hervey: Response of *Pythiomorpha gonapodyoides* to manganese, *Bull. Torrey Botan. Club* **71**: 258–266, 1944.

Roberg, M.: Ueber die Wirkung von Eisen-, Zink-, und Kupfersalzen auf Aspergillen. *Cent. Bakt.*, Abt. II. **74**: 333–371. 1928.

Roberg, M.: Weitere Untersuchungen über die Bedeutung des Zinks für *Aspergillus niger*. *Cent. Bakt.*, Abt. II, **84**: 196–230, 1931.

Roberts, C.: The effect of iron and other factors on the production of pigment by the yeast *Torulopsis pulcherrima*, *Am. Jour. Botany* **33**: 237–244, 1946.

Shu, P., and M. J. Johnson: The interdependence of medium constituents in citric acid production by submerged fermentation, *Jour. Bact.* **56**: 577–585, 1948.

Stark, W. H., P. J. Kolichov, and H. F. Willkie: Some factors affecting yeast propagation, *Proc. Am. Soc. Brewing Chemists* **4**: 49–56, 1941.

★Steinberg, R. A.: A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*, *Am. Jour. Botany* **6**: 330–372, 1919.

Steinberg, R. A.: The nutritional requirements of the fungus *Aspergillus niger*, *Bull. Torrey Botan Club* **62**: 81–90, 1935.

Steinberg, R. A.: Nutrient-solution purification for the removal of heavy metals in deficiency investigations with *Aspergillus niger*, *Jour. Agr. Research* **51**: 413–424, 1935*a*.

Steinberg, R. A.: Relation of accessory growth substances to heavy metals, including molybdenum, in the nutrition of *Aspergillus niger*, *Jour. Agr. Research* **52**: 439–448, 1936.

Steinberg, R. A.: Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*, *Jour. Agr. Research* **55**: 891–902, 1937.

Steinberg, R. A.: The essentiality of gallium to growth and reproduction of *Aspergillus niger*, *Jour. Agr. Research* **57**: 569–574, 1938.

Steinberg, R. A.: Correlations between biological essentiality and atomic structure of the chemical elements, *Jour. Agr. Research* **57**: 851–858, 1938*a*.

★Steinberg, R. A.: Growth of fungi in synthetic nutrient solutions, *Botan. Rev.* **5**: 327–350, 1939.

Steinberg, R. A.: Relation of carbon nutrition to trace element and accessory requirements of *Aspergillus niger*, *Jour. Agr. Research* **59**: 749–763, 1939*a*.

Steinberg, R. A.: A dibasal (minimal salt, maximum yield) solution for *Aspergillus niger;* acidity and magnesium optimum, *Plant Physiol.* **20**: 600–608, 1945.

Steinberg, R. A.: Specificity of potassium and magnesium for the growth of *Aspergillus niger*, *Am. Jour. Botany* **33**: 210–214, 1946.

★Steinberg, R. A.: Essentiality of calcium in the nutrition of fungi, *Science* **107**: 423, 1948.

Steinberg, R. A.: Growth on synthetic nutrient solutions of some fungi pathogenic to tobacco, *Am. Jour. Botany* 37: 711–714, 1950.

Stout, P. R., and D. I. Arnon: Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants, *Am. Jour. Botany* **26**: 144–149, 1939.

Sumner, J. B., and G. F. Somers: Chemistry and Methods of Enzymes, Academic Press, Inc., New York, 1947.

Thatcher, R. W.: A proposed classification of the chemical elements with respect to their function in plant nutrition, *Science* **79**: 463–466, 1934.

Waksman, S. A., and J. W. Foster: Respiration and lactic acid production by a fungus of the genus *Rhizopus*, *Jour. Agr. Research* **57**: 873–900, 1938.

Waring, W. S., and C. H. Werkman: Growth of bacteria in an iron-free medium. *Arch. Biochem.* **1**: 303–310, 1943.

Yoe, J. H., and L. A. Sarver: Organic Analytical Reagents, John Wiley & Sons, Inc., New York, 1941.

Yoshimura, F.: The action of some heavy metals upon the production of catalase in *Aspergillus*, *Japan. Jour. Botany* **10**: 75, 1939–1940.

Young, H. C., and C. W. Bennett: Growth of some parasitic fungi in synthetic culture media, *Am. Jour. Botany* **9**: 459–469, 1922.

CHAPTER 6

THE ESSENTIAL NONMETALLIC ELEMENTS OTHER THAN CARBON

Fungus mycelium and spores are composed mainly of compounds of the nonmetallic elements. As a rule, more than 95 per cent of the fungus consists of hydrogen, oxygen, carbon, nitrogen, sulfur, and phosphorus. The nonmetallic essential elements are both structural and functional. The cell wall, which is composed mainly of chitin or cellulose, appears to be the most stable structure of the fungus. Protoplasm is highly labile, and the constituent compounds of protoplasm are continually undergoing destruction, repair, and synthesis. The various structural and functional compounds of organisms are in a state of continual flux (Hevesy, 1947). The turnover of essential elements in functional compounds is more rapid than in structural compounds.

The terms utilization, assimilation, and dissimilation are frequently used in physiology. *Utilization* is a broad term and implies that an organism uses or gains some benefit from a specific substance. Fungi utilize water as a solvent but derive neither energy nor substance from it. *Assimilation* is the incorporation of substances or their degradation products into cellular materials. Assimilation implies synthesis. *Dissimilation* is the degradation, or breakdown, of complex compounds into simpler ones. This term is particularly applied to those processes such as alcoholic fermentation where intermediate metabolic products accumulate in the medium. Frequently dissimilation must precede assimilation and may be considered as the first phase of utilization.

HYDROGEN

Hydrogen enters into the composition of nearly all organic compounds of interest to physiology except carbon dioxide. This is true of the organic nutrients used by fungi as well as of the fungus protoplasm and other cellular compounds. Elemental hydrogen is not used by fungi. All the hydrogen utilized by fungi is in chemical combination. Certain bacteria (hydrogen bacteria), however, are able to obtain energy by oxidizing hydrogen.

The importance of *water* for all living organisms is so great that it seems impossible to conceive of life without water. The formula H_2O is really the formula of steam. In the liquid state these simple molecules

associate to form polymers. At room temperature water consists mostly of $(H_2O)_3$, which is sometimes called trihydrol. For a further discussion of water see Barnes (1937).

The chemistry of life processes is largely confined to reactions which take place in the presence of water or in solution. In addition to being a solvent of remarkable powers, water is associated with the colloids which comprise protoplasm. Gortner (1949) has distinguished between "free" and "bound" water. Free water is mobile within the cell and serves as a solvent and for the purpose of translocation of the various products of metabolism. Bound water is firmly adsorbed by protoplasm, and in this form water does not freeze. This property of bound water enables cells to withstand low temperatures. The ability of fungus spores to withstand low temperatures may well be due to their having most of their water content in the bound form.

Water ionizes to form hydrogen (H^+) and hydroxyl (OH^-) ions. The effects of these ions on biological processes are so important that they will be discussed in detail in Chap. 8.

OXYGEN

Apparently none of the fungi are obligate anaerobes. Many are strictly aerobic, and some are facultatively anaerobic. An aerobic organism requires uncombined oxygen, while a facultative anaerobe may use combined oxygen in addition to free oxygen. The amount of oxygen required for optimum growth varies with the species. It is common to express the amount of oxygen available in terms of millimeters of mercury. Approximately 21 per cent of air is oxygen. The amount of oxygen may be regulated by controlling the air pressure within the culture vessel. If the barometric pressure is 740 mm. Hg, the partial pressure due to oxygen is $^{21}/_{100} \times 740$, or 155.4 mm. Hg. If the pressure within a culture vessel is reduced to 100 mm. Hg, the partial pressure of oxygen amounts to 21 mm. Hg. Tamiya (1942) has reported that *Aspergillus oryzae* has a maximum rate of respiration when the partial pressure of oxygen is 500 to 630 mm. Hg. Such partial pressures of oxygen are readily obtained by using oxygen-nitrogen mixtures. Ternetz (1900) reported the following effects of reduced oxygen supply on *Ascophanus carneus:* at 10 mm. Hg the mycelium grew with difficulty; at 20 mm. Hg growth was good, but no spores formed; at 40 mm. Hg some fructification occurred; at 120 to 140 mm. Hg growth was somewhat better than at atmospheric pressure.

The ability of certain soil fungi to exist under conditions of low oxygen supply is important for survival. The amount of oxygen in soil depends upon the soil type and the amount of water present. Soil saturated with water contains but a trace of free oxygen. Hollis (1948) found *Fusarium oxysporum* to survive under essentially anaerobic conditions for 13 weeks,

while *F. eumartii* perished within 3 weeks when exposed to the same conditions. The mycelium of *F. oxysporum* grown under reduced oxygen tension was abnormal in its morphology. For further information on the effect of reduced oxygen tension, see Fellows (1928) and Scheffer and Livingston (1937).

Enormous amounts of sterile air must be supplied to the 10,000- to 15,000-gal. tanks used in the production of penicillin and other antibiotics. In the laboratory, aeration is provided by shaking machines of the rotating or reciprocal type. Aeration under these conditions is more uniform than is possible in stationary cultures, where submerged and aerial hyphae obtain different amounts of oxygen. This was shown by Tamiya (1942) who reported that the enzyme systems of submerged mycelium of *Aspergillus oryzae* are more easily poisoned by cyanide than are those of aerial mycelium.

In a broad sense, respiration denotes all the enzymatic processes which occur in cells involving a release of energy. There are two general ways in which energy is released by living cells: (1) Cells obtain energy from chemical reactions in which free oxygen is a reactant. The oxidation of metabolite molecules by this process is generally called *respiration*, or more specifically *aerobic respiration*. This process is characterized by the intake of free oxygen and the formation of carbon dioxide. If the compound being oxidized is composed of carbon, hydrogen, and oxygen only, the products are carbon dioxide, water, and energy. (2) Cells also obtain energy from chemical reactions in which free oxygen is not a reactant. This process is called *anaerobic respiration*, or *fermentation*. Metabolic processes of this kind are characterized by the production of carbon dioxide, the incomplete oxidation of substrate molecules, and the release of a small amount of energy.

The reactions involved in the aerobic respiration of glucose may be summarized in a single equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 673{,}000 \text{ cal.}$$

This equation gives no indication of the intermediate stages in this reaction or how the energy is utilized by the organism performing the oxidation. The number and variety of intermediate reactions do not affect the total amount of energy released. The reactions involved in the alcoholic fermentation of glucose are summarized in the following equation:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 + 25{,}000 \text{ cal.}$$

This equation, like the preceding one, gives no indication of the intermediate reactions involved. To obtain the same amount of energy, more of a compound must be fermented than when it is completely oxidized. Not all of the energy released by either of these processes is available to the organism (Chap. 4).

A knowledge of the amounts of oxygen consumed and carbon dioxide evolved by organisms is the basis of a useful method of study in many phases of physiology. The principles of such measurements are simple. In aerobic respiration both the oxygen and carbon dioxide may be measured. The ratio of the moles, or volumes, of carbon dioxide evolved and oxygen used is called the respiratory quotient (R.Q.) and is written CO_2/O_2. From the respiratory quotient the nature of the substrate being oxidized may be deduced. A respiratory quotient of 1 is characteristic of aerobic oxidation of carbohydrate. The complete oxidation of a fat may be represented as follows:

$$(C_{18}H_{35}O_2)_3C_3H_5 + 81.5O_2 \rightarrow 57CO_2 + 55H_2O$$

The respiratory quotient for this fat is 57/81.5, or 0.7. If fungus cells are suspended in a buffer in the absence of nutrients, and the respiratory quotient determined, it is possible to deduce the type of compound within the cells being used as a source of energy. Oxidation of the stored compounds within the cell is called *endogenous respiration.* The oxidation of substrate molecules from the medium is called *exogenous respiration.* Since both types of respiration may occur simultanously in the presence of nutrients, it is necessary, in order to determine exogenous respiration, to subtract the value for endogenous respiration from that obtained in the presence of nutrients.

The rate and amount of respiration are determined by instruments known as respirometers. Various types of respirometers have been used to investigate different phases of fungus metabolism and nutrition. In principle a respirometer is a closed vessel of known volume in which fungus cells are suspended in a buffer or other solution. The carbon dioxide evolved is absorbed in a concentrated solution of potassium hydroxide. The change in volume due to the consumption of oxygen is measured by the use of suitable manometers. At the end of the experiment the amount of carbon dioxide evolved is measured after the potassium hydroxide solution is treated with a mineral acid. Carbon dioxide alone may be measured by passing a stream of carbon dioxide–free air through a culture and absorbing the carbon dioxide evolved in barium hydroxide or other suitable reagent. The results of such experiments are reported on the basis of the volumes of oxygen used and carbon dioxide evolved per milligram of dry weight per hour. These values are reported as Q_{O_2} and Q_{CO_2} (see Umbreit *et al.,* 1945).

A modern respirometer is illustrated in Fig. 16. The various manipulative details will not be discussed. For an adequate treatment of these see Umbreit *et al.* (1945) and Dixon (1943). These methods are extremely useful in studying a wide range of problems. Hawker (1944) used manometric techniques in studying the effect of excess thiamine on

glucose utilization by *Melanospora destruens* and *Phycomyces nitens*. The papers of Siu and Mandels (1950) and Mandels and Siu (1950) should be consulted for details concerning a simple differential manometer. This manometer is designed to measure the respiration of intact growing cultures of filamentous fungi. Dorrell (1948) investigated the effect of

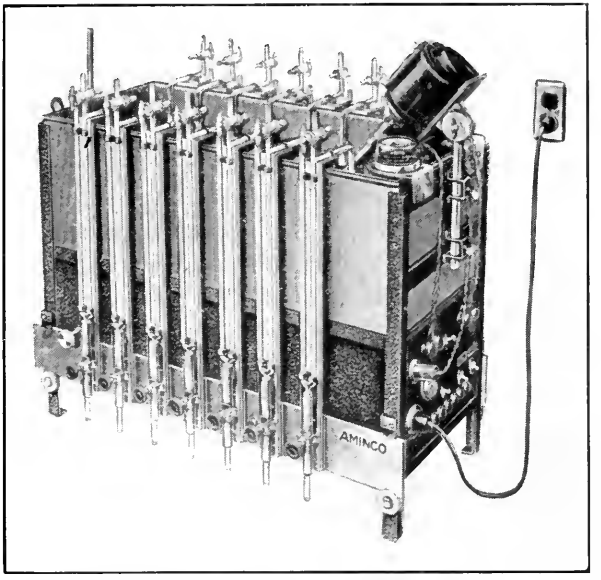


FIG. 16. A constant-temperature bath and shaking device for micro respiration studies. (Courtesy of American Instrument Company.)

dinitrophenol on endogenous and exogenous respiration of *Fusarium graminearum* (*Gibberella zeae*). As usually carried out, respiration experiments last only a few hours. The initial state of the cells or mycelium

TABLE 16. THE EFFECT OF AGE OF *Zygosaccharomyces acidifaciens* CELLS ON THE AMOUNT OF AEROBIC RESPIRATION

(Nickerson and Carroll, *Jour. Cellular Comp. Physiol.* **22,** 1943. Published by permission of the Wistar Institute of Anatomy and Biology.)

Age of cells, hr.	Q_{O_2}*	
	Glucose substrate	No substrate (endogenous)
24	60	16
48	35	7.3
72	35.5	7.0

* Q_{O_2} equals μl O_2 per hr. per mg. dry cells.

used has a great effect on the results obtained. Nickerson and Carroll (1943) have indicated that the culture history of the cells used influences the amount of aerobic respiration. Some of their data for *Zygosaccharomyces acidifaciens* are shown in Table 16.

SULFUR

Not all compounds which contain an essential element are equally useful. In fact, some compounds are useless because the essential element is unavailable. Among the factors which may affect availability is the state of oxidation of the essential element. This is particularly true of sulfur, phosphorus, and nitrogen. Among the organic compounds, structure is enormously important. The situation is further complicated in that not all fungi utilize the same compounds. Many examples of thi will be cited in connection with nitrogen and carbon nutrition. Attention must be given the *sources of the essential elements* as well as the uses fungi make of them.

Sources of sulfur. This element is present in many types of compounds, both inorganic and organic. The state of oxidation of sulfur, as well as the specific structure of organic sulfur compounds, affects utilization. Sulfate sulfur, $SO_4^=$, is the most common source of sulfur used in media. Some fungi, however, require specific organic sources of sulfur. Steinberg (1936, 1941) has made an exhaustive study of sulfur sources for *Aspergillus niger* and reached the general conclusions that inorganic sulfur compounds containing oxidized sulfur are utilized, while sulfide and disulfide sulfur are not utilized. Of the organic compounds containing sulfur, the alkyl thioalcohols, sulfides, and disulfides are not used. Alkyl sulfonates and sulfinates are excellent sources of sulfur. Steinberg is of the opinion that oxidized sulfur is reduced to sulfoxylate before it enters the normal metabolic channels. An exception to the nonutilization of reduced sulfur was noted for compounds which occur as normal metabolites, such as cysteine, cystine, methionine, and homocystine. These are assumed to enter normal metabolic channels without preliminary modification. An exception to this statement was noted with thiamine (thiazole sulfur), but the enormous (physiologically) amounts used may have upset the metabolic activities of the fungus.

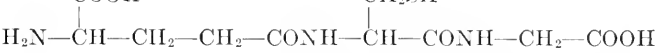
In spite of the general utility of sulfate sulfur in fungus nutrition, many fungi either utilize organic sulfur contained in natural metabolites to better advantage or require these compounds as a source of sulfur. Leonian and Lilly (1938) reported that the addition of cystine to a synthetic medium was necessary for the growth of *Saprolegnia mixta, Achlya conspicua, Isoachlya monilifera,* and *Aphanomyces camptostylus.* Since other naturally occurring sulfur-containing amino acids were not tested, it should not be concluded that these species are deficient for cystine.

Volkonsky (1933, 1934) observed that certain of the aquatic Phycomycetes failed to utilize sulfate sulfur. These species were *Saprolegnia parasitica, Isoachlya monilifera, Achlya prolifera, A. polyandra, A. oblongata, A. conspicua, Dichtyuchus monosporus,* and *Aphanomyces* sp. A total of 26 isolates failed to utilize sulfate sulfur. This investigator (1933*a*) designates ability to utilize 6-valent sulfur as *euthiotrophy* and inability to utilize sulfate sulfur and ability to utilize reduced sulfur as *parathiotrophy.*

Fries (1946) was able to induce mutation in *Ophiostoma* (*Ceratostomella*) *multiannulatum* by irradiating the ascospores with X rays. Among these mutants 13 strains were unable to utilize sulfate sulfur. Only five of these strains regained this ability when cultivated on media containing sulfate. These parathiotrophic strains of *O. multiannulatum* utilized ammonium sulfide as well as cystine and cysteine as sources of sulfur. From the fact that these mutants could utilize sulfide sulfur, it is evident that these strains were not deficient for specific sulfur-containing amino acids. Bonner (1946) has, however, found induced mutants of *Penicillium* to be deficient for specific sulfur-containing amino acids. *Blastocladia pringsheimii* has been reported to require methionine (Cantino, 1949).

Fries (1948) has reported the occurrence of *natural mutants* of *Ophiostoma multiannulatum* which require reduced sulfur, and also mutants which are unable to synthesize methionine. Of a total of 51,037 single-conidium cultures, 2 required reduced sulfur and 30 required methionine.

The role of sulfur. The use fungi make of sulfur may be deduced from the sulfur-containing compounds which are known to occur in mycelium and spores. Among these are the proteins. In Chap. 4 it was noted that the activity of many enzymes depends upon the sulfhydryl or thiol group, —SH. On hydrolysis, fungus protein yields the following sulfur-containing amino acids: cystine, cysteine, and methionine. Sulfur is thus a structural element. Another sulfur-containing compound is the tripeptide, glutathione, which is abundant in yeast. The formula for glutathione is given below:



This compound is sometimes represented by the symbol GSH. In spite of intensive investigation the role of this compound is not fully understood. Perhaps one of its functions is to protect sulfhydryl enzymes from inactivation.

The probable mechanism of the biosynthesis of cystine has been studied using mutants of *Aspergillus nidulans* (Hockenhull, 1949). All these cystine-deficient mutants were able to utilize thiosulfate sulfur, methio-

nine, and cystine. It was postulated that sulfate sulfur was first reduced to sulfite and then to sulfoxylate, which was assumed to dimerize to thiosulfate. The next reaction was believed to be between serine and thiosulfate to form cysteine S-sulfonate, which is then converted to cysteine. Cysteine on being oxidized forms cystine.

Two vitamins, thiamine and biotin, contain sulfur. The role of these compounds will be considered in Chap. 9. In addition to the sulfur-containing amino acids and vitamins there is evidence that other types of organic sulfur compounds are formed by fungi. Raistrick and Vincent (1948) found that many strains and species of *Aspergillus* and *Penicillium* converted essentially all of the sulfate sulfur into organic sulfur compounds, but not all of these compounds were found in the fungus proteins. *Penicillium chrysogenum* excretes into the medium various unidentified organic sulfur compounds (Plumlee and Pollard, 1949). The function of these compounds is unknown.

The reactions whereby a fungus transforms a single source of sulfur into these various compounds are obscure. When sulfate or other sources containing oxidized sulfur are utilized, it is necessary for the fungus to reduce the sulfur to its lowest valence. *Schizophyllum commune* has been shown to reduce sulfate to methyl mercaptan, CH_3SH (Birkinshaw *et al.*, 1942). This substance contributes to the characteristic odor of this fungus.

PHOSPHORUS

Raulin (1869) found phosphorus to be an essential element for *Aspergillus niger*. Omission of phosphate from his synthetic medium reduced the yield approximately 50 per cent. Phosphorus is essential for all forms of life. Phosphorus may be classified as a structural element in the sense that definite compounds containing this element have been isolated from fungi. Phosphorus compounds play an important role in the functions of chemical transformations and energy transfer.

Sources of phosphorus. Apparently phosphorus is utilized only when it is in the form of phosphate. This element is taken up as phosphate and functions in this form, mainly in the form of phosphate esters. It will be recalled that there are several different phosphates. The formulas for the potassium salts are K_3PO_4, potassium orthophosphate; KPO_3, potassium metaphosphate; and $K_4P_2O_7$, potassium pyrophosphate. More complex phosphates than pyrophosphate occur. Orthophosphoric acid may be neutralized in three steps to produce the following types of salts: KH_2PO_4, monopotassium orthophosphate; K_2HPO_4, dipotassium orthophosphate; and K_3PO_4, tripotassium orthophosphate. All these salts furnish utilizable phosphate, but the effects of these three salts on the acidity of the medium are quite different. In addition to inorganic phosphates, the

organic phosphates (esters) may also be used as sources of this element. Dox (1911–1912) investigated the assimilation of various phosphorus compounds by *Aspergillus niger* with the following results: Ortho-, meta-, and pyrophosphates supported excellent growth, as did such organic compounds of phosphorus as phytin, sodium glycerophosphate, sodium nucleinate, casein, and ovovitellin. Sodium hypophosphite ($NaH_2PO_2 \cdot H_2O$) and sodium phosphite ($Na_2HPO_3 \cdot 5H_2O$) were not utilized and appeared to be toxic.

Smith (1949) studied the phosphorus metabolism of *Merulius lacrymans* and *Marasmius chordalis* in connection with the utilization of different carbon sources. In glucose medium *M. lacrymans* grew better when supplied with inorganic phosphate, while *M. chordalis* grew more rapidly when supplied with organic phosphorus (adenylic acid). On cellobiose medium *M. lacrymans* grew faster when supplied with organic phosphorus.

The role of phosphorus. An idea of the manifold ways in which phosphorus enters into fungus metabolism may be gained from the studies of Mann (1944, 1944*a*). *Aspergillus niger* was grown on a glucose-nitrate medium containing varying amounts of dipotassium orthophosphate. Some of Mann's data on the effect of two concentrations of phosphate are given in Table 17.

TABLE 17. THE EFFECT OF TWO CONCENTRATIONS OF ORTHOPHOSPHATE UPON THE APPEARANCE, SPORULATION, AND OTHER METABOLIC FUNCTIONS OF *Aspergillus niger* (Mann, *Biochem. Jour.* **38,** 1944. Published by permission of the Cambridge University Press.)

Characteristics of 5-day-old cultures	Grown in presence of 0.02% K_2HPO_4	Grown in presence of 0.2% K_2HPO_4
Mycelium	Thin, white, smooth. Conidiophores present	Thick, yellowish. No conidiophores
Dry weight, mg.	460	1,092
Q_{O_2} of intact mycellium, μl	6.12	11.4
Total N, mg.	8.1	23.7
Total P, mg.	1.5	12.1
Thiamine, μg	3.2	19.0
Riboflavin, μg	16.1	78.7
Nicotinic acid, μg	19.4	302.0
Medium	Colorless	Yellow

From Table 17 it may be seen that suboptimal amounts of phosphorus affect the metabolism of *A. niger* in many ways besides diminishing growth. Nitrogen utilization was affected, and the synthesis of three vitamins (thiamine, riboflavin, and nicotinic acid) was greatly decreased. The ability of phosphorus-starved mycelium to utilize oxygen was diminished, as shown by the lower Q_{O_2}. Mann also showed that utilization of

phosphate by *A. niger* takes place only in the presence of oxygen. The utilization of phosphorus by yeasts, and presumably by other fungi which are capable of anaerobic respiration, may take place in the absence of oxygen. Various respiratory inhibitors such as iodoacetate, azide, and cyanide inhibited both respiration and phosphorus metabolism. This points to an intimate connection between carbohydrate and phosphorus metabolism. By analysis, ortho-, meta-, and pyrophosphates were found in the mycelium. Since only orthophosphate was supplied in the medium, it is shown that *A. niger* is capable of these transformations.

Phosphorus appears to participate in almost every step in the anaerobic dissimilation of glucose into alcohol by yeast. Some of these steps may be common to other fungi. It is remarkable that the formation of alcohol by yeast and lactic acid in muscle should follow almost the same pathways. Phosphorus is required in the enzymatic transformation of glucose into alcohol and carbon dioxide (Harden, 1932). Sumner and Somers (1947) and Tauber (1949) have summarized the enzymatic reactions involved.

Either starch or glycogen may be transformed into glucose-1-phosphate by enzymatic esterification. The shift of the phosphate radical to the other end of the glucose molecules leads to glucose-6-phosphate, which may also be formed by direct esterification of glucose. Glucose-6-phosphate is transformed into fructose-6-phosphate and then into fructose-1,6-diphosphate. Scission of a molecule of fructose-1,6-diphosphate yields dihydroxyacetone-1-phosphate and D-1-phosphoglyceric aldehyde. An equally long series of transformations leads to pyruvic acid, CH_3—CO—COOH, which on decarboxylation by the enzyme carboxylase yields acetaldehyde, which is enzymatically reduced by DPN·H_2 to ethyl alcohol. Cocarboxylase and diphosphopyridine nucleotide (DPN) are coenzymes, both of which contain phosphorus.

Gould *et al.* (1942) studied the formation of alcohol by *Fusarium tricothecioides* and found the limited production of alcohol by this species was due to insufficient synthesis of diphosphopyridine nucleotide. Alcohol production was increased 20- to 25-fold by the addition of either yeast extract or DPN to the medium. The paper of Semeniuk (1943–1944), which deals with the relation of phosphorus to glucose dissimilation by *Chaetomium funicola*, has an extensive bibliography (117 references). Nord and Mull (1945) have summarized a long series of papers on the physiology and biochemistry of *Fusarium lini* and reached the conclusion that fermentation by this fungus follows a pathway which does not involve the sugar phosphates. The review of Barron (1943) on the mechanisms of carbohydrate metabolisms contains much information about the role of phosphorus (219 references) in carbohydrate metabolism. The role of phosphorus compounds in the transfer of energy was noted in Chap. 4.

Phosphorus enters into the composition of the nucleoproteins, which are found in the nucleus and cytoplasm of every cell. The nucleoproteins are conjugated proteins which consist of a protein moiety in combination with purine or pyrimidine nucleotides (nucleic acids). These nucleotides are important functional compounds and may be classified according to their heterocyclic components.

The preliminary hydrolysis of purine and pyrimidine nucleotides involves the removal of phosphoric acid and the formation of nucleosides. Nucleosides on hydrolysis yield sugars, purines (adenine, guanine) or pyrimidines (cytosine, thymine, uracil). The nucleotides are also classified according to the sugar moiety, *i.e.*, D-ribose or D-desoxyribose.

The nucleoproteins which contain D-ribose are mainly found in the cytoplasm, while D-desoxyribose characterizes the nucleoproteins of the nucleus. The Feulgen stain is used by cytologists to detect the presence of D-desoxyribose nucleic acid. Viruses, chromosomes, and genes consist largely of nucleoproteins. For a review of the role of nucleoproteins see Mirsky (1943).

NITROGEN

This essential element is used by fungi for functional as well as structural purposes. The cell wall of many species, with the exception of the Oomycetes and yeasts, appears to be composed of chitin (Brian, 1949). Chitin is a linear polymer, similar to cellulose, of D-glucosamine. The amino group of glucosamine in chitin is acetylated. This substance makes up the exoskeleton of insects and Crustacea. It is interesting that the chitin formed by fungi, insects, and Crustacea appears to be the same substance. Protein, the basis of protoplasm, is composed of nitrogenous substances. Purines, pyrimidines, and some of the vitamins are also nitrogen-containing compounds.

Not all nitrogen sources are equally suitable for all fungi. Fungi may be specific in the nitrogen sources they utilize. Our information on this subject, while extensive, is far from complete. The reports in the literature which indicate that specific fungi are able to grow on a given source of nitrogen may be accepted with confidence, but the reported negative results are to be viewed with caution. Failure of a fungus to grow upon a given nitrogen source may mean only that the medium used did not contain the necessary growth factors, as in the case of *Ophiobolus graminis* (See Chap. 2).

Classification according to nitrogen sources used. Robbins (1937), Steinberg (1939, 1950), and others have classified the fungi according to their ability to utilize different sources of nitrogen. In the main Robbins's classification is as follows: (1) fungi able to utilize atmospheric nitrogen, nitrate nitrogen, ammonium nitrogen, and organic nitrogen; (2) fungi able to utilize nitrate nitrogen, ammonium nitrogen, and organic

nitrogen but not able to utilize atmospheric nitrogen; (3) fungi able to utilize ammonium and organic nitrogen but unable to utilize atmospheric or nitrate nitrogen; (4) fungi which are able to utilize only organic nitrogen and unable to utilize atmospheric, nitrate, or ammonium nitrogen. Robbins recognized that the experimental conditions might affect the classification of some fungi. In spite of admitted imperfections the above classification is very useful in preparing media and in discovering the causes of failure of some fungi to grow on certain media.

Nitrogen-fixing fungi. It has been shown to the satisfaction of all competent investigators that various genera of bacteria (*Rhizobium, Azotobacter, Clostridium*) contain species which are able to fix nitrogen.

TABLE 18. NITROGEN FIXATION BY *Phoma betae* AND *Azotobacter vinlandii* (Duggar and Davis, *Ann. Missouri Botan Garden* **3**, 1916.)

Organism	Inoculated flasks		Uninoculated flasks		Mg. N fixed per flask
	Mg. N per flask	Ave.	Mg. N per flask	Ave.	
Aspergillus niger (30 days)	62.510 62.545 63.140	62.732	62.510 62.335 62.300	62.382	0.350
Phoma betae (89 days)	31.010 31.360	31.185	25.585 25.655	25.620	5.565
Azotobacter vinlandii (28 days)	46.515 46.480 46.445	46.480	5.810 6.405	6.108	40.372

No such agreement exists regarding fungi. Much of the early work on nitrogen fixation by fungi was done without using proper precautions. However, in several instances the experimental methods appear to be beyond reproach. Duggar and Davis (1916) cultured *Phoma betae* and *Aspergillus niger* in Kjeldahl flasks and determined the nitrogen content after growth without removing either the mycelium or medium prior to digestion. Two types of controls were used. A number of uninoculated flasks which had been stored under the same conditions as the inoculated flasks were analyzed for nitrogen at the end of the experiment. A culture of *Azotobacter vinlandii* served as a positive control. The data in Table 18 show that *A. niger* did not fix nitrogen, while *P. betae* and *A. vinlandii* did. However, the nitrogen-fixing power of *P. betae* was slight compared with that of *A. vinlandii*. In addition, the following fungi were tested for ability to fix nitrogen, with negative results: *Macrosporium commune, Penicillium digitatum, P. expansum,* and *Glomerella gossypii*. For further

references to nitrogen fixation by filamentous fungi see Wolf and Wolf (1947) and Buchanan and Fulmer (1930).

So far as we are aware, only one study of nitrogen fixation by fungi using modern isotopic techniques (Tove *et al.*, 1949) has been published. *Phoma causarina* was grown on a sucrose-salts medium in oxygen and nitrogen enriched with N^{15}. Growth was slow and sparse under these conditions, but some N^{15} was fixed. These authors state that the isotopic method is about 100 times more sensitive than the Kjeldahl procedure used by other investigators.

While it is probable that only a relatively few fungi are able to fix nitrogen, the importance of biological nitrogen fixation is so great that further investigations with modern techniques are desirable. Long ago Ternetz (1907) reported that five species of *Phoma* isolated from roots of Ericaceae fixed significant amounts of nitrogen. For a discussion of nitrogen fixation by bacteria see Wilson (1940).

Fungi utilizing nitrate nitrogen. Nitrates occur in the soil and thus are a "natural" source of nitrogen. A fungus which utilizes nitrate nitrogen (NO_3^-) must be able to reduce the nitrogen to the oxidation level of ammonia. We may assume that failure of a fungus to utilize nitrate nitrogen is coupled with inability to perform this reduction. According to Robbins (1937) no instances have been recorded in the literature of an organism being able to utilize nitrate nitrogen and unable to utilize ammonium nitrogen. This does not mean that fungi which are able to utilize nitrate nitrogen will grow at the same rate on ammonium nitrogen, or that all sources of organic nitrogen will be as favorable as nitrate nitrogen. Yeasts as a rule do not utilize nitrate nitrogen.

The following is a partial list of fungi which have been reported or observed to utilize nitrate nitrogen:

Armillaria mellea
Ascobolus denudata
A. leveillei
Ascochyta pisi
Aspergillus spp.
Botryotinia convoluta
Botrytis allii
B. cinerea
Cephalothecium roseum
Cercospora apii
C. beticola
Chaetomium cochlioides
C. convolutum
C. globosum
Colletotrichum lagenarium
C. lindemuthianum
Collybia tuberosa
C. velutipes
Cordyceps militaris
Dendrophoma obscurans
Dothidella quercus
Fusarium spp.
Glomerella cingulata
Gymnoascus setosus
Helminthosporium spp.
Lambertella corni-maris
Lentinus tigrinus
Macrosporium sarcinaeforme
Marasmius fulvobulbillosus
Neocosmopara vasinfecta
Ophiobolus graminis
O. miyabeanus
Penicillium spp.
Phoma apiicola

P. betae
Pleurage curvicolla
Pyronema confluens
Pythiomorpha gonapodyoides
Pythium debaryanum
P. intermedium
P. irregulare
Rhizoctonia solani
Sclerotinia minor
S. sclerotiorum
Sclerotium bataticola
Septoria nodorum
Sordaria fimicola
Sphaerobolus stellatus
Sphaeropsis malorum
Trichoderma lignorum
Verticillium albo-atrum
Xylaria mali

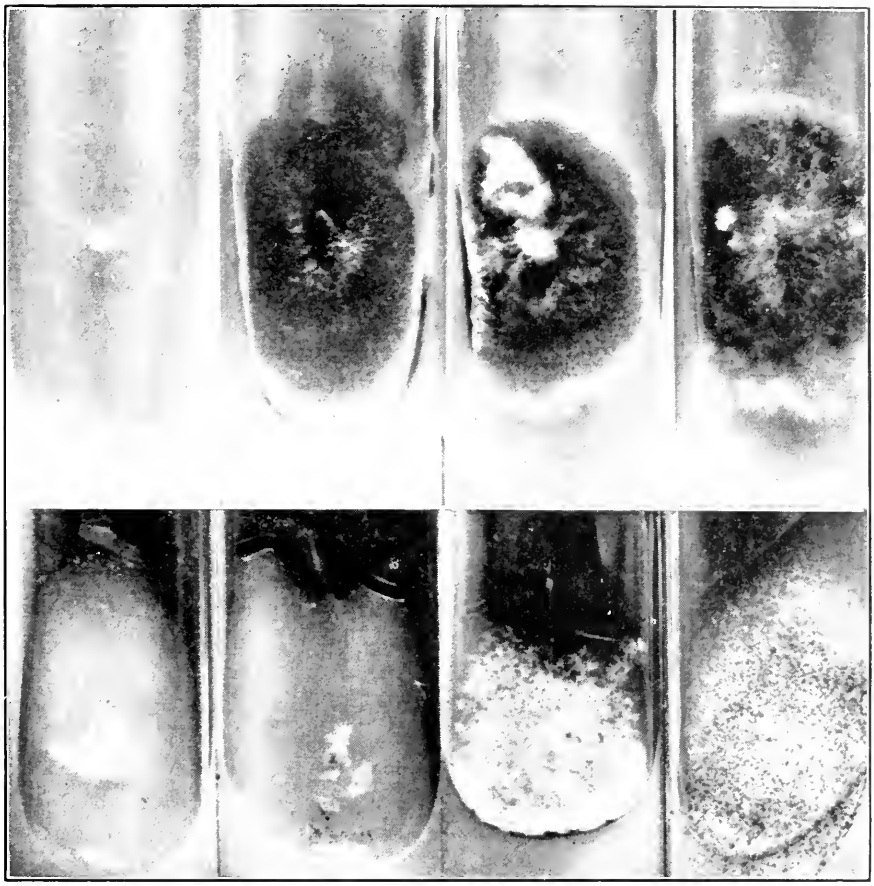
Several of the species in the above list were reported by Young and Bennett (1922) and others by Robbins and Kavanagh (1942). Some reports are found in the papers of various authors, while some of the fungi have been observed in our laboratory (see Fig. 17 for illustrations).

Fungi which utilize ammonium nitrogen. In the nitrogenous compounds found in fungi the nitrogen is in the same state of oxidation as in ammonium compounds. The following is a partial list of fungi which have been reported or observed to require ammonium or organic nitrogen and to be unable to assimilate nitrate nitrogen:

Absidia coerulea
A. cylindrospora
A. dubia
A. glauca
A. orchidis
Basidiobolus ranarum
Ceratostomella fimbriata
C. ulmi
Choanephora cucurbitarum
Cyathus striatus
Endothia parasitica
Lenzites trabea
Marasmius alliaceus
M. androsaceus
M. chordalis
M. epiphyllus
M. foetidis
M. graminum
M. perforis
M. personatus
M. putillus
M. ramealis
M. rotula
M. scorodonius
Monilinia fructicola
Mortierella rhizogena
Mucor flavus
M. hiemalis
M. nodosus
M. pyriformis
M. saturninus
M. stolonifer
M. strictus
Phycomyces blakesleeanus
Pleurotus ostreatus
Rhizophlyctis rosea
Rhizopus nigricans
R. oryzae
Sporodina grandis
Zygorrhynchus moelleri

A number of fungi in the above list were listed by Robbins (1937). The studies on *Marasmius* are reported by Lindeberg (1944). Others are reported by various authors, while some have been observed in our laboratory. Obviously, this list is far from complete, and numerous common fungi have been omitted from both this and the previous list because of lack of definite information regarding their ability to utilize nitrate nitrogen.

Fungi which utilize only organic nitrogen. Certain fungi are unable to utilize nitrogen except in the form of amino acids, peptides, and mixtures of these compounds such as peptone. The use of organic nitrogen does not extend to all organic compounds which contain this element. Many



A *B* *C* *D*

FIG. 17. Growth of two fungi on four media differing in nitrogen source. *A*, no nitrogen added; *B*, potassium nitrate; *C*, ammonium tartrate; *D*, asparagine. Above, *Helminthosporium sativum;* below, *Ceratostomella fimbriata.*

of the early reports claiming utilization of organic nitrogen only by various species have been found to be in error.

All the early work where peptone was the nitrogen source used is to be suspected because the need for growth factors was not recognized. The use of other complex nitrogen sources such as proteins makes interpretation doubtful for the same reason. However, in the case of amino-acid-deficient fungi a portion of the nitrogen source must be supplied in the

form of a particular amino acid. Cantino (1949) found that *Blastocladia pringsheimii* is deficient for methionine and perhaps other amino acids. Presumably other amino acids are used to supply a portion of the metabolic nitrogen of this species. The same situation may exist in the nitrogen utilization of amino-acid-deficient mutants of *Neurospora*. Leonian and Lilly (1938) reported *Coprinus lagopus* and *Pleurotus corticatus* to grow on a mixture of five amino acids and not on ammonium nitrate as a source of nitrogen.

Inorganic sources of nitrogen. The *nitrates* commonly used in preparing media are potassium nitrate, sodium nitrate, and calcium nitrate. These salts are equivalent in so far as they supply the same kind of nitrogen. They are not equivalent in that different cations are involved. Calcium ion may precipitate a varying amount of phosphate, depending upon the concentrations of the two ions and the pH of the medium.

Some fungi utilize *nitrite* (NO_2^-) nitrogen. *Blakeslea trispora* makes some growth on nitrite nitrogen (Leonian and Lilly, 1938). Owing to the instability of nitrites in acid solution and the destructive effect of nitrous acid on proteins and amino acids, nitrite nitrogen is little used in making media. Nitrite is produced by many fungi from nitrate and may accumulate in the medium under certain conditions. The toxic effect is related to the pH of the medium, being greatest at low pH. Wirth and Nord (1942) attributed the accumulation of pyruvic acid in the nitrate medium on which *Fusarium lini* grew to the presence of the nitrite, which inactivated thiamine pyrophosphate (cocarboxylase).

Yeasts utilize nitrate nitrogen poorly as a general rule. Pirschle (1930) studied the relative value of nitrate and ammonium nitrogen for a yeast and concluded that poor utilization of nitrate nitrogen was due in part to the accumulation of nitrite in the medium. This was shown by the yields of aerated and nonaerated cultures on media containing nitrate and ammonium nitrogen as well as by analyses of the culture medium for nitrite. Aeration prevented the accumulation of toxic amounts of nitrite or its decomposition product nitrogen trioxide. In other experiments Pirschle showed that nitrite inhibited the growth of yeast on ammonium nitrogen. By adding sufficient nitrite to a medium containing ammonium sulfate, growth was depressed below that obtained on potassium nitrate. How far these conclusions may be applied to other fungi which do not utilize nitrate nitrogen is not known.

Inorganic and organic *ammonium* salts are equivalent in that they furnish inorganic nitrogen; *i.e.*, ammonium ion. The nitrogen of all ammonium salts is the same, but the physiological effects of the anions are not. The ammonium salts of strong inorganic acids generally tend to make a culture medium more strongly acidic than when an ammonium salt of a weak acid is used. However, the situation is far more compli-

cated than this simple theory would predict. It should be emphasized that nitrates and ammonium salts have opposite effects on the acidity of culture media. Other conditions being equal, as nitrate ions are consumed, the culture medium becomes more alkaline, while as ammonium ions are utilized, the culture medium becomes more acid.

Before considering the ammonium salts of the organic acids, the use of ammonium nitrate should be mentioned. Both ions contain nitrogen, a feature which has led many investigators to use it in media. If a fungus is able to utilize both kinds of nitrogen, the pH of the medium will be somewhat stabilized. This salt should not be used if the purpose of an experiment is to determine whether a fungus can utilize either one or the other or both forms of nitrogen. Some fungi apparently use nitrate nitrogen in preference to ammonium nitrogen when both are supplied in the medium. *Fusarium lini* appears to be such a fungus (Wirth and Nord, 1942).

TABLE 19. THE EFFECT OF VARIOUS ORGANIC ACIDS ON THE GROWTH OF FOUR FUNGI ON MEDIA CONTAINING AMMONIUM NITRATE

Initial pH 5.5. Figures are milligrams of mycelium produced. (Leonian and Lilly, *Am. Jour. Botany* **27,** 1940.)

Organic acids, 0.02*M*	*Mucor ramannianus*	*Phythium ascophallon*	*Pythiomorpha gonapodyoides*	*Phycomyces blakesleeanus*
Control	77	8	8	27
Acetic	144	19	40	144
Lactic	154	34	113	121
Succinic	142	117	157	165
Glutaric	135	23	76	144
Fumaric	158	156	180	189
Tartaric	174	56	112	149
Citric	152	0	0	171

Metarrhizium glutinosum (*Myrothecium verrucaria*) grew well on nitrate nitrogen alone and poorly on ammonium nitrogen (Brian *et al.*, 1947). Ammonium nitrogen inhibited growth of this fungus, whether nitrate was present or not. Growth was equally poor on ammonium nitrate and ammonium sulfate. Since this fungus grew well on media containing nitrate as the sole source of nitrogen, these authors have questioned the common belief that all fungi which are able to utilize nitrate nitrogen can also utilize ammonium nitrogen. Most fungi appear to utilize ammonium nitrogen before nitrate nitrogen when both are supplied in the medium, but this is not universal. Rippel (1931) found the pH of the medium to determine which form of nitrogen was utilized by *Aspergillus niger* and *A. oryzae*. Additional examples are given by Foster (1949).

The utilization of ammonium and some forms of organic nitrogen may

be modified by the presence of other compounds in the medium. Among these the organic acids, especially the four-carbon dicarboxylic acids, play an important role. This subject has been studied by Leonian and Lilly (1940), Burkholder and McVeigh (1940), Brian *et al.* (1947), and Bernhard and Albrecht (1947). The data in Table 19 illustrate the effect of organic acids on the amount of growth of four fungi.

Succinic and fumaric acids were most uniform in their effect on nitrogen assimilation. Figure 18 shows the reciprocal effect of varying amounts of

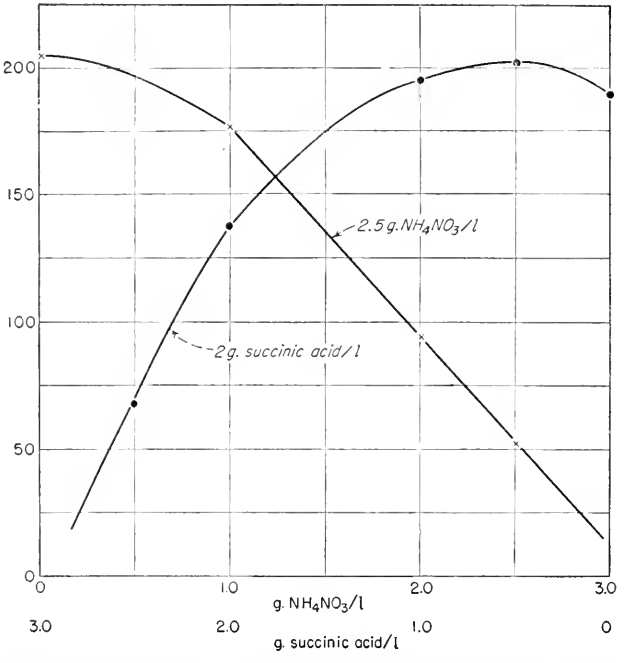


FIG. 18. The reciprocal effect of varying amounts of succinic acid (ammonium nitrate constant) and ammonium nitrate (succinic acid constant) on the growth of *Phycomyces blakesleeanus*. (Drawn from data of Leonian and Lilly, *Am. Jour. Botany* **27**: 22, 1940.)

succinic acid and ammonium nitrogen on the growth of *Phycomyces blakesleeanus*, which does not utilize nitrate nitrogen. The amount of growth, within certain limits, is directly proportional to the amount of succinic acid in the medium.

Brian *et al.* (1947) have suggested on the basis of studies on *Myrothecium verrucaria* that a definite antagonism exists between the metabolic pathways involved in nitrate and ammonium utilization, and in the presence of ammonium nitrogen the nitrate pathway is blocked. Ammonium nitrogen is poorly utilized unless certain organic acids are present in the medium. Malic acid has no effect on utilization of nitrate nitrogen.

These authors suggest that different pathways of carbohydrate utilization may be followed, depending upon whether nitrate or ammonium nitrogen is present.

Organic sources of nitrogen. Of the vast number of organic compounds which contain nitrogen the ones of interest in fungus nutrition are those which occur naturally. A few exceptions will be noted later. In practice, this means proteins and the products of protein hydrolysis. The following steps in protein hydrolysis have been recognized: protein → metaprotein → proteoses → peptones → peptides → amino acids. Peptone, which is a complex mixture of peptides and amino acids, is frequently used as a nitrogen source in media. According to Gortner (1929), peptones are neither coagulated by heat nor precipitated by saturating a solution with ammonium sulfate, properties which distinguish peptones from proteins, metaproteins, and proteoses. Since peptides having some 11 amino-acid residues are precipitated by ammonium sulfate, it may be deduced that the peptides in peptone have on the average 10 or less amino-acid residues. Peptone is a useful source of nitrogen when it is desired to culture a large number of species upon a single medium. A part of its virtue may be ascribed to its complex nature, for a mixture of nitrogen sources may be better utilized than a single source. Peptone also contains most of the water-soluble vitamins (Stokes *et al.*, 1944).

Most of the amino acids which have been isolated from proteins are listed in Table 20. In addition, the amides of aspartic and glutamic acids are included. These compounds are found free in many plants and are thus available to the fungi in nature.

These amino acids are not of equal value in fungus nutrition. The relative value of 24 amino acids for 14 fungi was tested by Leonian and Lilly (1938) who found no one amino acid was best for all these species. Steinberg (1942) made an extensive study of growth of *Aspergillus niger* on 22 amino acids. Seven were excellent sources of nitrogen for *A. niger:* alanine, arginine, aspartic and glutamic acids, glycine, proline, and hydroxyproline. Steinberg expressed the opinion that the seven amino acids which supported the most growth of *A. niger* are those which are synthesized first (primary amino acids) by this fungus and from which the other amino acids (secondary amino acids) are normally formed. It is assumed that the "primary" amino acids enter directly into the metabolic pathways, while the "secondary" amino acids must undergo preliminary deamination before use. The primary amino acids are probably not the same for all fungi. Lilly and Leonian (1942) investigated the effect of nitrogen source on the growth of 10 strains of *Saccharomyces cerevisiae.* The data in Table 21 show clearly that different amino acids vary in effectiveness, and that different strains of the same organism respond differently to the same source of nitrogen.

TABLE 20. COMMON NAMES AND FORMULAS OF SOME ALPHA-AMINO ACIDS ISOLATED FROM PROTEINS AND OF SOME AMIDES FOUND IN PLANTS

Monoamino dicarboxylic acids:

Aspartic acid: $HOOC—CH_2—CH(NH_2)—COOH$

Glutamic acid: $HOOC—CH_2—CH_2—CH(NH_2)—COOH$

Amides of monoamino dicarboxylic acids:

Asparagine: $NH_2OC—CH_2—CH(NH_2)—COOH$

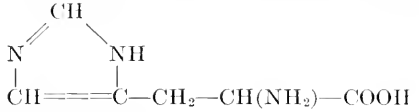
Glutamine: $NH_2OC—CH_2—CH_2—CH(NH_2)—COOH$

Basic amino acids:

Arginine*: $NH_2—C(=NH)—NH—CH_2—CH_2—CH_2—CH(NH_2)—COOH$

Lysine*: $NH_2—CH_2—CH_2—CH_2—CH_2—CH(NH_2)—COOH$

Histidine*:



Monoamino monocarboxylic acids:

Glycine: $CH_2(NH_2)—COOH$

Alanine: $CH_3—CH(NH_2)—COOH$

Valine*: $(CH_3)_2—CH—CH(NH_2)—COOH$

Leucine*: $(CH_3)_2—CH—CH_2—CH(NH_2)—COOH$

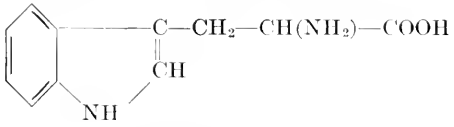
Isoleucine*: $CH_3—CH_2—CH(CH_3)—CH(NH_2)—COOH$

Phenylalanine*: $C_6H_5—CH_2—CH(NH_2)—COOH$

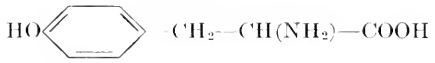
Serine: $CH_2(OH)—CH(NH_2)—COOH$

Threonine*: $CH_3—CH(OH)—CH(NH_2)—COOH$

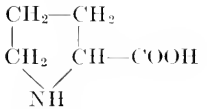
Tryptophane*:



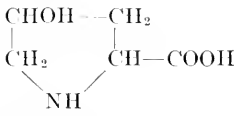
Tryosine:



Proline:



Hydroxyproline:



Sulfur-containing amino acids:

Cysteine: $CH_2(SH)—CH(NH_2)—COOH$

Cystine: $HOOC—CH(NH_2)—CH_2—S—S—CH_2—CH(NH_2)—COOH$

Methionine*: $CH_2(SCH_3)CH_2—CH(NH_2)—COOH$

* The 10 amino acids reported by Rose (1938) as essential for the nutrition of the white rat.

Physiological specificity extends to the configuration as well as the composition of the molecule. Optical isomers (enantiomorphs) usually have different physiological properties. A mixture of amino acids may or may not be utilized better than a single amino acid. The effect of one amino acid on the utilization of another varies with the amino acids

involved and the specific fungus used. Leonian and Lilly (1940) tested the growth of *Phycomyces blakesleeanus* upon five single amino acids and upon a mixture of these five amino acids with the following results: mixture of five amino acids, 214; asparagine, 209; DL-alanine, 151; arginine, 50; aspartic acid, 203; glycine, 201; and glutamic acid, 189 mg., respectively. Arginine is a poor nitrogen source for *P. blakesleeanus*, but the presence of arginine in the amino-acid mixture did not depress growth. More complex relations were found with yeast (Lilly and Leonian, 1942). Ten strains of yeast were grown upon media containing a mixture of six amino acids (aspartic and glutamic acids, arginine, asparagine, alanine, and leucine). Upon this mixture of amino acids two strains grew as well as or better than upon the best single amino acid (aspartic acid). The

TABLE 21. COMPARISON OF VARIOUS SOURCES OF NITROGEN FOR SIX STRAINS OF YEAST

Milligrams of dry yeast cells produced in 72 hr. Each culture received 8 mg. of N. (Lilly and Leonian, *Proc. West Va. Acad. Sci.* **16**, 1942.)

Nitrogen source	Yeast strain					
	2	3	4	5	6	7
Ammonium sulfate	18.7	21.2	22.3	17.5	21.7	23.8
Urea	33.2	31.5	32.9	27.3	32.0	35.1
L-Aspartic acid	60.7	59.9	65.6	62.0	52.4	70.6
L-Aspargine	49.4	45.8	50.0	49.2	47.6	35.0
Glycine	3.0	1.2	2.0	2.1	1.0	1.1
DL-Norleucine	29.3	17.6	33.0	18.4	1.2	4.2

amount of growth of one strain was 70.6 mg. on aspartic acid alone, while on the amino-acid mixture only 38.6 mg. was produced. Omission of asparagine from the mixture increased the yield to 52.0 mg. These results show that the effects of multiple nitrogen sources upon growth, and perhaps other functions, are complex.

Organic acids, especially the four-carbon dicarboxylic acids, affect the utilization of some amino acids much as they do that of ammonium compounds. *Phycomyces blakesleeanus* on a medium containing arginine produced 43 mg. of mycelium per flask. Addition of 0.1 per cent succinic acid to the medium increased the yield to 192 mg. (Leonian and Lilly, 1940).

Nitrogen utilization by the fungi has been studied for almost a century, but many of the problems involved are not yet solved. Brenner (1914) has reviewed the early work in this field, especially with reference to the divergent views of Raciborski and Czapek on the mode of utilization of amino acids. Raciborski held that amino acids were deaminated before

utilization, while Czapek believed that amino acids were utilized directly. Both processes are doubtless involved, and only prolonged study of specific fungi and various nitrogen sources will permit elucidation of these questions.

One of the main uses of nitrogen is in the synthesis of proteins. With the exception of certain amino acids (primary amino acids) and ammonia, most nitrogen sources undergo modification before entering the synthetic metabolic pathways. Nitrates, nitrites, and hydroxylamine are presumably reduced to ammonia before assimilation. Those amino acids (secondary amino acids) which do not enter directly into the metabolic pathways leading to the synthesis of protein are probably *deaminated*. Burk and Horner (1939) have listed the types of deamination performed by fungi as follows:

1. Deamination by hydrolysis:

$$R{-}CH(NH_2){-}COOH \xrightarrow{H_2O} R{-}CH(OH){-}COOH + NH_3$$

2. Deamination by hydrolysis followed by decarboxylation:

$$R{-}CH(NH_2){-}COOH \xrightarrow{H_2O} R{-}CH_2OH + CO_2 + NH_3$$

3. Oxidative deamination:

$$R{-}CH(NH_2){-}COOH \xrightarrow{^1/_2O_2} R{-}CO{-}COOH + NH_3$$

The production of higher alcohols, "fusel oil," is due to hydrolytic deamination and decarboxylation of various amino acids, especially leucine, which yields isoamyl alcohol. Various species of filamentous fungi, especially those which produce alcohol, are capable of the same reactions. The following amino acids are converted by yeasts into alcohols having one less carbon than the parent amino acid: leucine, isoleucine, phenylalanine, trytophane, and valine. Wirth and Nord (1942) indicate that *Fusarium lini* oxidatively transforms alanine into pyruvic acid. For further information on the process of deamination by yeast, see Thorn (1937). The process of deamination releases nitrogen in the form of ammonia, which is utilized by most fungi.

It seems probable that the synthesis of amino acids is the next step in protein formation. The formation of primary amino acids may result from the reaction of ammonia with certain alpha-keto acids (pyruvic, oxalacetic, and ketoglutaric); this is essentially the reverse of oxidative deamination. This process may be formulated as follows:

$$R{-}CO{-}COOH + NH_3 \rightarrow R{-}C(=NH){-}COOH + H_2 \rightarrow R{-}CH(NH_2){-}COOH$$

In addition, yeasts are able to add ammonia to fumaric acid to form aspartic acid (Haehn and Leopold, 1937). The role of the four-carbon dicarboxylic acids in nitrogen assimilation may be explained on the basis that these acids are transformed into keto acids. Brian *et al.* (1947) have assumed that those fungi, such as *Phycomyces blakesleeanus* and *Myro-*

thecium verrucaria, which make limited growth on ammonium nitrogen do so because they are unable to synthesize in adequate amounts the necessary three-, four-, and five-carbon keto acids. The interrelation among various dicarboxylic acids is shown in schemes IV, VIII, and IX.

The reactions discussed above account for the synthesis of only a few of the 20 or so amino acids found in fungus protein. Another type of reaction may account for the synthesis of secondary amino acids. This is called the *transamination* reaction and may be represented as follows:

$$R{-}CO{-}COOH + R'{-}CH(NH_2)COOH \rightarrow R{-}CH(NH_2)COOH + R'{-}CO{-}COOH$$

According to Roine (1947), *Torulopsis utilis* has the necessary enzymatic mechanisms for the synthesis of the following amino acids by transamina-

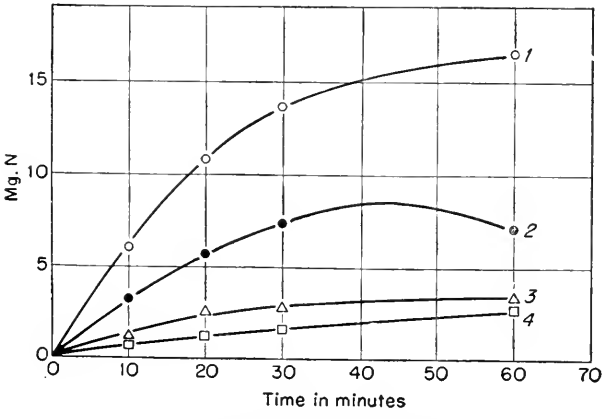


FIG. 19. Amounts of soluble nitrogen compounds found in the trichloroacetic acid extract as a function of time. Data are based on 100 ml. of yeast suspension, or about 5 g. fresh yeast. Curve 1 represents total soluble nitrogen, curve 2 total amide nitrogen, curve 3 alanine nitrogen, and curve 4 dicarboxylic-amino-acid nitrogen. (Courtesy of Roine, *Ann. Acad. Sci. Fennicae* **26**: 63, 1947.)

tion: aspartic acid, glutamic acid, alanine, valine, leucine, and isoleucine. For a general review of the transamination reaction, see Herbst (1944).

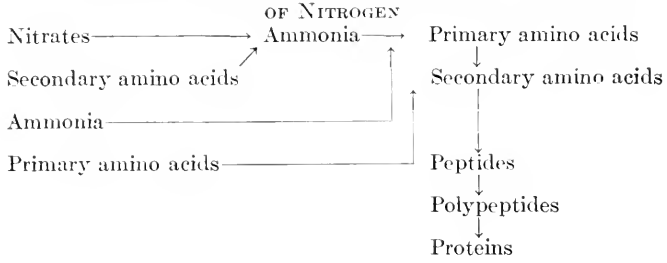
Roine (1947) has obtained experimental evidence which indicates that in *Torulopsis utilis* the primary amino acids are formed first and that the secondary amino acids are then formed from them. This evidence was obtained by analyzing the nonprotein nitrogen fraction which was extracted from cells of various ages with trichloroacetic acid (a protein precipitant). Nitrogen-starved cells of *T. utilis* were suspended in carbohydrate-free medium which contained ammonium nitrogen. The culture was aerated. Every 10 min. a portion of the crop was harvested, and the distribution of nitrogen compounds in the trichloroacetic acid extract was determined. Figure 19 show clearly that the *first stages* of protein synthesis consist in the formation of monoamino dicarboxylic acids, their

amides, and alanine. It may be assumed that the amides of glutamic and aspartic acid function in yeast as nitrogen carriers, as they do in green plants.

Preformed amino acids are probably used in *protein synthesis.* In principle this process is the reverse of hydrolysis. Many complex chemical reactions are involved. Proteins vary in complexity, the simplest having molecular weights in the neighborhood of 16,000 to 17,000. The molecular weight of some proteins is said to be greater than 1,000,000, and tobacco mosaic virus protein is estimated to have a molecular weight of 40,000,000. In spite of these enormous molecular weights, a good deal is known about the structure of proteins. Fundamentally, a protein consists of amino-acid residues joined together by peptide linkages, $-CH_2-NH-CO-$. Since different proteins have highly specific properties which depend upon the molecular structure, the synthesis of these compounds involves a systematic linking together of amino-acid residues in a definite pattern. For reviews of protein structure the reader is referred to Bull (1941) and Astbury (1943).

The general pathways of nitrogen utilization by fungi are shown in scheme III.

Scheme III. Possible Pathways of Protein Synthesis from Various Sources of Nitrogen



OTHER NONMETALLIC ELEMENTS

It is not known whether fungi require nonmetallic elements other than hydrogen, oxygen, sulfur, phosphorus, and nitrogen. Boron and iodine are frequently added to culture media, but good evidence of their essentiality for fungi appears to be lacking. Sodium chloride is frequently added to media, but neither sodium nor chlorine, so far as is known, is essential for the fungi.

In nature fungi come in contact with many nonessential elements. Some of these may be metabolized. Others may modify the life processes of the fungi by their toxic action or by other means. *Chlorine* is found in various compounds synthesized by fungi, *e.g.*, non-ionic chlorine is found in chloramphenicol, one of the newer antibiotics. Many species of fungi metabolize *arsenic. Penicillium brevicaule*, among other species, pro-

duces a volatile, toxic, organic arsenic compound, trimethylarsine, $(CH_3)_3As$, which has an odor resembling garlic. In the past *P. brevicaule* has been recommended for the detection of arsenic compounds in forensic medicine. This microbiological test for the presence of arsenic is said to be many times as sensitive as the Marsh test. The early work on the utilization of arsenic compounds by fungi is reviewed by La Far (1911) and more critically by Challenger *et al.* (1933). *P. brevicaule* also produces dimethyl selenide from selenium compounds (Challenger and North, 1934).

SUMMARY

The classification of essential elements as structural or functional may be misleading in that an element usually plays many roles. This is especially true of the essential nonmetallic elements.

With the exception of carbon dioxide all the organic compounds used by or contained in fungi contain hydrogen. One of the most important hydrogen-containing compounds is water. This compound is associated with proteins in the form of bound water, and it functions as a solvent in which most if not all biochemical reactions take place. Water enters into many reactions, particularly in the hydrolytic processes of "digestion." Apparently fungi do not utilize free hydrogen.

None of the fungi appear to be obligate anaerobes. Many are facultative anaerobes, while some appear to be strict aerobes. Free oxygen is used by the fungi in respiration, chiefly as an acceptor of hydrogen. The facultative anaerobes have another mechanism of oxidation which does not involve free oxygen. This is called anaerobic respiration, or fermentation. The rate and amount of growth and sporulation and the metabolic by-products of a given fungus are affected by the oxygen supply.

The problem of specificity arises in connection with the form of sulfur utilized. Most fungi utilize sulfate sulfur, but some require reduced sulfur. Other species are unable to synthesize specific sulfur-containing amino acids, especially methionine. Sulfur enters into the composition of enzymes and other proteins, peptides, and at least two vitamins.

The fungi utilize phosphorus in the form of phosphate salts and esters. Some specificity in the different sources of phosphate has been found Phosphate esters enter into a wide variety of enzymatic reactions, and many coenzymes are phosphate esters.

It is thought that certain phosphate esters act to transfer chemical energy to certain enzymatic reactions. Phosphorus enters into the composition of proteins, especially the nucleoproteins, which are found in the nucleus or cytoplasm of every cell. Viruses and genes are thought to consist largely of nucleoproteins.

Fungi differ in ability to utilize different forms of nitrogen. A few utilize atmospheric nitrogen; many utilize nitrate nitrogen; and a still

greater number utilize ammonium nitrogen. All species are able to utilize some form of organic nitrogen. Other constituents in media, especially the four-carbon dicarboxylic acids, modify the availability of ammonium nitrogen and certain amino acids. Not all amino acids are of equal value in fungus nutrition. The primary amino acids are those which enter directly metabolic pathways, while secondary amino acids are deaminated before the nitrogen is used.

Most of the nitrogen utilized by fungi enters into the synthesis of proteins. The primary amino acids are formed first, and the secondary amino acids are formed from primary amino acids. Proteins are the most complex compounds synthesized by living cells. Many of the vitamins and other essential metabolites also contain nitrogen.

REFERENCES

Astbury, W. T.: X-rays and the stoichiometry of the proteins, *Advances in Enzymol.* **3**: 63–108, 1943.

Barnes, T. C.: Textbook of General Physiology, The Blakiston Company, Philadelphia, 1937.

Barron, E. S. G.: Mechanisms of carbohydrate metabolism. An essay in comparative biochemistry, *Advances in Enzymol.* **3**: 149–189, 1943.

Bernhard, K., and H. Albrecht: Stoffwechselprodukte des Mikroorganismus *Phycomyces Blakeslecanus* in glucose haltiger Nährlösung und Untersuchungen über das Wachstum dieses Schimmelpilzes bei verschiedenen Stickstoffquellen, *Helv. Chim. Acta* **30**: 627–632, 1947.

Birkinshaw, J. H., W. P. K. Findlay, and R. A. Webb: Biochemistry of wood-rotting fungi. 3. The production of methyl mercaptan by *Schizophyllum commune* Fr., *Biochem. Jour.* **36**: 526–529, 1942.

Bonner, D.: Production of biochemical mutants in *Penicillium*, *Am. Jour. Botany* **33**: 788–791, 1946.

Brenner, W.: Die Stickstoffnahrung der Schimmelpilze, *Cent. Bakt.*, Abt II, **40**: 555–647, 1914.

Brian, P. W.: Studies on the biological activity of griseofulvin, *Ann. Botany* **13**: 59–77, 1949.

Brian, P. W., P. J. Curtis, and H. G. Hemming: Glutinosin: a fungistatic metabolic product of the mould *Metarrhizium glutinosum* S. Pope., *Proc. Roy. Soc.* (*London*), Ser. B, **135**: 106–132, 1947.

Buchanan, R. E., and E. I. Fulmer: Physiology and Biochemistry of Bacteria, Vol. II, the Williams & Wilkins Company, Baltimore, 1930.

Bull, H. B.: Protein structure, *Advances in Enzymol.* **1**: 1–42, 1941.

Burk, D., and C. K. Horner: Nitrogen assimilation by yeast, *Wallerstein Labs. Communs.* **2**: 5–23, 1939.

Burkholder, P. R., and I. McVeigh: Growth of *Phycomyces blakeslecanus* in relation to varied environmental conditions, *Am. Jour. Botany* **27**: 634–640, 1940.

★Cantino, E. C.: The physiology of the aquatic Phycomycete, *Blastocladia Pringsheimii*, with emphasis on its nutrition and metabolism, *Am. Jour. Botany* **36**: 95–112, 1949.

Challenger, F., C. Higginbottom, and L. Ellis: The formation of organo-metalloidal compounds by microorganisms. I. Trimethylarsine and dimethylethylarsine, *Jour. Chem. Soc.* **1933**: 95–101.

CHALLENGER, F., and H. E. NORTH: The formation of organo-metalloidal compounds by microorganisms. II. Dimethylselenide, *Jour. Chem. Soc.* **1934**: 68–71.

DIXON, M.: Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes, Cambridge University Press, New York, 1943.

DORRELL, W. W.: The oxidative respiration of *Fusarium graminearum*, thesis, University of Wisconsin, 1948.

DOX, A. W.: The phosphorus assimilation of *Aspergillus niger*, *Jour. Biol. Chem.* **10**: 77–80, 1911–1912.

★DUGGAR, B. M., and A. R. DAVIS: Studies in the physiology of the fungi. I. Nitrogen fixation, *Ann. Missouri Botan. Garden* **3**: 413–437, 1916.

FELLOWS, H.: The influence of oxygen and carbon dioxide on the growth of *Ophiobolus graminis* in pure culture, *Jour. Agr. Research* **37**: 349–355, 1928.

FOSTER, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

FRIES, N.: X-ray induced parathiotrophy in Ophiostoma, *Svensk Botan. Tidskrift* **40**: 127–140, 1946.

FRIES, N.: Spontaneous physiological mutations in *Ophiostoma*, *Hereditas* **34**: 338–350, 1948.

GORTNER, R. A.: Outlines of Biochemistry, 1st ed., John Wiley & Sons, Inc., New York, 1929.

GORTNER, R. A.: Outlines of Biochemistry, 3d ed., John Wiley & Sons, Inc., New York, 1949.

GOULD, B. S., A. A. TYTELL, and H. JAFFE: Biochemistry of Fusaria. The influence of diphosphopyridine nucleotide on alcoholic fermentation (*in vivo*), *Jour. Biol. Chem.* **146**: 219–224, 1942.

HAEHN, H., and H. LEOPOLD: Ueber eine Aspartasewirkung der Hefe, *Biochem. Zeit* **292**: 380–387, 1937.

HARDEN, A.: Alcoholic Fermentation, Longmans, Roberts and Green, London, 1932.

HAWKER, L. E.: The effect of vitamin B_1 on the utilization of glucose by *Melanospora destruens* Shear., *Ann. Botany* **8**: 79–90, 1944.

HERBST, R. M.: The transamination reaction, *Advances in Enzymol.* **4**: 75–97, 1944.

HEVESY, G.: Some applications of radio-active indicators in turnover studies, *Advances in Enzymol.* **7**: 111–214, 1947.

HOCKENHULL, D. J. D.: The suphur metabolism of mould fungi: the use of "biochemical mutant" strains of *Aspergillus nidulans* in elucidating the biosynthesis of cystine, *Biochim. et Biophys. Acta* **3**: 326–335, 1949.

★HOLLIS, J. P.: Oxygen and carbon dioxide relations of *Fusarium oxysporum* Schlecht. and *Fusarium eumartii* Carp., *Phytopathology* **38**: 761–775, 1948.

LA FAR, F.: Technical Mycology. Vol. II, Eumycetic Fermentation (trans. C. T. C. Salter), Charles Griffin & Co., Ltd., London, 1911.

LEONIAN, L. H., and V. G. LILLY: Studies on the nutrition of fungi. I. Thiamin, its constituents, and the source of nitrogen, *Phytopathology* **28**: 531–548, 1938.

LEONIAN, L. H., and V. G. LILLY: Studies on the nutrition of fungi. IV. Factors influencing the growth of some thiamin-requiring fungi, *Am. Jour. Botany* **27**: 18–26, 1940.

LILLY, V. G., and L. H. LEONIAN: Nitrogen metabolism in *Saccharomyces cerevisiae*, *Proc. West Va. Acad. Sci.* **16**: 60–70, 1942.

LINDEBERG, G.: Ueber die Physiologie Ligninabbauender Bodenhymenomyzeten, *Symbolae Botan. Upsalienses* **8**(2): 1–183, 1944.

MANDELS, G. R., and R. G. H. SIU: Rapid assay for growth: Determination of microbiological susceptibility and fungistatic activity, *Jour. Bact.* **60**: 249–262, 1950.

★Mann, T.: Studies in the metabolism of mould fungi. I. Phosphorus metabolism in moulds, *Biochem. Jour.* **38**: 339–345, 1944.

Mann, T.: Studies in the metabolism of mould fungi. II. Isolation of pyrophosphate and metaphosphate from *Aspergillus niger*, *Biochem. Jour.* **38**: 345–351, 1944*a*.

Mirsky, A. E.: Chromosomes and nucleoproteins, *Advances in Enzymol.* **3**: 1–34, 1943.

Nickerson, W. J., and W. R. Carroll: The effect of culture history on the metabolic activities of cells of *Zygosaccharomyces*, *Jour. Cellular Comp. Physiol.* **22**: 21–32, 1943.

Nord, F. F., and R. P. Mull: Recent progress in the biochemistry of Fusaria, *Advances in Enzymol.* **5**: 165–205, 1945.

Pirschle, K.: Biologische Beobachtungen über Hefewachstum mit besonders Berücksichtigen von Nitraten als Stickstoffquelle, *Biochem. Zeit.* **218**: 412–444, 1930.

Plumlee, C. H., and A. L. Pollard: Studies on bromine-oxidizable sulfur-containing compounds in mold metabolism, *Jour. Bact.* **57**: 405–407, 1949.

Raistrick, H., and J. M. Vincent: Studies in the biochemistry of micro-organisms. 77. A survey of fungal metabolism of inorganic sulphates, *Biochem. Jour.* **43**: 90–99, 1948.

Raulin, J.: Etudes chimiques sur la végétation, *Ann. sci. nat.*, Ser. V, **11**: 93–299, 1869.

Rippel, K.: Quantitative Untersuchungen über die Abhängigkeit der Stickstoffassimilation von der Wasserstoffionenkonzentration bei einigen Pilzen, *Arch. Mikrobiol.* **2**: 72–135, 1931.

★Robbins, W. J.: The assimilation by plants of various forms of nitrogen, *Am. Jour. Botany* **24**: 243–250, 1937.

Robbins, W. J., and V. Kavanagh: Vitamin deficiencies of the filamentous fungi, *Botan. Rev.* **8**: 411–471, 1942.

★Roine, P.: On the formation of primary amino acids in the protein synthesis by yeast, *Ann. Acad. Sci. Fennicae* **26**: 1–83, 1947.

Rose, W. C.: The nutritive significance of the amino acids, *Physiol. Revs.* **18**: 109–136, 1938.

Scheffer, T. C., and B. E. Livingston: Relation of oxygen pressure and temperature to growth and carbon dioxide production in the fungus *Polystictus versicolor*, *Am. Jour. Botany* **24**: 109–119, 1937.

Semeniuk, G.: The dissimilation of glucose by *Chaetomium funicola* Cke. III. Some phosphorus relationships of *Chaetomium funicola*, *Iowa State Coll. Jour. Sci.* **18**: 325–308, 1943–1944.

Siu, R. G. H., and G. R. Mandels: Rapid method for determining mildew susceptibility of materials and disinfecting activity of compounds, *Textile Research Jour.* **20**: 516–518, 1950.

Smith, V. M.: On the mechanism of enzyme action. XXXIX. A comparative study of the metabolism of carbohydrates, in the presence of inorganic and organic phosphates, by *Merulius lacrymans* and *Marasmius chordalis*, *Arch. Biochem.* **23**: 446–472, 1949.

Steinberg, R. A.: Effects of barium salts upon *Aspergillus niger* and their bearing upon the sulfur and zinc metabolism of the fungus in an optimum solution, *Botan. Gaz.* **97**: 666–671, 1936.

Steinberg, R. A.: Growth of fungi in synthetic nutrient solutions, *Botan. Rev.* **5**: 327–350, 1939.

STEINBERG, R. A.: Sulfur and trace-element nutrition of *Aspergillus niger*, *Jour. Agr. Research* **63**: 109–127, 1941.

STEINBERG, R. A.: The process of amino acid formation from sugars by *Aspergillus niger*, *Jour. Agr. Research* **64**: 615–633, 1942.

★STEINBERG, R. A.: Growth of fungi in synthetic nutrient solutions. II. *Botan. Rev.* **16**: 208–228, 1950.

STOKES, J. L., M. GUNNESS, and J. W. FOSTER: Vitamin content of ingredients of microbiological culture media, *Jour. Bact.* **47**: 293–299, 1944.

SUMNER, J. B., and G. F. SOMERS: Chemistry and Methods of Enzymes, 2d ed., Academic Press, Inc., New York, 1947.

TAMIYA, H.: Atmung, Gärung und die sich daran beteiligenden Enzyme von *Aspergillus*, *Advances in Enzymol.* **2**: 183–238, 1942.

TAUBER, H.: The Chemistry and Technology of Enzymes, John Wiley & Sons, Inc., New York, 1949.

TERNETZ, C.: Protoplasma bewegung und Fruchtkörperbildung bei *Ascophanus carneus* Pers., *Jahrb. wiss. Botan.* **35**: 273–312, 1900.

TERNETZ, C.: Ueber die Assimilation des atmosphärischen Stickstoff durch Pilze, *Jahrb. wiss. Botan.* **44**: 353–408, 1907.

THORN, R. S. W.: The assimilation of nitrogen from amino acids by yeast, *Jour. Inst. Brewing* **43**: 288–293, 1937.

TOVE, S. R., H. F. NISS, and P. W. WILSON: Nitrogen fixation by the fungus, *Phoma causarina*, 49th meeting Soc. Am. Bact. (Abs. No. A-26, p. 59), 1949.

UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER: Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Burgess Publishing Co., Minneapolis, 1945.

VOLKONSKY, M.: Sur les conditions de culture et le pouvoir de synthèse de *Saprolegnia* sp. Etude qualitative de l'alimentation carbonée, azotée et sulfurée, *Ann. inst. Pasteur* **50**: 703–730, 1933.

VOLKONSKY, M.: Sur l'assimilation des sulfates par les champignons: euthiotrophie et parathiotrophie, *Compt. rend. acad. sci.* **197**: 712–714, 1933*a*.

VOLKONSKY, M.: Sur la nutrition de quelques champignons saprophytes et parasites, *Ann. inst. Pasteur* **52**: 76–101, 1934.

WILSON, P. W.: The Biochemistry of Symbiotic Nitrogen Fixation, University of Wisconsin Press, Madison, 1940.

WIRTH, J. C., and F. F. NORD: Essential steps in the enzymatic breakdown of hexoses and pentoses. Interaction between dehydrogenation and fermentation, *Arch. Biochem.* **1**: 143–163, 1942.

WOLF, F. A., and F. T. WOLF: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

YOUNG, H. C., and C. W. BENNETT: Growth of some parasitic fungi in synthetic culture media, *Am. Jour. Botany* **9**: 459–461, 1922.

CHAPTER 7

CARBON SOURCES AND CARBON UTILIZATION

Carbon occupies a unique position among the essential elements required by living organisms. Almost half of the dry weight of fungus cells consists of carbon. Protoplasm, enzymes, the cell wall, and reserve nutrients stored within the cells are compounds of carbon. Carbon compounds are equally important in fugus nutrition. Fungi secure energy by oxidizing organic compounds. In addition to being the main structural elements, carbon compounds play an equally important functional role. The number of carbon compounds known far exceeds the total of known compounds of all the other elements, because of the property of carbon of forming compounds in which carbon is linked to carbon in the form of chains and rings. Various other elements such as nitrogen, oxygen, and sulfur may serve as linking elements. While many carbon compounds are stable at ordinary temperatures, others are extraordinarily sensitive to a wide range of chemical reagents and to slight changes in the physical environment.

Organic compounds differ in composition, structure, and configuration. These are key factors which must be considered in relation to utilization of organic compounds by fungi. Since more is known about carbohydrates and related compounds as carbon sources, and about the manner in which they are dissimilated and assimilated, than about any other class of organic compounds, most of the discussion in this chapter will be devoted to these topics. In the main, only naturally occurring organic compounds will be considered.

MONOSACCHARIDES AND RELATED COMPOUNDS

The simple sugars, or monosaccharides, have the general formula $C_n(H_2O)_n$. The carbon chain is unbranched except in a few, very rare sugars. The functional groups present are primary (—CH_2OH) and secondary (—CHOH—) alcohol groups, and an aldehyde (—CHO) or ketone (—CO—) group, actual or potential, is always present. The primary alcohol and aldehyde groups are restricted to the end positions of the carbon chain, while the ketone group is usually on the second

carbon in the chain. Sugars having an aldehyde group are called *aldoses*, those having ketone group, *ketoses;* the ending *-ose* denotes a sugar. In addition, the sugars are further classified according to the number of carbon atoms in the chain, *e.g.*, pentoses, hexoses, or more specifically as aldopentoses, ketohexoses, etc. While it will be necessary in the discussion to follow to include some information about the chemistry and structure of the sugars, the reader is advised to consult suitable texts for further information. Those of Gilman (1943) and Pigman and Goepp (1948) are recommended.

Compounds which have the same composition and the same molecular weight are called *isomers*. There are 16 aldohexoses (32, if the alpha and beta forms are considered), which have the same percentage composition and the same functional groups as glucose (dextrose). There are eight possible ketohexoses isomeric with fructose. Two kinds of isomers exist among the sugars: First, there are those which have the same physical properties but differ in the direction in which they rotate plane-polarized light (*enantiomorphs*). Isomers of this kind occur in pairs, and the configuration of the functional groups of one isomer is the mirror image of the configuration of the other. Enantiomorphs usually differ physiologically. One such isomer may be utilized and the other not, or one may be utilized much more rapidly than the other. Pasteur (1860) was the first to demonstrate that fungi are able to distinguish between such isomers. *Penicillium glaucum* utilized *d*-tartrate more rapidly than *l*-tartrate (*d* and *l* refer to optical rotation). Second, there are those isomers which, although they have the same functional groups, have these groups arranged in a different order, so that one isomer is not the mirror image of the other (diastereoisomers). It is usually safe to assume that one member of a pair of enantiomorphs will be better utilized than the other, but such an assumption about utilization of diastereoisomers is not possible.

Since not all sugars of a group such as the aldohexoses are utilized by fungi, it is of interest to compare chemical structure or configuration with utilization. Not all fungi are able to utilize exactly the same sugars (Fig. 20). Whether a sugar is utilized or not depends upon both the configuration of the sugar and the particular abilities of the specific fungus. By configuration is meant the spatial arrangement of the hydrogen and hydroxyl groups. The long history of chemical investigation which established the configuration of the simple sugars must be passed by. Inasmuch as glucose is the key compound in sugar chemistry, as well as in physiology, particular emphasis will be devoted to this aldose.

The structures of the glucose enantiomorphs are given at the top of page 119.

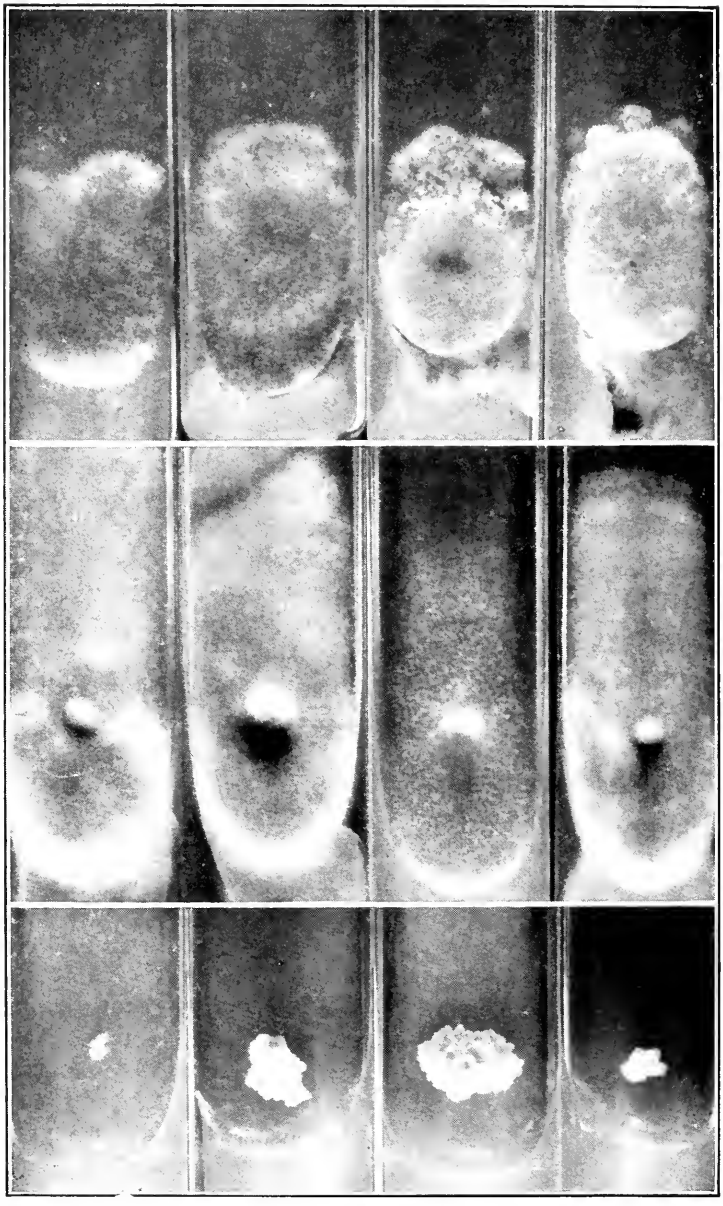
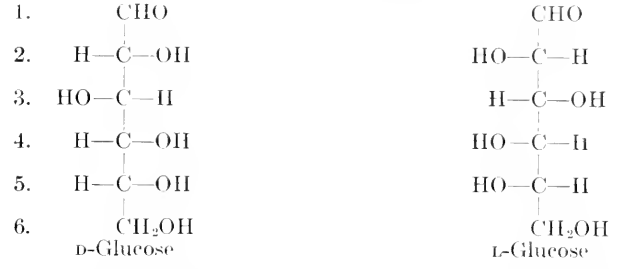
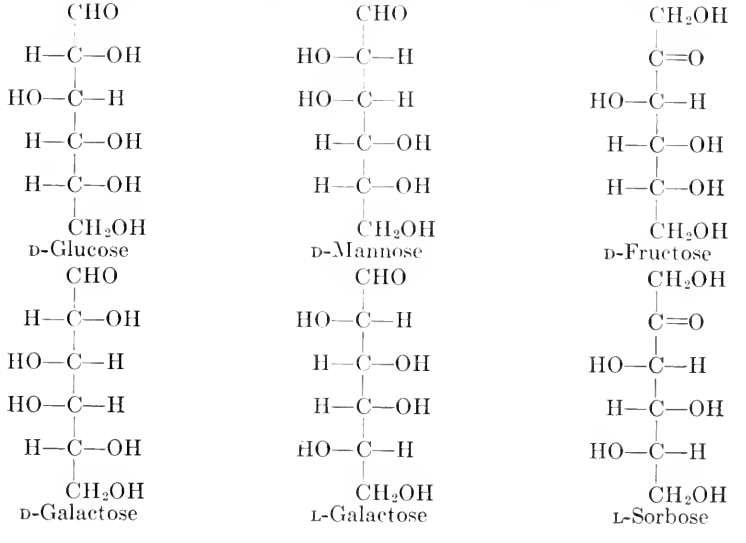


FIG. 20. Growth of three fungi on four sugars. *A*, glucose; *B*, fructose; *C*, sucrose; *D*, maltose. Top row, *Monilinia fructicola* (8 days); middle, *Mucor ramannianus* (8 days); bottom, *Ustilago striiformis*, fragmenting strain (20 days).

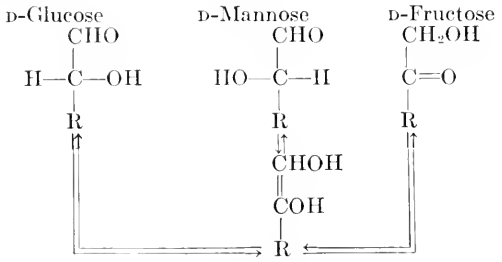


The letters D and L indicate that these sugars belong to different series; they do not indicate optical rotation. The small letters *d* and *l* have been used in the past to express two separate ideas, optical rotation or configuration. The use of *d* and *l* in the old literature makes it difficult at times to discover which enantiomorph was meant. The configuration of the secondary hydroxyl group farthest from the carbonyl group determines to which series a sugar belongs. D-Glucose is the form which occurs naturally and is meant when glucose is used without qualification. Not all naturally occurring sugars belong to the D series; *e.g.*, L-arabinose. For the sake of clearness and accuracy, the series designation should always be used where there is any chance of confusion and misinterpretation. Pigman and Goepp (1948) point out that only sugars of the galactose type occur naturally as both enantiomorphs. D-Galactose is fermented by some yeasts, while L-galactose is not.

Hexoses. The following hexoses occur naturally: D-glucose, D-mannose, D-galactose, L-galactose, D-fructose, and L-sorbose. It is doubtful if L-sorbose occurs in green plants, but it is formed from sorbitol by bacterial (*Acetobacter suboxydans*) oxidation (Bertrand, 1904).



The configuration of glucose, mannose, and fructose is the same for carbons 3 to 6. In the presence of dilute alkali these sugars undergo enolization to produce the same enol form.



Common enol form

Other effects of alkali and heat on sugars were noted in Chap. 2.

Many fungi will utilize these three sugars if configuration is important in determining availability. However, these sugars are not equivalent for all fungi. The fact that galactose is not utilized by all fungi which utilize the three closely related sugars is illustrated by the data in Table 22.

Glucose is utilized by more fungi than 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 which are unable to utilize glucose, or any sugar, as a carbon source. *Leptomitus lacteus* (Schade, 1940; Schade and Thimann, 1940) is unable to utilize glucose, fructose, galactose, or sucrose. Skoog and Lindegren (1947) have reported the behavior of 12 strains of *Saccharomyces cerevisiae* which did not utilize glucose when first isolated. These strains became adapted to glucose on sufficiently long exposure to this sugar. Cheo (1949) found certain isolates of *Ustilago striiformis* to be unable to grow on glucose when freshly transferred from a sucrose medium. After 2 to 4 weeks these isolates began to grow. This behavior suggests the formation of an adaptive enzyme which was not formed when these isolates were cultured on sucrose medium. Some fungi, such as *L. lacteus*, apparently lack the ability to form adaptive enzymes for glucose utilization and must be classed as absolutely incapable of glucose utilization, while the yeasts of Skoog and Lindegren and the isolates of *U. striiformis* are facultatively able to utilize glucose. The differences among these fungi probably lie in the ability to form adaptive enzymes.

No carbon source can be utilized if the medium is lacking in any essential element or compound. Kinsel (1937) and Stevens and Larsh (1939) reported that *Diplodia macrospora* would grow only on disaccharides and not on media containing glucose or other monosaccharides.

The explanation of this anomalous situation was given by Margolin (1940) and confirmed by Wilson (1942), who found that *D. macrospora* was deficient for biotin. It is probable that other vitamin-deficient fungi have been reported in the past as unable to utilize certain sugars owing to the absence of specific growth factors. Negative results reported in the literature are therefore to be viewed with caution.

Wolf and Shoup (1943) studied the oxidation of carbohydrates by *Allomyces arbuscula*, *A. javanicus*, *A. moniliformis*, and *A. cystogenus*. All four species oxidized dextrin (degraded starch), while *A. arbuscula* oxidized maltose and sucrose in addition. The other common naturally occurring sugars, including glucose and fructose, were not oxidized. It has since been shown that *A. arbuscula* is deficient for methionine and thiamine (Yaw, 1950).

While there is an immense amount of information scattered throughout the literature to the effect that a certain sugar is utilized by various species, much of this information deals with relatively few sugars. Critical studies on the utilization of the sugars are rare. Margolin (1942) studied the amount of growth of 21 fungi on four hexoses. These data (Table 22) were obtained under uniform conditions. A mixed nitrogen source (ammonium nitrate and amino acids) was used, and vitamins were supplied to the deficient fungi. The time chosen for harvest in this study was the time maximum weight was attained on glucose. This work suffers from the common defect that the yields are compared on the basis of a fixed time of harvest. The ideal way of determining the value of different sugars for fungi would be to study both the rate and amount of growth as a function of time of incubation.

The following generalizations about utilization of the common hexoses may be drawn from the data in Table 22: (1) There is no single sugar which supports the maximum amount of growth for all of these fungi. (2) All of these fungi utilize glucose, although the maximum amount of growth was not always attained on this sugar. (3) The more closely the configuration of another sugar approaches that of glucose, the more fungi utilize it. It is believed that these generalizations are valid for all fungi which utilize sugars.

Steinberg (1939) found D-glucose, D-fructose, D-mannose, L-sorbose, and sucrose to be equally effective in the nutrition of *Aspergillus niger* while D-galactose, lactose, glycerol, and mannitol were poor sources of carbon for this fungus. Herrick (1940) reported that two isolates of *Stereum gausapatum* grew on glucose, fructose, mannose, and galactose. One isolate made significantly better growth on fructose; the other grew equally well on all four sugars. This indicates that not all isolates of a species are alike in ability to utilize a given sugar. The utilization of different carbon sources by *A. oryzae* was investigated in detail by Tamiya

(1932). This paper should be consulted for the experimental details and references to the literature. One hundred twenty-three carbon compounds were investigated, and of the hexoses, mannose supported

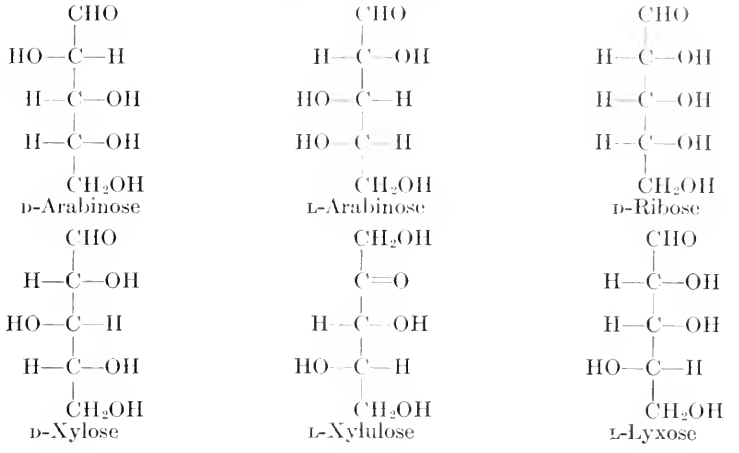
TABLE 22. MILLIGRAMS OF MYCELIUM PRODUCED BY 21 FUNGI GROWN ON MEDIA CONTAINING DIFFERENT SUGARS

All the sugars were used at a rate which supplied 8 g. of carbon per liter. Each 125-ml. flask contained 20 ml. of medium. Cultures were incubated at 25°C. Each weight in the table is the average of 12 cultures. (Margolin, thesis, West Virginia University, 1942.)

Fungus	Days of incubation	Mg. mycelium						
		D-Glucose	D-Fructose	D-Mannose	D-Galactose	Maltose	Sucrose	Lactose
Blakeslea trispora	6	91	94	98	123	113	10	7
Diplodia macrospora	15	83	55	71	55	94	58	21
D. natalensis	8	199	154	89	50	190	199	17
Fusarium lycopersici	6	108	101	100	126	119	74	18
Helicostylum pyriforme	5	126	81	126	99	102	11	40
Helminthosporum sativum	8	75	128	83	46	96	100	40
Mucor ramannianus	8	89	118	115	116	128	12	124
Pilaira moreaui	7	40	32	45	44	44	11	44
Phycomyces blakesleeanus	7	138	130	139	74	101	111	6
Phytophthora cactorum	14	119	40	16	11	157	77	4
P. erythroseptica	12	79	20	81	17	114	93	10
P. fagopyri	6	89	51	19	11	20	130	13
Pythiomorpha gonapodyoides	6	152	122	79	14	76	142	12
Pythium ascophallon	6	85	56	84	10	111	116	27
Rhizopus nigricans	4	121	114	117	121	121	7	5
R. suinus	6	130	128	136	135	30	12	8
Rosellinia arcuata	6	73	58	49	33	63	38	34
Sordaria fimicola	6	121	162	147	28	127	16	52
Syncephalastrum racemosum	5	131	141	126	140	132	15	13
Thielavia basicola	10	60	54	55	78	57	61	6
Typhula variabilis	12	181	122	113	23	202	126	15

the most growth. Quantitative data on the utilization of L-sorbose by fungi is less abundant than for the other hexoses. Observations in this laboratory indicate that many fungi either do not utilize sorbose or do so slowly.

Pentoses. The pentoses shown below occur naturally, mostly in the form of polysaccharides or other complex compounds. L-arabinose and D-xylose are the most easily available and have been more extensively used than the other pentoses. The formulas for the naturally occurring pentoses are given below:



Aspergillus niger utilizes D-xylose and L-arabinose but not their enantiomorphs, as is shown in Table 23. Many of the pentoses listed in Table 23 are difficult to obtain in quantity, which accounts for the varied amounts used per culture.

TABLE 23. THE AMOUNT OF GROWTH AND SPORULATION OF *Aspergillus niger* ON VARIOUS PENTOSES

Time of incubation, 4 days. Cultures incubated at 35°C. (Steinberg, *Jour. Agr. Research* **64,** 1942.)

Pentose	Pentose, g. per culture	Mg. mycelium	Sporulation
D-Lyxose	1.0	0.2	0
D-Xylose	2.0	860.2	10
L-Xylose	0.5	6.2	1
D-Arabinose	2.0	0	0
L-Arabinose	2.0	205.1	6
D-Ribose	0.25	0	0
L-Ribose	0.25	5.2	0

Herrick (1940) found *Stereum gausapatum* to utilize xylose better than arabinose, while *Aspergillus oryzae* utilizes arabinose better than xylose, (Tamiya, 1932). *Lentinus lepideus* utilizes xylose (Nord and Vitucci, 1947). A comparative study of five fungi on xylose and arabinose indicated that xylose was utilized either more completely or more rapidly than arabinose (Margolin, 1942). The data of Margolin are given in Table 24; for comparable growth of these species on other sugars, see Table 22.

Methylpentoses. D-Isorhamnose, L-fucose, and L-rhamnose are related to D-glucose, L-galactose, and L-mannose in that carbon 6 with its primary alcohol group in these hexoses has been replaced by a methyl group. These methylpentoses have not been thoroughly investigated in nutritional studies involving many fungi. *Aspergillus niger* utilizes L-rhamnose to some extent, but L-fucose is not utilized (Steinberg, 1942). *A. oryzae* makes much poorer growth on L-rhamnose than on D-xylose or

TABLE 24. MILLIGRAMS OF MYCELIUM PRODUCED BY FIVE FUNGI GROWN UPON XYLOSE AND ARABINOSE

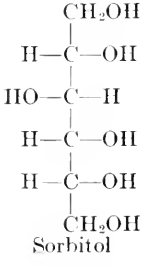
These sugars were used at concentrations which supplied 8 g. of carbon per liter. Each 125-ml. flask contained 20 ml. of medium. Cultures were incubated at 25°C. (Margolin, thesis, West Virginia University, 1942.)

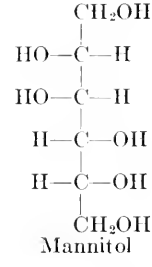
Fungus	Days of incubation	D-Xylose*	L-Arabinose
Blakeslea trispora	6	77	49
Mucor ramannianus	8	77	74
Phycomyces blakesleeanus	7	126	85
Phytophthora erythroseptica	12	15	7
Pythiomorpha gonapodyoides	6	33	18

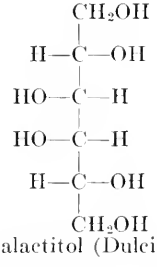
* This sugar was called *l*-xylose in the earlier literature.

L-arabinose (Tamiya, 1932). Of the five fungi listed in Table 24 only *Mucor ramannianus* utilizes L-rhamnose (Margolin, 1942). *Stereum gausapatum* utilizes rhamnose about as well as arabinose (Herrick, 1940).

Sugar alcohols. Reduction of the aldehyde or keto group of the simple sugars converts them into alcohols. Several sugar alcohols are widely distributed in nature. Only the formulas for three of the naturally occurring sugar alcohols will be given.


Sorbitol


Mannitol


Galactitol (Dulcitol)

Most fungi appear to utilize the corresponding sugars with greater facility than the sugar alcohols. Data for the comparative growth of five fungi on these sugar alcohols and the parent sugars are given in Table 25.

Sugar acids. Three types of sugar acids may be produced from aldoses by oxidizing the terminal groups. Oxidation of the aldehyde group yields aldonic acids, such as D-gluconic acid from glucose, while oxidation of the primary alcohol group yields uronic acids, such as D-galacturonic acid from D-galactose. Oxidation of both the aldehyde and primary alcohol groups yields saccharic acids. The uronic acids are widely distributed in natural polysaccharides such as plant gums and mucilages and in pectin. The fungi in nature must frequently come in contact with uronic acids, but data on utilization of these and other sugar acids are rare. Steinberg (1942) cultured *Aspergillus niger* on media which con-

TABLE 25. MILLIGRAMS OF MYCELIUM PRODUCED BY FIVE FUNGI GROWN UPON GLUCOSE, MANNOSE, AND GALACTOSE AND THE CORRESPONDING SUGAR ALCOHOLS

These compounds were used at a rate which supplied 8 g. of carbon per liter. Each 125-ml. flask contained 20 ml. of medium. Cultures were incubated at 25°C. (Margolin, thesis, West Virginia University, 1942.)

Fungus	D-Glucose	Sorbitol	D-Mannose	Mannitol	D-Galactose	Galactitol
Blakeslea trispora	90	12	98	9	123	10
Mucor ramannianus	89	93	115	149	116	6
Phycomyces blakesleeanus	138	59	139	108	74	6
Phytophthora erythroseptica	79	10	81	8	17	8
Pythiomorpha gonapodyoides	152	13	79	9	14	10

tained 1 g. of the calcium salts of the following sugar acids per culture (the weight of mycelium in milligrams is given in parentheses): 2-keto-D-gluconic (201), 5-keto-D-gluconic (25), D-gluconic (32), D-glucuronic (206), and mucic (102). Tamiya (1932) reports that *A. oryzae* utilizes D-gluconic acid. While such compounds as the sugar acids are little used in making media, they are of interest in attempting to discover the relation between structure and configuration on the one hand and utilization on the other.

Mixed carbon sources. In nature the fungi usually come in contact with mixed carbon sources rather than a single source of carbon. Certain fungi make more growth when supplied with a mixture of carbon sources. This increased utilization may be expected only if one or both carbon sources are poorly utilized. Horr (1936) investigated the growth of *Aspergillus niger* upon mixtures of glucose and galactose. Some of these data are given in Table 26. If these two carbon sources were utilized independently, and without one affecting the utilization of the other, the weight of mycelium produced on the combination of 18 g. of galactose and 2 g. of glucose should be 42.4 + 145.6, or 188 mg. The actual yield was 577.4 mg. The experiment indicates that *A. niger* is able to

utilize galactose to better advantage in the presence of glucose. The experiments of Steinberg (1939) on the effect of two poor carbon sources on the growth of *A. niger* were made at 35°C. Some combinations of poor carbon sources supported more growth than when these sources were used singly. Thus, the calculated weight of mycelium for the combination, D-mannitol–lactose was 21.4; the actual yield was 233.6 mg. Some combinations of poor carbon sources resulted in a decrease in amount of mycelium formed (glycerol–D-galactose: calculated yield, 243.7 mg.; actual yield, 154.7 mg.). The effect of mixed carbon sources in the amount of growth of *Phycomyces blakesleeanus* and *Pythiomorpha gonapodyoides* appeared to be purely additive (Margolin, 1942).

TABLE 26. THE EFFECT OF GALACTOSE AND GLUCOSE, SINGLY AND IN COMBINATION, UPON THE AMOUNT OF GROWTH OF *Aspergillus niger*

Cultures incubated 7 days at 20°C. (Horr, *Plant Physiol.* **11**, 1936.)

Grams of Sugars Used per Liter	Yield, Mg. per Culture
10 galactose	45.1
18 galactose	42.4
20 galactose	44.3
2 glucose	145.6
10 glucose	411.0
18 galactose + 2 glucose	577.4
10 galactose + 10 glucose	1,151.6

All these results indicate that the effect of mixed carbon sources is highly specific. A mixture of poor carbon sources may or may not result in increased growth, depending on the carbon sources involved as well as the fungus concerned.

The favorable effects of mixtures of poor carbon sources on the rate and amount of growth have been ascribed to the ease with which a fungus is able to synthesize certain key intermediates. If the synthesis of intermediate X from carbon source A is slow and difficult, and the synthesis of X is rapid from carbon source B, it is probable that growth will be more rapid on media which contain both carbon sources.

ORGANIC ACIDS

An organic acid is characterized by having one or more carboxyl (—COOH) groups. Some organic acids are utilized as sources of carbon and in other ways. Two series of organic acids are especially interesting from the standpoint of physiology. The fatty acids are monocarboxylic acids; the higher members, when esterified with glycerol, form fats. The dicarboxylic acids, especially those which contain four carbon atoms, enter into the metabolic pathways of the fungi in various ways; *e.g.*, utilization of ammonium nitrogen (Chap. 6).

The form in which an organic acid exists (free acid or salt) is a function of the pH of the medium or cells. The free acid is the predominant form at low pH values. The terms for an acid and its salt (*e.g.*, fumaric acid, fumarate) are used in the literature somewhat loosely. The effect of a free acid and its anion may be different (Chap. 8).

Leptomitus lacteus, which does not utilize sugars, grows on various fatty acids—acetic, butyric to capric—but not on formic or propionic acids (Schade, 1940). *Apodachlya brachynema* utilizes the same fatty acids as *L. lacteus* and also, fumarate, succinate and malate. *Aspergillus niger*, according to Steinberg (1942), makes some growth on acetate, lactate, tartrate, malate, and fumarate. Growth was very poor compared with that on sucrose. Dulaney (1949) reported that little streptomycin was produced when organic acids were used by *Streptomyces griseus*. Yeasts use acetate to synthesize fat (White and Werkman, 1947). Tamiya (1932) investigated the utilization of many organic acids by *Aspergillus oryzae*. Growth was poor on most of these compounds except quinic acid. While an organic acid may serve as the sole source of carbon for fungi, in general acids do not allow as much or as rapid growth as carbohydrates.

An amino acid may serve as a source of both nitrogen and carbon. Peptone may serve as a source of carbon and nitrogen for many fungi. *Aspergillus niger*, when grown on peptone as the sole source of carbon, deaminates the peptides and amino acids and releases ammonia in quantities greater than the fungus can use. The utilization of amino acids as carbon sources by *A. niger* was investigated by Steinberg (1942*a*), who found certain combinations of "primary" amino acids to be utilized about three-fourths as efficiently as sucrose.

The utilization of individual amino acids by *Penicillium roqueforti* and *Fusarium oxysporum* var. *lycopersici* was studied by Gottlieb (1946). Not all the naturally occurring amino acids were utilized as carbon sources by these fungi. The six-carbon straight-chain amino acids norleucine and lysine and the sulfur-containing amino acids cysteine and methionine were not utilized as carbon sources. Glycine and valine were poor carbon sources for *P. roqueforti*, while *F. oxysporum* var. *lycopersici* grew well on these amino acids. *Alternaria solani*, *Helminthosporium sativum*, *Rhizoctonia solani*, *Fusarium moniliforme*, *Chaetomium globosum*, and *Aspergillus niger* were unable to utilize the naturally occurring sulfur-containing amino acids as a source of carbon.

Yeasts differ in ability to utilize different amino acids as the sole source of carbon (Schultz *et al.*, 1949). Glutamic acid and proline were available to more species than other amino acids. It is characteristic of fungi cultivated on amino-acid media as the sole source of carbon that the medium becomes alkaline. This is probably due to accumulation of

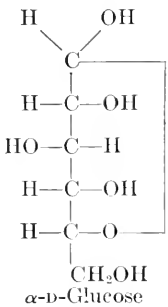
ammonia which results from deamination. In general, the amino acids appear to be poor sources of carbon.

GLYCOSIDES

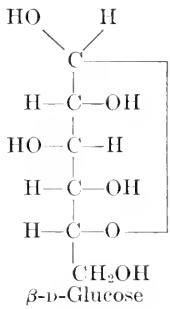
The carbon sources to be discussed in this and the next two sections differ from those previously considered in that they undergo hydrolysis. The complex carbohydrates and carbohydrate-like compounds yield simple sugars when hydrolyzed. In some instances, other compounds are also formed. In most instances, fungi utilize these compounds only after hydrolysis. Therefore, utilization will be dependent upon the production of the necessary hydrolytic enzymes. If a fungus is unable to perform this preliminary "digestion," such complex carbohydrates will be unavailable.

Many of the compounds to be considered in this section are isomers. The simple sugars exist mainly in the form of ring structures, rather than the open-chain forms which were depicted in the previous sections of this chapter. The chemical evidence may be reviewed in Pigman and Goepp (1948) or other text dealing with the sugars. Glucose exists in aqueous solution as an equilibrium mixture of α-D-glucose and β-D-glucose. These formulas contain a six-membered ring of which one atom is oxygen (pyranose). Some sugars, however, contain a five-membered ring (furanose).

The formulas for these two forms of glucose are given below:



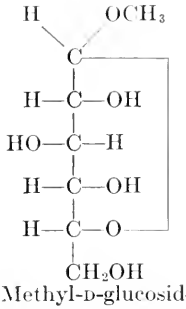
α-D-Glucose



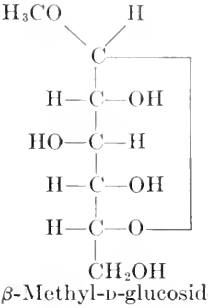
β-D-Glucose

The simple glycosides are a widely distributed group of naturally occurring compounds which contain a sugar moiety and an alcohol or phenol moiety. The form *glucoside* was formerly used to designate compounds of this type irrespective of the sugar moiety. Specific glycosides are designated by adding the ending *oside* to the name of the sugar involved; *e.g.*, glucoside, mannoside, etc.

Two glucosides are formed when glucose is treated with methanol under appropriate conditions. The formulas are given below:



α-Methyl-D-glucoside



β-Methyl-D-glucoside

These formulas correspond to the alpha and beta isomers of glucose. The proof that α-methylglucoside and α-glucose have the same structure was furnished by Armstrong (1903), who followed the enzymatic hydrolyses of these glucosides polarimetrically.

Our interest in the glycosides is not in the chemical structure per se, but in the fact that utilization of these and other compounds having the same type of glycoside linkage is dependent upon configuration. Different enzymes are required for the hydrolysis of the α- and β-glycoside linkages. Some fungi possess both types of hydrolytic enzymes, others but one, and some fungi appear to lack both. Thus, certain yeasts ferment α-methylglucoside but not β-methylglucoside. These yeasts have an enzyme or enzymes which catalyze the hydrolysis of the α-glycoside linkage but not the β-glycoside linkage (lactose-fermenting yeasts are able to hydrolyze β-glycosides).

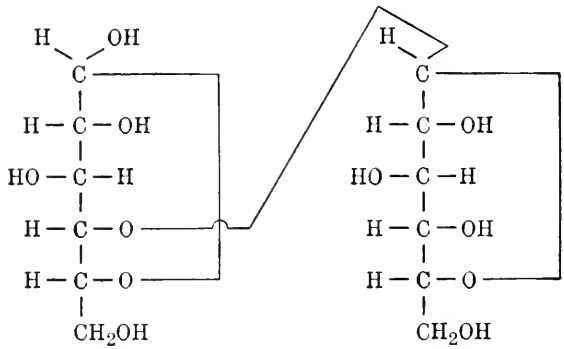
The use of the methylglucosides is not always a safe guide in predicting which complex sugars will be utilized by fungi. *Aspergillus niger* utilizes β-methylglucoside rapidly and completely, while α-methylglucoside is poorly utilized (Dox and Neidig, 1912). Attempts to adapt *A. niger* to utilize α-methylglucoside as a sole source of carbon were without much success, although the fungus apparently utilized this compound in the presence of sucrose (Dox and Roark, 1920). *A. niger* utilizes lactose poorly. Tamiya (1932) found *A. oryzae* made only a trace of growth on α-methylglucoside. β-Methylglucoside was not tested. This fungus grows well on maltose. These results are, perhaps, not unexpected in view of the specificity of enzymes. The utilization of the naturally occurring simple glycosides by fungi has been investigated but slightly.

OLIGOSACCHARIDES

These sugars are derived from two, three, or four hexose sugars by the elimination of water. On hydrolysis, the individual sugars are regenerated. Five factors which determine the structure of the oligosaccharides are (1) the component sugars; (2) the component sugar which functions

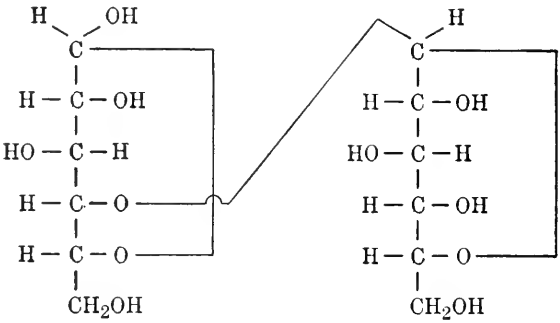
as the alcohol; (3) the stereochemical nature of the glycoside linkage; (4) the carbon of the alcohol moiety which forms the glycoside linkage; and (5) the ring structure of the component sugars (see Gilman, 1943).

Maltose. It is doubtful whether this disaccharide occurs free in nature. It is formed when starch is enzymatically hydrolyzed; on further hydrolysis two molecules of glucose are formed. This disaccharide is utilized by many fungi. The glycoside linkage is alpha in maltose.



Maltose

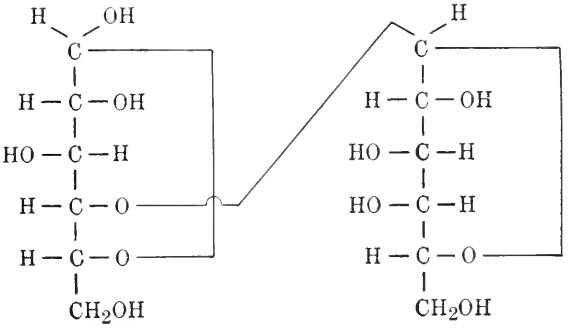
Cellobiose. The occurrence of this sugar as a repeating unit in cellulose makes it important. Cellobiose differs from maltose only in the nature of the glycoside linkage. With few exceptions only fungi which produce enzymes which attack the β-glycoside linkage will utilize this sugar.



Cellobiose

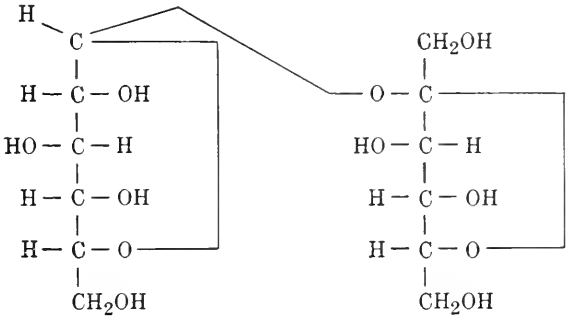
Since cellobiose and maltose differ only in the nature of the glycoside linkage, it would be interesting to compare the utilization of these two sugars by a large number of fungi. Celiobiose has been studied so infrequently that the necessary data are lacking.

Lactose. This sugar is probably present in the milk of all animals. Hydrolysis of lactose by acids or lactase yields a molecule each of glucose and galactose. This sugar is hydrolyzed by *emulsin* and is therefore a β-glycoside.



Lactose

Sucrose. This sugar is of common occurrence in plants. On hydrolysis one molecule of glucose and one of fructose are formed; a mixed α- and β-glycoside linkage unites the sugar moieties. Sucrose apparently is utilized by fewer fungi than maltose, but more extensively than lactose (see Table 22).

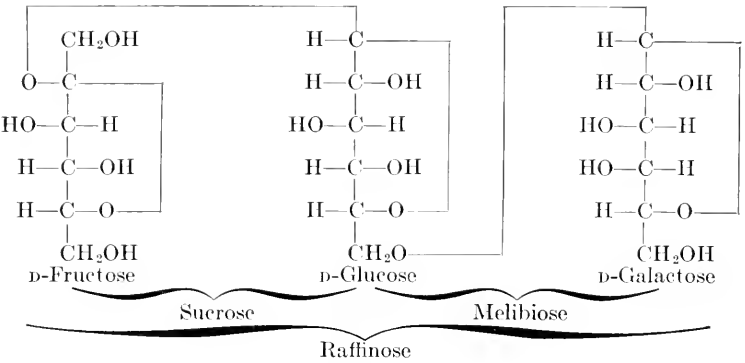


Sucrose

In addition to the three common disaccharides (maltose, lactose, and sucrose), many other oligosaccharides are known. Owing to cost and relative unavailability, these sugars have not been studied intensively. Some of these "rare" sugars are used in differential media in bacteriology. Brief mention will be made here of some of these sugars. The nonreducing disaccharide trehalose (mushroom sugar) is synthesized by various fungi and is fermented by many yeasts. Trehalose on hydrolysis yields glucose; it differs from maltose in the position of the

glycoside linkage. Tamiya (1932) reported that *Aspergillus oryzae* utilized trehalose and raffinose. The trisaccharide raffinose is obtained as a by-product of beet-sugar manufacture. On complete hydrolysis galactose, glucose, and fructose are formed in equivalent amounts. The structure for raffinose is given below.

Volkonsky (1934) found raffinose to be utilized readily by *Pythium debaryanum* and a species of *Sporotrichum*. One isolate of *Phytophthora parasitica* utilized raffinose rapidly, while another isolate utilized this sugar slowly. *Phytophthora cactorum* and *P. palmivora* utilized this sugar slowly. The great majority of fungi tested by Volkonsky did not utilize raffinose.



Oligosaccharides and polysaccharides are utilized by fewer fungi than is glucose. All microorganisms which can utilize a given polysaccharide are also able to utilize its hydrolytic products (Van Niel, 1944). Not all polysaccharides yield glucose on hydrolysis, but the majority of them do.

While the evidence at hand does not exclude the direct utilization of disaccharides by some fungi, it is probable that these sugars are hydrolyzed before utilization in most instances. Smith (1949) suggests that *Marasmius chordalis* attacks cellobiose by a route that involves neither preliminary hydrolysis nor phosphorylation.

The failure of a fungus to utilize an oligosaccharide may be due either to the lack of the necessary hydrolytic enzyme or to inability to utilize the component sugars. Failure to synthesize the necessary hydrolytic enzymes appears to be by far the most common cause of nonutilization. This is borne out by the data in Table 22. Of the 21 fungi studied by Margolin, two failed to grow on maltose, while eight did not utilize sucrose. Since all these fungi grew well on glucose and fructose, it is evident that failure to utilize maltose and sucrose was due to the fact that these fungi could not hydrolyze these sugars. The nonutilization of lactose by *Syncephalastrum racemosum* is evidently due to the failure of

this fungus to synthesize lactase, for this fungus makes good growth on either glucose or galactose. The same argument applies to *Blakeslea trispora*, *Fusarium lycopersici*, *Rhizopus nigricans*, and *R. suinus*. Non-utilization of a complex carbohydrate is usually due to the lack of the necessary hydrolytic enzymes.

The hydrolysis of oligosaccharides by fungi is easily demonstrated. *Phycomyces blakesleeanus* utilizes sucrose while *Mucor ramannianus* does not. If the mycelium of *P. blakesleeanus* is removed from a flask of sucrose medium after several days' incubation and the flask reinoculated with *M. ramannianus*, the latter fungus will grow. *P. blakesleeanus* excretes sucrase, which catalyses the hydrolysis of sucrose to D-glucose and D-fructose, both of which are utilized by *M. ramannianus*.

A complex carbohydrate and its hydrolytic products are not necessarily equivalent in all respects. Hawker (1947) reported that the amount of mycelium produced by *Melanospora destruens* was different when this fungus was grown on equivalent amounts of glucose, fructose, mixtures of glucose and fructose, and sucrose. More mycelium was produced from glucose than from an equivalent amount of sucrose, and this was true whether the concentrations of these sugars were low or high. On the other hand, perithecia were produced more abundantly on sucrose than on glucose media. Indeed, hydrolysis of the same lot of sucrose to glucose and fructose allowed the production of no more perithecia than other samples of these sugars. The conclusion seems inescapable that the particular structure of sucrose was in some way favorable for the production of perithecia. While a fungus may utilize an oligosaccharide and its hydrolytic products, it is not safe to assume that both are used with the same efficiency for all purposes.

POLYSACCHARIDES

The chemistry of the polysaccharides resembles that of the oligosaccharides except that the number of sugar residues is much larger. These substances constitute the bulk of carbohydrate materials synthesized by plants and animals. The most important polysaccharides are cellulose, starch, and glycogen. On hydrolysis simple sugars are formed. The molecular weights of polysaccharides may be very large; cellulose from different sources may have a molecular weight ranging from 200,000 to 400,000. The molecular weights of many polysaccharides are much less than that of cellulose. In general, polysaccharides are insoluble or only colloidally soluble. The utilization of these substances by fungi is dependent upon the excretion of the necessary hydrolytic enzymes. Pigman and Goepp (1948) classify polysaccharides on the basis of the hydrolytic products as homopolysaccharides, which yield only one monosaccharide on hydrolysis, and heteropolysaccharides, which yield two or

more monosaccharides or related compounds on hydrolysis. Cellulose, starch, and glycogen are members of the first class and yield glucose on hydrolysis. Polysaccharides are frequently named by replacing the ending *-ose* of the parent monosaccharide by *-an*. Fructan (levulan) designates a polysaccharide which yields fructose on hydrolysis. A hexosan is a polysaccharide which yields hexose sugars on hydrolysis, and a pentosan yields pentoses. Pectins are polymers of galacturonic acid.

The heteropolysaccharides occur in lesser amounts than the homopolysaccharides. Among them are the hemicelluloses, which on hydrolysis yield D-xylose as the principal sugar, the plant gums, and agar.

Cellulose. Chemically, cellulose is a linear polymer of D-glucose. The glucose residues are joined together through β-glycoside linkages as in cellobiose, and cellulose may be thought of as consisting of repeating cellobiose units. Norman and Fuller (1942) postulate that the majority of fungi are able to utilize cellulose. In spite of the importance of cellulose utilization by fungi in the economy of nature much remains to be learned about this process.

It is commonly accepted that the first stage in utilization of cellulose is hydrolysis, although Campbell (1932) has suggested oxidation. The hydrolysis of cellulose may be expressed schematically as follows: cellulose → cellodextrins → cellotetrose → cellobiose → D-glucose. Fungus cellulases appear to have been infrequently studied. Grassmann *et al.* (1933) separated cellulase and cellobiase from *Aspergillus oryzae*. This cellulase was inactive in hydrolyzing cellulose degradation products having a molecular weight less than 1,000 (six glucose residues), while the cellobiase hydrolyzed cellulose fragments containing from two to six glucose residues.

Fungi differ widely in ability to utilize cellulose. In general, the rate of utilization of cellulose is less than that of glucose. This is probably due to the insolubility of cellulose, which limits the action of cellulase to the surface, or to an inadequate rate of enzyme synthesis.

The principal source of cellulose available to fungi in nature is wood and other plant remains. While cellulose is the chief constituent in such materials, hemicelluloses, gums, tannins, and lignin are also present. The wood-rotting fungi have been classified according to whether they cause white or brown rots. The fungi which cause brown rots attack cellulose in preference to lignin. The fungi which preferentially attack the noncellulosic constituents of wood cause white rots. The latter species are apparently more numerous than those which cause brown rots. The following are some of the fungi listed by Nobles (1948) as causing white rots: *Armillaria mellea, Ganoderma lobatum, Lenzites betulinus, Pleurotus ostreatus, Polyporus abietinus, P. cinnabarinus, P. pargamenus.* A few fungi causing brown rots are *Daedalea quercina, Lentinus lepideus,*

Lenzites trabea, *Merulius lacrymans*, *Polyporus betulinus*, and *Trametes americana*.

The effect of a typical fungus causing white rot on the composition of wood is given in Table 27. *Polyporus pargamenus* was allowed to act on blocks of aspen wood for 20 months. At the end of this time the wood block showed three degrees of attack. The tan-colored portion was altered least. The pink-colored portion was intermediate, while the white portion had lost the most lignin. *P. pargamenus* also degraded the cellulose somewhat, as shown by lower degree of polymerization.

TABLE 27. THE EFFECT OF *Polyporus pargamenus* IN ALTERING THE COMPOSITION OF ASPEN WOOD

Time of incubation 20 months. (Heuser *et al.*, *Arch. Biochem.* **21**, 1949. Published by permission of Academic Press, Inc.)

Portion of wood block	Lignin, %	Pentosans, %	Cellulose, % (calculated)
Original	17.5	19.3	60.68
Tan	10.4	12.8	73.84
Pink	4.5	8.3	84.20
White	3.4	8.4	85.32

The effect of fungi causing brown rots on the composition of coniferous woods has been studied by Schubert and Nord (1950). *Lenzites sacpiaria* in 13 months caused a decrease in cellulose in pine sawdust from 45.5 to 18.5 per cent. During this period the apparent lignin content increased from 33.9 to 50.1 per cent. Similar results were obtained with *Lentinus lepideus* and *Poria vaillantii*. For a recent review of the microbiological degradation of cellulose see Nord and Vitucci (1948).

Starch. Like cellulose, starch is a polymer of D-glucose. The glucose residues are joined through α-glycoside linkages, and starch (and glycogen) may be thought of as consisting of repeating units of maltose. Starch consists of two types of molecules. The linear portion of starch is called amylose, while the branched-chain fraction is known as amylopectin. Starch is synthesized by green plants, while glycogen is formed by animals and fungi. The enzymes which catalyze the hydrolysis of starch are known as amylases and were discussed in Chap. 4. The enzymatic hydrolysis of starch may be represented schematically as follows: starch → dextrins → maltose → D-glucose. The branched-chain dextrins are incompletely hydrolyzed by amylase, while the straight-chain dextrins are completely converted to maltose (Myrbäck, 1948).

Starch is insoluble in water. Only those fungi which produce amylase are able to utilize starch. This ability is common among fungi but not universal. Volkonsky (1934) found 26 isolates and species of the

Saproliginales to utilize starch and its hydrolytic products (dextrin, maltose, and glucose). Thirteen other carbon sources, including fructose, were not utilized. Margolin (1942) found that 19 out of 21 fungi which utilized maltose also utilized dextrin.

The nonutilization of starch by *Sclerotinia libertiana* has been suggested as the basis of a method of preparing potato starch (Kakeura, 1946). Few yeasts utilize starch, although maltose and glucose are readily utilized.

All the fungi listed in Table 22 except *Pythium ascophallon* and *Phytophthora fagopyri* utilized dextrin. A comparison of the ability of fungi to utilize glycogen and starch has not been investigated thoroughly. Tamiya (1932) found the yield of mycelium of *Aspergillus oryzae* to be greater on glycogen than on dextrin. Dextrin was a better carbon source than starch.

The role of the pectin-destroying enzymes in parasitism and the rotting of fruits and vegetables is discussed in Chap. 17. Presumably these fungi utilize some or all of the hydrolytic products of pectin (D-galacturonic acid and methyl alcohol). None of the fungi, in so far as is known, utilize agar as a source of carbon. *A. niger* utilizes the arabo-galactan from western larch as a source of carbon (Ratajak and Owens, 1942).

HETEROTROPHIC UTILIZATION OF CARBON DIOXIDE

The assimilation of carbon dioxide is not restricted to green plants. Carbon dioxide fixation has been demonstrated in bacteria, fungi, protozoa, liver slices, barley roots, and intact green plants in the absence of light. The basis for classifying organisms according to the way they utilize carbon dioxide is discussed by Werkman and Wood (1942). By the use of carbon isotopes an elegant method is available for demonstrating carbon dioxide assimilation. In addition, the mechanism of fixation can be studied. This involves isolation and degradation studies of the compounds synthesized while the organisms were exposed to isotopic carbon dioxide. Either stable or radioactive carbon isotopes may be used. The finding of isotopic carbon in compounds synthesized is proof of assimilation.

Aspergillus niger and *Rhizopus nigricans* were shown to assimilate carbon dioxide (Foster *et al.*, 1941). Radioactive carbon dioxide ($C^{11}O_2$) was used in these experiments. Mycelium of *R. nigricans* was suspended in 5 per cent glucose solution and agitated in a closed system containing isotopic carbon dioxide. At the end of the experiment the mycelium and the medium were analyzed for radioactivity. More than one-third of the carbon dioxide assimilated was incorporated into cell constituents which were not decomposed by boiling for 1 hr. with 2*M* hydrochloric acid. Carbon dioxide was assimilated under aerobic and anaerobic conditions. The data of such an experiment are given in Table 28.

TABLE 28. DISTRIBUTION OF RADIOACTIVE CARBON (C^{11}) IN THE CULTURE MEDIUM AND MYCELIUM OF *Rhizopus nigricans* EXPOSED TO $C^{11}O_2$ IN THE GAS PHASE FOR 30 MINUTES

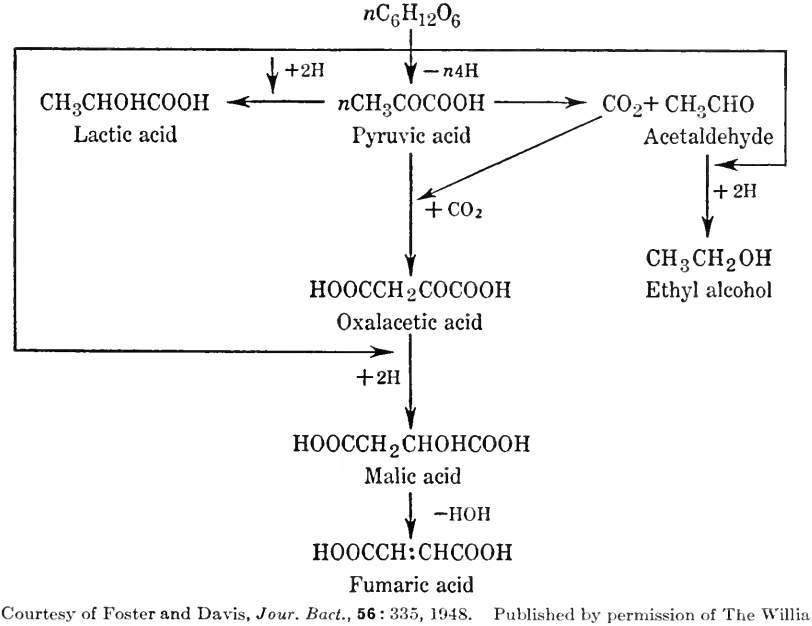
Results are expressed as percentage of $C^{11}O_2$ assimilated. (Foster *et al.*, *Proc. Natl. Acad. Sci., U.S.* **27**, 1941.)

Substance tested	Aerobic	Anaerobic
Total C* in supernatant solution after removing cells	19.5	29.0
Fumaric* acid in this solution	8.0	25.0
C* in neutral volatile distillate	0.1	0.2
Total C* in acid extract of cells	44.0	30.0
Fumaric* acid in this solution	6.5	12.0
C* remaining in cells after acid extraction	36.5	41.0

* Designates radioactive carbon.

It is probable that carbon dioxide enters into various metabolic processes. Foster and Davis (1948) postulate that strains of *Rhizopus nigricans* which produce fumaric acid anaerobically do so according to scheme IV. Cantino (1949), in studying the metabolism of *Blastocladia*

SCHEME IV. A SCHEME FOR THE ANAEROBIC TRANSFORMATION OF GLUCOSE INTO FUMARIC ACID BY *Rhizopus nigricans**



* Courtesy of Foster and Davis, *Jour. Bact.*, **56**: 335, 1948. Published by permission of The Williams & Wilkins Company.

pringsheimii, found that, by increasing the carbon dioxide in the gaseous phase, the formation of lactic acid was decreased, while the amount of

succinic acid was increased. It was surmised that this fungus utilizes carbon dioxide, since none was set free.

The formation of oxalacetic acid by the reaction between pyruvic acid and carbon dioxide suggests that heterotrophic carbon dioxide fixation may play a role in amino-acid synthesis. Support of this hypothesis may be found in the work of Ajl and Werkman (1949), who found the carbon dioxide requirement of *Aerobacter aerogenes* could be replaced by oxalacetic, α-ketoglutaric, fumaric, or aspartic acid. For further information on carbon dioxide utilization by fungi see Foster (1949).

UTILIZATION OF CARBON

Carbon compounds are utilized by fungi for two general purposes, as a source of energy and as a source of the chief structural element. These two processes may be the same until a number of chemical transformations have taken place but may then diverge after certain intermediate compounds are formed. The over-all use of carbon is quite easily determined, but it is a problem of a different order to trace all the chemical transformations which occur when a compound is utilized.

TABLE 29. THE DISTRIBUTION OF CARBON FROM ARABINOSE AMONG THE PRODUCTS OF METABOLISM OF *Fusarium lini*

(White and Willaman, *Biochem. Jour.* **22,** 1928. Published by permission of Cambridge University Press.)

Age of culture, days	Mycelium, %	CO_2, %	Alcohol, %	Lead precipitate, %	Sugar, %	Total carbon, %
5	0.8	0.6	7.6	—	90.6	99.6
10	3.4	4.4	7.6	0.6	85.2	101.2
15	4.6	6.1	6.6	1.0	80.4	98.7
25	4.0	9.4	3.3	1.5	81.2	99.4
40	10.4	20.8	9.9	1.7	55.2	98.3

Carbon balances. A general idea of the way a carbon source is utilized may be gained by following the amounts of mycelium synthesized, carbon dioxide evolved, and other metabolic products formed. If the initial amount of carbon is known, its distribution can be followed by analysis. From 95 to 99 per cent of the carbon is usually accounted for in such experiments. The accompanying data from White and Willaman (1928) illustrate this distribution of carbon from arabinose by *Fusarium lini* (Table 29).

While the analytical difficulties in experiments of this kind are considerable, chemical analysis of the mycelium and the other metabolic products reveals how the carbon originally present in the carbon source is distributed. Such analyses are useful in detecting the major metabolic

products. Carbon balances are especially useful in determining the efficiency with which a fungus produces metabolic products of value, such as alcohol and citric acid. For further examples see Raistrick *et al.* (1931).

Utilization ratios. The relations of the amounts of fungus metabolic products to the amount of carbon source (or other substance) used are frequently expressed as ratios. However, these ratios are valid only for the fungi and the experimental conditions used. These ratios should be considered as absolute values only for the conditions under which they were obtained. The various utilization ratios are of less value than complete carbon balances, but the analytical determinations are fewer. To be of most value, these ratios should be determined at various intervals during incubation, because these ratios change with age.

The most useful of these ratios is the *economic coefficient*, which is obtained by dividing the weight of mycelium and spores by the weight of sugar or other carbon source used. The residual carbon source in the medium must be determined at the end of an experiment. In general an efficient fungus will convert half the weight of sugar supplied in the medium into cellular material. The efficiency of most fungi when grown on laboratory media is much less. This is due in part to the use of unbalanced media and to the type of carbon metabolism taking place. The carbon which is not utilized for the synthesis of cellular material appears either as carbon dioxide or as intermediate metabolic products, such as alcohol and organic acids. In industrial applications it is desirable to employ cultural conditions which divert a large part of the carbon used into the desired intermediate products, rather than into the production of mycelium and carbon dioxide.

The economic coefficient of *Fusarium sambucinum* under various cultural conditions has been studied by Holzapfel (1925). This fungus utilized sucrose (0.33) and fructose (0.36) more efficiently than glucose (0.24). The economic coefficient varied with the concentration of the carbon source and with the source of nitrogen, as well as with the age of the cultures.

For a discussion of other utilization ratios and examples, see Steinberg (1942), Peterson *et al.* (1922), White and Willaman (1928), and Fries (1938).

Intermediary metabolism. The problem to be considered here is the way fungi utilize the various sources of carbon available to them. From the data and discussion in the earlier part of this chapter it is clear that structure and configuration play an important role in determining which compounds may serve as a source of carbon for a given fungus. The availability of complex natural compounds, such as the carbohydrates, was found to depend upon the production of the necessary extracellular hydrolytic enzymes. The utilization of simple compounds, such as the

monosaccharides, is likewise an enzymatically catalyzed chain of metabolic processes. It may be assumed that the chemical composition of the fungus will be about the same, irrespective of the carbon source utilized. Therefore, at some place along the path of synthesis the initial carbon sources are converted into the same compounds. It is probable that the original compounds are converted into the same intermediate compounds before synthesis. Thus, galactose is apparently transformed by *Saccharomyces fragilis* into galactose-1-phosphate, which is then converted into glucose-1-phosphate (Caputto *et al.*, 1949). These intermediate compounds then enter the various metabolic reaction chains which lead to the production of materials which make up the fungus. We may suppose that the first steps in utilization are those which transform a carbon source into key intermediates.

The intermediate metabolic products should also serve as a source of carbon for the fungus in question. If a fungus transforms compound *A* into compound *B*, then compound *B* should serve as a source of carbon. Nonutilization of compound *B* indicates that this compound is not part of the metabolic pathway. This simple hypothesis neglects two important considerations: compound *B* may not enter the fungus cells with the same facility as compound *A*, or compound *B* may be toxic in the concentrations present. As an example of this approach, the work of Steinberg (1942) may be consulted. Since *Aspergillus niger* made only a trace of growth on D-gluconic acid, it seems probable that the first step in the utilization of glucose by this fungus is not the oxidation of the aldehyde group. The isolated enzymes from a fungus may also be studied to determine the reactions catalyzed, or the effect of specific enzyme inhibitors on the intact fungus may be studied.

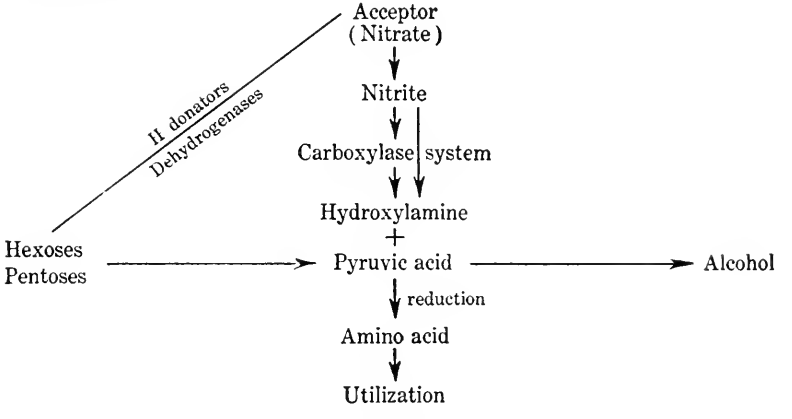
In some instances intermediates of sugar dissimilation are excreted into the medium and may be isolated. Thus, the production of acetaldehyde may be demonstrated by adding bisulfite to the medium. Acetaldehyde forms an insoluble addition product with this reagent. The excretion of intermediate metabolites may be due to slowness of the next step in the metabolic process. These products are usually utilized in the course of time. Among such intermediates which have been identified are acetaldehyde, ethyl alcohol, and pyruvic acid.

On the basis of the evidence now available we may not assume that all fungi utilize a sugar or other carbon source in exactly the same way, or that a fungus has only one metabolic pathway for the utilization of a sugar. Nord and Mull (1945) consider that species of *Fusarium* dissimilate carbohydrates by oxidation, by splitting the carbon chain, and by a phosphorylation mechanism. The relative importance of these three methods of attack depends upon the fungus involved and upon the environmental conditions. Identity of a metabolic product formed by

two fungi is not proof that the reaction mechanism is the same in both instances. Yeast and certain species of *Fusarium* produce alcohol, but the pathways from glucose to alcohol appear to be different. The mechanism of carbohydrate dissimilation by *Fusarium lini*, when grown upon a nitrate medium, is believed to take place as shown in scheme V. An essential feature of this scheme is the formation of pyruvic acid from both pentoses and hexoses. The intermediate steps in this biosynthesis by *Fusarium lini* have not been elucidated. A portion of the hydrogen derived from the dissimilation of carbohydrate is enzymatically transferred and used for the reduction of nitrate ion which acts as a hydrogen acceptor. The nitrite produced inhibits the carboxylase enzyme system which transforms pyruvic acid into carbon dioxide and acetaldehyde. Pyruvic acid does not accumulate in the culture medium when ammonium nitrogen is used.

SCHEME V. THE PATHWAY OF HEXOSE AND PENTOSE UTILIZATION BY *Fusarium lini* GROWN ON NITRATE MEDIUM

(Courtesy of Wirth and Nord, *Arch. Biochem.* **1**: 155, 1942. Published by permission of Academic Press, Inc.)



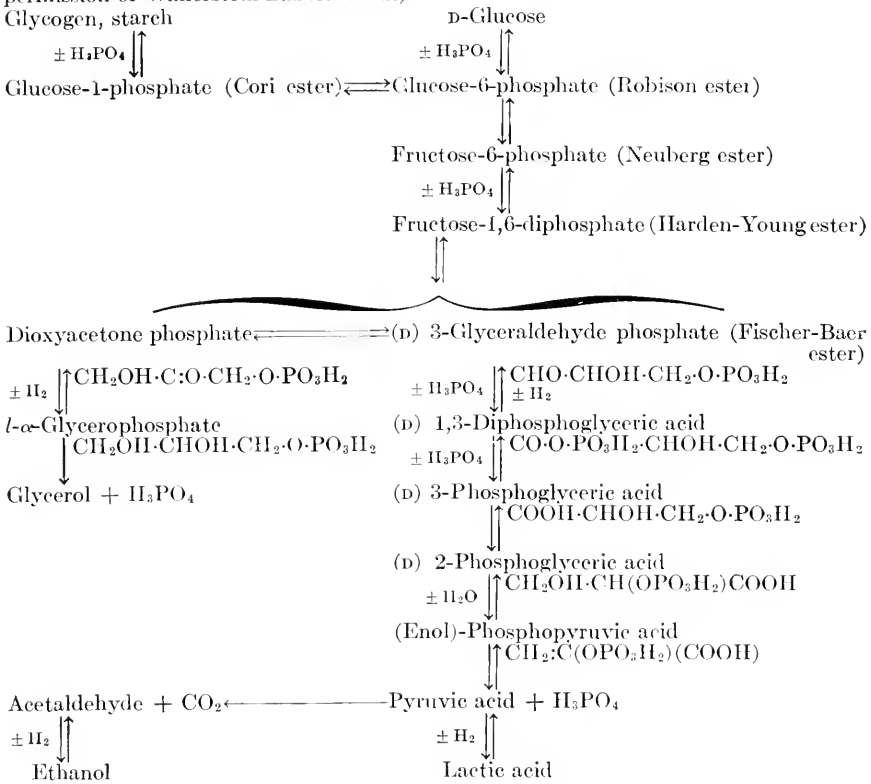
Pyruvic acid is the key intermediate compound formed in the dissimilation of hexoses and pentoses by *F. lini*. The transformation of pyruvic acid into alcohol by *F. lini* and yeasts appears to follow the same pathway and to require the same coenzymes, cocarboxylase and codehydrogenase I.

The anaerobic dissimilation (fermentation) of glucose by yeast and the comparable process in muscle (glycolysis) have been intensively studied. These are perhaps the best understood of all metabolic processes. Although it does not function in glucose dissimilation by *F. lini* in the same way as in yeast, phosphate plays a role in all these transformations until pyruvic acid is formed. Many investigators have contributed

to the scheme of glucose dissimilation presented in scheme VI (Meyerhof, 1938, 1949). Further information about these reactions may be found in Sumner and Somers (1947), Tauber (1949), and Prescott and Dunn (1949).

SCHEME VI. THE PATHWAY OF GLUCOSE DISSIMILIATION BY YEAST (ALCOHOLIC FERMENTATION) AND MUSCLE (GLYCOLYSIS)

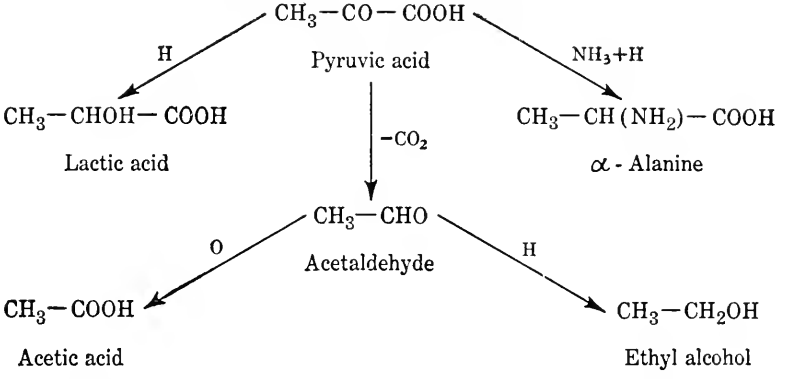
(Courtesy of Meyerhof, *Wallerstein Labs. Communs.* **12**: 256, 1949. Published by permission of Wallerstein Laboratories.)



Pyruvic acid is a key intermediate compound in metabolism. Pyruvic acid serves as a source of carbon for many fungi, although the rate of growth on this substance is frequently slow. This is in accord with the hypothesis that intermediate metabolites are able to replace the original carbon source. The accumulation of this compound in the culture medium may be demonstrated by the formation of iodoform in the cold by adding a solution of iodine and making the medium strongly alkaline. The sensitive color test of Lu (2,4-dinitrophenylhydrazine) may also be used [see Friedemann and Haugen (1943) for details]. Acetaldehyde also yields iodoform under these conditions, but gentle heating will drive off

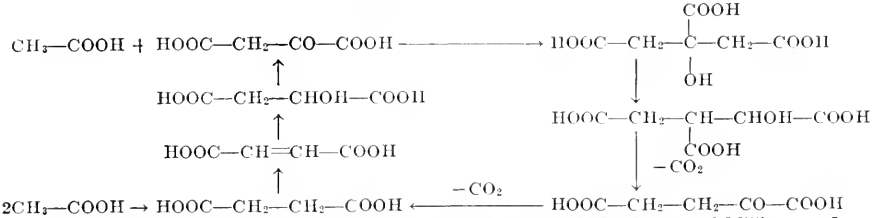
this substance. We have noted in this laboratory that pyruvic acid ordinarily disappears from culture medium as the time of incubation is increased. The disappearance of the pyruvic acid in the culture medium is usually correlated with a rise in pH. Some typical reactions of pyruvic acid are shown in scheme VII. For a review of pyruvate metabolism see Stotz (1945).

SCHEME VII. SOME TYPICAL TRANSFORMATIONS OF PYRUVIC ACID



It is probable that most intermediates used in the synthesis of protoplasm are synthesized from low-molecular-weight compounds. Acetate is used by yeasts for the synthesis of fats and other cellular constituents. Weinhouse and Millington (1947) studied the metabolism of isotopic acetate by yeast depleted of endogenous nutrients. Acetate was rapidly utilized. The distribution of the carbon from the isotopic acetate was

SCHEME VIII. OXIDATION OF ACETATE BY YEAST BY MEANS OF THE KREBS CITRIC ACID CYCLE*



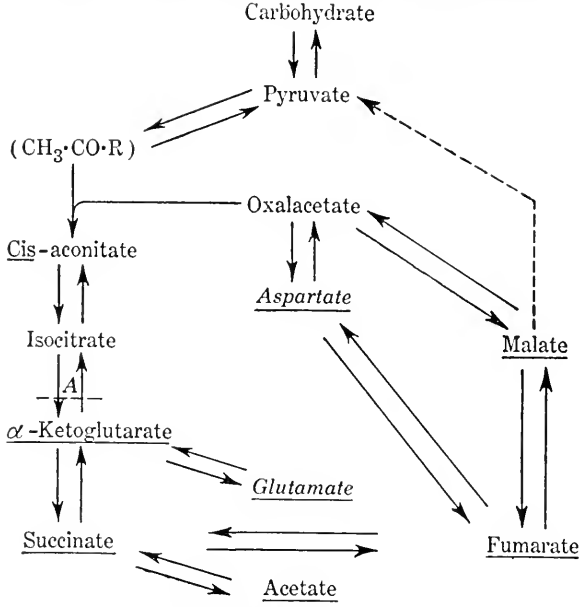
* Original scheme modified according to Weinhouse. Courtesy of Weinhouse and Millington, *Jour. Am. Chem. Soc.* **69**: 3093, 1947. Published by permission of the American Chemical Society.

determined by analysis. A portion of the acetate was oxidized; another portion was found in the lipide fraction and cell residue; some was converted to citric acid. It was calculated that from one-fourth to one-third of the lipides found in the yeast cells at the end of the experiment (a 7-hr. period) were newly synthesized from acetate. The cell residue (after extraction of the fats) contained only a little isotopic carbon. This is not surprising, since nitrogen was not furnished during these experiments.

The mechanism of acetate oxidation by yeast is postulated by these authors to follow a modified Krebs citric acid cycle (scheme VIII). The oxidation of acetate is thus the result of a rather complex cyclic process.

While the four-carbon dicarboxylic acids of the Krebs cycle are poor sources of carbon for most fungi, they are important in intermediary metabolism. These acids are readily interconvertible. The role of the keto acids in amino-acid synthesis was noted in Chap. 6. Lewis (1948) studied the metabolism of mutants of *Neurospora crassa* which were unable to synthesize either aspartic or glutamic acids. These amino

Scheme IX. A Generalized Krebs Isocitric Acid Cycle Proposed to Illustrate the Pathways of Conversion of Carbohydrate into Aspartic and Glutamic Acids by *Neurospora**



* Courtesy of Lewis, *Am. Jour. Botany* **35**: 294, 1948.

acids could be replaced by α-ketoglutaric, succinic, malic, or fumaric acids. A generalized Krebs cycle was proposed by Lewis which indicates the pathway of synthesis of aspartic and glutamic acids from glucose (scheme IX). Compounds utilized by the *Neurospora* mutants are printed in italics. The probable location of the genetic block which prevents the biosynthesis of aspartic and glutamic acids is indicated by *A*.

SUMMARY

Organic compounds are utilized by fungi for the synthesis of structural and functional compounds and as sources of energy. The fungi utilize a wide range of natural organic compounds including those of great com-

plexity. Not all fungi utilize all natural organic compounds, nor do all species utilize a given compound with the same facility. The composition, structure, and configuration of organic compounds affect utilization, but the effect of these factors may be different for different fungi.

The carbohydrates are the most common and important sources of carbon for the fungi. Sugars (and other compounds) having the same structure, but with mirror-image configuration, differ physiologically. Usually only one enantiomorph is utilized, or one is utilized much more rapidly than the other. Glucose is utilized by more fungi than any other sugar. Few fungi are unable to utilize glucose. A few species are apparently unable to utilize any sugar; *e.g.*, *Leptomitus lacteus*. The species that utilize the pentoses, sugar alcohols, acids, and other simple organic compounds are fewer in number than those which utilize glucose.

The oligo- and polysaccharides are utilized by fewer species than is glucose. The nature of the glycoside linkage as well as the sugar residues is important in determining whether these compounds are utilized by a given fungus. It is probable that most fungi hydrolyze oligosaccharides before utilization occurs. This does not exclude direct utilization in some instances. An oligosaccharide and its hydrolytic products are not always physiologically equivalent. The general order of availability of the three common disaccharides appears to be maltose, sucrose, and lactose. Among the polysaccharides, cellulose and starch are the most abundant. These compounds are insoluble and must be hydrolyzed or otherwise degraded to low-molecular-weight compounds before utilization. Only those fungi which form cellulase and amylase are able to utilize these compounds. This "digestion" is accomplished by enzymes. Ability to utilize other polysaccharides is also dependent upon possession of the necessary hydrolytic enzymes.

Some fungi utilize carbon dioxide, but not as a sole source of carbon. It is postulated that carbon dioxide combines with pyruvic acid and other keto acids to form key intermediate products which are necessary for the formation of amino acids.

The fate of the carbon supplied to a fungus is best determined by carbon-balance studies; *i.e.*, by complete chemical analyses of the mycelium and other metabolic products, including the carbon dioxide produced.

The first step in utilization of sugars and other carbon sources is the formation of certain key intermediate metabolic compounds. These key compounds are in part utilized for synthesis and in part oxidized to provide energy. The metabolic pathways leading to the formation of key intermediates differ, depending upon the environmental conditions and the fungus involved. Among the key intermediates pyruvic acid is especially noteworthy. Reduction of this compound yields lactic acid, while amination and reduction leads to alanine. Decarboxylation pro-

duces acetaldehyde, which in turn may yield either ethyl alcohol or acetic acid. Acetate is utilized by yeast and other fungi for the synthesis of fats and other cellular constituents. A fungus utilizes a compound by a series of step-by-step transformation. Among the best understood of these metabolic activities is the transformation of glucose into alcohol by yeasts.

REFERENCES

Ajl, S. J., and C. H. Werkman: On the mechanism of carbon dioxide replacement in heterotrophic metabolism, *Jour. Bact.* **57**: 579–593, 1949.

Armstrong, E. F.: Studies in enzyme action. I. The correlation of the stereoisomeric *alpha* and *beta* glucosides with the corresponding glucoses, *Jour. Chem. Soc.* **83**: 1305–1313, 1903.

Bertrand, G.: Etude biochemique de la bactérie du sorbose, *Ann. chim. et phys.* **3**: 181–288, 1904.

Campbell, W. G.: The chemistry of the white rots of wood. III. The effect on wood substance of *Ganoderma applanatum* (Pers.) Pat., *Fomes fomentarius* (Linn.) Fr., *Polyporus adustus* (Willd.) Fr., *Pleurotus ostreatus* (Jacq.) Fr., *Armillaria mellea* (Vahl.) Fr., *Trametes pini* (Brot.) Fr., and *Polystictus abietinus* (Dicks.) Fr., *Biochem. Jour.* **26**: 1829–1838, 1932.

Cantino, E. C.: The physiology of the aquatic Phycomycete, *Blastocladia Pringsheimii*, with emphasis on its nutrition and metabolism, *Am. Jour. Botany* **36**: 95–112, 1949.

Caputto, R., L. F. Leloir, R. E. Trucco, C. E. Cardini, and A. C. Paladini: The enzymatic transformation of galactose into glucose derivatives, *Jour. Biol. Chem.* **179**: 497–498, 1949.

Cheo, P. C.: Stripe smut of blue grass (*Ustilago striiformis forma Poae-pratensis*): Spore germination, artificial inoculation, pathological histology and growth in artificial media, thesis, West Virginia University, 1949.

Dox, A. W., and R. E. Neidig: Spaltung von α- und β-Methylglucosid durch *Aspergillus niger*, *Biochem. Zeit.* **46**: 397–402, 1912.

Dox, A. W., and G. W. Roark, Jr.: The utilization of *alpha*-methylglucoside by *Aspergillus niger*, *Jour. Biol. Chem.* **41**: 475–481, 1920.

Dulaney, E. L.: Observations on *Streptomyces griseus*. III. Carbon sources for growth and streptomycin production, *Mycologia* **41**: 1–10, 1949.

Foster, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

Foster, J. W., S. F. Carson, S. Ruben, and M. D. Kamen: Radioactive carbon as an indicator of carbon dioxide utilization. VII. The assimilation of carbon dioxide by molds, *Proc. Natl. Acad. Sci. U.S.* **27**: 590–596, 1941.

Foster, J. W., and J. B. Davis: Anaerobic formation of fumaric acid by the mold *Rhizopus nigricans*, *Jour. Bact.* **56**: 329–338, 1948.

Friedemann, T. E., and G. E. Haugen: Pyruvic acid. II. The determination of keto acids in blood and urine, *Jour. Biol. Chem.* **147**: 415–442, 1943.

Fries, N.: Ueber die Bedeutung von Wuchsstoffen für das Wachstum verscheidener Pilze, *Symbolae Botan. Upsalienses* **3**: 2, 1938.

Gilman, H.: Organic Chemistry, an Advanced Treatise, Vol. II, John Wiley & Sons, Inc., New York, 1943.

Gottlieb, D.: The utilization of amino acids as a source of carbon by fungi, *Arch. Biochem.* **9**: 341–351, 1946.

Grassmann, W., L. Zeichmeister, G. Toth, and R. Stadder: Ueber den enzymatischen Abbau der Cellulose und ihrer Spaltprodukte. 2. Mitteilung über enzymatische Spaltung von Polysacchariden, *J. Leibigs Ann. d. Chem.* **503**: 167–179, 1933.

★Hawker, L. E.: Further experiments on growth and fruiting of *Melanospora destruens* Shear in the presence of various carbohydrates, with special reference to the effects of glucose and of sucrose, *Ann. Botany* (N.S.) **11**: 245–260, 1947.

Herrick, J. A.: The carbon and nitrogen metabolism of *Stereum gausapatum* Fries., *Ohio Jour. Sci.* **40**: 123–129, 1940.

★Heuser, E., B. F. Shema, W. Shockley, J. W. Appling, and J. F. McCoy: The effect of lignin-destroying fungi upon the carbohydrate fraction of wood, *Arch. Biochem.* **21**: 343–350, 1949.

Holzapfel, H. H.: Untersuchung über die C- und N-Quellen einiger Fusarien, *Cent. Bakt.*, Abt. II., **64**: 174–222, 1925.

★Horr, W. H.: Utilization of galactose by *Aspergillus niger* and *Penicillium glaucum*, *Plant Physiol.* **11**: 81–99, 1936.

Kakeura, M.: Starches by using parasitic fungi, Japanese patent 172,381, Mar. 6, 1946. *Chem. Abs.* **43**: 6850, 1949.

Kinsel, K.: Carbohydrate utilization by the corn Diplodias, *Phytopathology* **27**: 1119–1120, 1937.

Lewis, R. W.: Mutants of *Neurospora crassa* requiring succinic acid or a biochemically related acid for growth, *Am. Jour. Botany* **35**: 292–295, 1948.

Margolin, A. S.: The carbohydrate requirements of *Diplodia macrospora*, *Proc. West Va. Acad. Sci.* **14**: 56–59, 1940.

Margolin, A. S.: The effect of various carbohydrates upon the growth of some fungi, thesis, West Virginia University, 1942.

Meyerhof, O: The intermediary reactions of fermentation, *Nature* **141**: 855–858, 1938.

Meyerhof, O.: Glycolysis of animal tissue extracts compared with the cell-free fermentation of yeast, *Wallerstein Labs. Communs.* **12**: 255–264, 1949.

Myrbäch, K.: The structure of starch, *Wallerstein Labs. Communs.* **11**: 209–219, 1948.

Nobles, M. K.: Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi, *Can. Jour. Research*, Sec. C, **26**: 281–431, 1948.

Nord, F. F., and R. P. Mull: Recent progress in the biochemistry of Fusaria, *Advances in Enzymol.* **5**: 165–205, 1945.

Nord, F. F., and J. C. Vitucci: On the mechanism of enzyme action. XXX. The formation of methyl-*p*-methoxycinnamate by the action of *Lentinus lepideus* on glucose and xylose, *Arch. Biochem.* **14**: 243–247, 1947.

Nord, F. F., and J. C. Vitucci: Certain aspects of the microbiological degradation of cellulose, *Advances in Enzymol.* **8**: 253–298, 1948.

Norman, A. G., and W. H. Fuller: Cellulose decomposition by microorganisms, *Advances in Enzymol.* **2**: 239–264, 1942.

Pasteur, L.: Note relative au *Penicillium glaucum* et à la dissymétrie moléculaire des produits organiques naturels, *Compt. rend. acad. sci.* **51**: 298–299, 1860.

Peterson, W. H., E. B. Fred, and E. G. Schmidt: The fermentation of pentoses by molds, *Jour. Biol. Chem.* **54**: 19–34, 1922.

Pigman, W. W., and R. M. Goepp: Chemistry of the Carbohydrates, Academic Press, Inc., New York, 1948.

Prescott, S. C., and C. G. Dunn: Industrial Microbiology, 2d ed., McGraw-Hill Book Company, Inc., New York, 1949.

Raistrick, H., J. H. Birkinshaw, J. H. V. Charles, P. W. Clutterbuck, F. P. Coyne, A. C. Hetherington, C. H. Lilly, M. L. Rintoul, W. Rintoul, R. Robinson, J. A. R. Stoyle, C. Thom, and W. Young: Studies in the biochemistry of micro-organisms, *Trans. Roy. Soc.* (*London*), Ser. B, **220**: 1–367, 1931.

Ratajak, E. J., and H. S. Owens: Optimal conditions for the hydrolysis of arabogalactan by *Aspergillus niger*, *Botan. Gaz.* **104**: 329–337, 1942.

Schade, A. L.: The nutrition of *Leptomitus*, *Am. Jour. Botany* **27**: 376–384, 1940.

Schade, A. L., and K. V. Thimann: The metabolism of the water mold *Leptomitus lacteus*, *Am. Jour. Botany* **27**: 659–670, 1940.

Schubert, W. J., and F. F. Nord: Investigations on lignin and lignification. I. Studies on softwood lignin, *Jour. Am. Chem. Soc.* **72**: 977–981, 1950.

Schultz, A. S., D. K. McManus, and S. Pomper: Amino acids as carbon source for the growth of yeasts, *Arch. Biochem.* **22**: 412–419, 1949.

Skoog, F. K., and C. C. Lindegern: Adaptation in a yeast unable to ferment glucose *Jour. Bact.* **53**: 729–742, 1947.

Smith, V. M.: On the mechanism of enzyme action. XXXIX. A comparative study of the metabolism of carbohydrates, in the presence of inorganic and organic phosphates, by *Merulius lacrymans* and *Marasmius chordalis*, *Arch. Biochem.* **23**: 446–472, 1949.

Steinberg, R. A.: Relation of carbon nutrition to trace-element and accessory requirements of *Aspergillus niger*, *Jour. Agr. Research* **59**: 749–763, 1939.

Steinberg, R. A.: The process of amino acid formation from sugars in *Aspergillus niger*, *Jour. Agr. Research* **64**: 615–633, 1942.

Steinberg, R. A.: Effects of trace elements on growth of *Aspergillus niger* with amino acids, *Jour. Agr. Research* **64**: 455–475, 1942*a*.

Stevens, N. E., and H. W. Larsh: Carbohydrate utilization by *Diplodia maeospora*, *Trans. Illinois State Acad. Sci.* **32**: 82, 1939.

★Stotz, E.: Pyruvate metabolism, *Advances in Enzymol.* **5**: 129–164, 1945.

Sumner, J. B., and G. F. Somers: Chemistry and Methods of Enzymes, Academic Press, Inc., New York, 1947.

Tamiya, H.: Ueber die Verwendbarkeit von verschiedenen Kohlenstoffverbindungen im Bau- und Betreibsstoffwechsel der Schimmelpilze. Studien über die Stoffwechselphysiologie von *Aspergillus oryzae*. IV. *Acta Phytochim. (Japan)* **6**: 1–129, 1932.

Tauber, H.: The Chemistry and Technology of Enzymes, John Wiley & Sons, Inc., New York, 1949.

Van Niel, C. B.: Recent advances in our knowledge of the physiology of microorganisms, *Bact. Revs.* **8**: 225–234, 1944.

Volkonsky, M.: Sur la nutrition de quelques champignons saprophytes et parasites, *Ann. inst. Pasteur* **52**: 76–101, 1934.

Weinhouse, S., and R. H. Millington: Acetate metabolism in yeast, studied with isotopic carbon, *Jour. Am. Chem. Soc.* **69**: 3089–3093, 1947.

★Werkman, C. H., and H. G. Wood: Heterotropic assimilation of carbon dioxide, *Advances in Enzymol.* **2**: 135–182, 1942.

White, A. G. C., and C. H. Werkman: Assimilation of acetate by yeast, *Arch. Biochem.* **13**: 27–32, 1947.

White, M. G., and J. J. Willaman: Biochemistry of plant diseases. X. Fermentation of pentoses by *Fusarium lini*, *Biochem. Jour.* **22**: 583–591, 1928.

★Wilson, W. E.: Physiological studies on two species of *Diplodia* parasitic on corn, *Phytopathology* **32**: 130–140, 1942.

Wirth, J. C., and F. F. Nord: Essential steps in the enzymatic breakdown of hexoses and pentoses. Interaction between dehydrogenation and fermentation, *Arch. Biochem.* **1**: 143–163, 1942.

Wolf, F. T., and C. S. Shoup: The effects of certain sugars and amino acids upon the respiration of *Allomyces*, *Mycologia* **35**: 192–200, 1943.

Yaw, K.: Personal communication, 1950.

CHAPTER 8

HYDROGEN-ION CONCENTRATION

The growth of fungi and bacteria may be inhibited or prevented by media which are too acidic or too alkaline. A completely satisfactory medium may be made useless by the addition of relatively small amounts of strong acids or bases but may have its former usefulness restored if the excess acid or base is neutralized. This suggests that the ions which characterize acids and bases are particularly active in life processes. It is necessary to understand certain fundamental ideas about acidity and ways of measuring concentration of these ions before discussing in detail the effects of acids and bases on the activities of the fungi.

IONIZATION OF COMPOUNDS

Since water is the universal solvent for all life processes, our discussion will be confined to aqueous solutions. The chemical compounds which comprise natural and synthetic media may be divided into two classes, those which form ions in solution (acids, bases, and salts), and those which do not ionize (organic compounds in general, except organic acids and bases). Water is a compound of the first class, although it forms ions to a very slight degree. The ionization of water may be represented by the following equation:

(1) $\mathrm{HOH} \rightleftarrows \mathrm{H^+} + \mathrm{OH^-}$ or $\mathrm{HOH} + \mathrm{HOH} \rightleftarrows \mathrm{H_3O^+} + \mathrm{OH^-}$

For each molecule of water ionized one hydrogen and one hydroxyl ion are formed.

In any aqueous solution the product of the concentrations of the hydrogen and the hydroxyl ions (in moles) is equal to a constant (K_w). Water is a neutral compound, *i.e.*, the concentrations of hydrogen and hydroxyl ions are equal. A solution which contains a greater concentration of hydrogen than of hydroxyl ions is acidic; a solution which contains a greater concentration of hydroxyl ions than of hydrogen ions is basic, or alkaline. Since all aqueous solutions contain hydrogen and hydroxyl ions, the deleterious effects of these ions must be due to their relative concentrations. At room temperature (23 to 25°C.) the concentration of hydrogen and hydroxyl ions in water is 1×10^{-7} mole per liter, or 1 mole each of these ions in 10 million liters. The degree of ionization of water increases with temperature. However, water is a neutral substance at

at temperatures because equal numbers of hydrogen and hydroxyl ions are present. The value of K_w at 23 to 24°C. is obtained by multiplying the concentrations of hydrogen and hydroxyl ions present.

2 $$K_w = 1 \times 10^{-7} \times 1 \times 10^{-7} = 1 \times 10^{-14}$$

For the purpose of this discussion we will consider an acid to be a compound whose aqueous solution contains a greater concentration of hydrogen than of hydroxyl ions. A base is a compound whose aqueous solution contains a greater concentration of hydroxyl ions than of hydrogen ions. These definitions will include a number of compounds which are ordinarily considered as salts. A solution of an acid contains a greater concentration of hydrogen ions than pure water by virtue of the ionization of the acid. Hydrochloric acid ionizes as follows:

3 $$HCl = H^+ + Cl^-$$

In a solution of hydrochloric acid there are two sources of hydrogen ions, the acid and water. Enough of the hydrogen ions will combine with the hydroxyl ions to reduce the concentration of this ion so that Eq. 2 will be satisfied. The ionization of a base may be represented as follows:

4 $$NaOH = Na^+ + OH^-$$

The situation is exactly the same as given above for an acid except that the concentration of hydrogen ions is less in a solution of a base than in pure water.

A strong acid, such as hydrochloric, is considered to be completely ionized even in concentrated solutions. A weak acid, such as acetic, in 1*N* solution is ionized only slightly, about 1 per cent. The percentage of ionization of acetic acid increases as the dilution increases. The concentration of hydrogen ions in equal volumes of a normal hydrochloric and acetic acids is thus the chief difference between these acids. Thus the strength of an acid may be expressed in two ways. 1 the total acidity, designated by titratability, which includes both the ionized and unionized molecules of the acid, i.e. the measure of titratability, and 2 the actual acidity, or any instant, which is a function of the concentration of hydrogen ions present. This concentration of hydrogen ions is a function of the concentration and degree of ionization of the acid involved. It is the actual acidity which affects biological processes. It is also necessary to consider the physiological effects of the anions or cations which are associated with particular acids or bases, for it is impossible to add just hydrogen or hydroxyl ions to a medium.

THE MEANING OF pH

The concentration of hydrogen ions in a solution can be expressed in various ways. A derived unit, pH, is most used in biological work. The

calculated concentrations of hydrogen and hydroxyl ions in solutions of hydrochloric acid and potassium hydroxide are given in Table 30. Complete ionization was assumed in these calculations.

TABLE 30. CALCULATED CONCENTRATIONS OF HYDROGEN AND HYDROXYL IONS IN PURE WATER AND IN SOLUTIONS OF HYDROCHLORIC ACID AND POTASSIUM HYDROXIDE OF VARIOUS NORMALITIES

Concentrations are expressed in moles per liter.

Normality	Hydrogen-ion concentration	pH	Hydroxyl-ion concentration
HCl			
1.0	1×10^{0}	0	1×10^{-14}
0.1	1×10^{-1}	1	1×10^{-13}
0.01	1×10^{-2}	2	1×10^{-12}
0.001	1×10^{-3}	3	1×10^{-11}
0.0001	1×10^{-4}	4	1×10^{-10}
0.00001	1×10^{-5}	5	1×10^{-9}
0.000001	1×10^{-6}	6	1×10^{-8}
H_2O	1×10^{-7}	7	1×10^{-7}
KOH			
0.000001	1×10^{-8}	8	1×10^{-6}
0.00001	1×10^{-9}	9	1×10^{-5}
0.0001	1×10^{-10}	10	1×10^{-4}
0.001	1×10^{-11}	11	1×10^{-3}
0.01	1×10^{-12}	12	1×10^{-2}
0.1	1×10^{-13}	13	1×10^{-1}
1.0	1×10^{-14}	14	1×10^{0}

Sorensen devised a logarithmic scale to express the concentration of hydrogen ions in solutions. The name applied to this scale is the "potential of hydrogen," which is abbreviated to pH. The relation between pH and hydrogen-ion concentration is given below.

$$\text{pH} = \log \frac{1}{[\text{H}^+]} \tag{5}$$

In words this equation means pH is equal to the logarithm (base 10) of the reciprocal of the hydrogen-ion concentration. From Table 30 it is seen that the hydrogen-ion concentration of 0.000001*N* hydrochloric acid is 1×10^{-6} mole per liter. Substituting this value in Eq. (5) we have

$$\text{pH} = \log \frac{1}{1 \times 10^{-6}} = \log 10^{6} = 6 \tag{6}$$

One obvious advantage of the pH scale lies in the fact that hydrogen-ion concentrations are expressed as positive numbers rather than as decimal

fractions. If a solution contains 4.23×10^{-6} mole per liter of hydrogen ion, this concentration of hydrogen ions may be expressed in terms of pH by use of Eq. (5).

$$\text{pH} = \log \frac{1}{4.23 \times 10^{-6}} = \log 1 + \log 10^{6} - \log 4.23 = 5.37 \tag{7}$$

By reversing the above calculations, the hydrogen-ion concentration may be calculated if the pH value is known.

The pH scale has three features which may be perplexing: (1) alkaline solutions are designated on the same scale as acidic solutions; (2) increasing acidity is expressed by smaller pH values; and (3) the logarithmic nature of the scale. A difference of one pH unit indicates a tenfold difference in hydrogen-ion concentration, while a difference of 0.3 pH unit means a twofold difference. A solution having a pH value of 4 contains 10,000 times as many hydrogen ions per unit volume as a solution having a pH of 8. A solution having a pH value of 6.2 has twice the hydrogen-ion concentration of a solution having a pH value of 6.5. The student should remember that pH is the name of a logarithmic scale used to measure hydrogen-ion concentration and not an entity in itself.

BUFFERS AND BUFFER CAPACITY

A medium having pH values between 5 and 6 at the time of inoculation is suitable for most fungi, but more acidic or more alkaline media are used at times. It is often important that the pH of the culture medium does not change too greatly as the result of metabolic activities of the organism. The ease with which the pH of a medium is modified depends upon the composition of the medium.

Substances which tend to maintain the pH of a solution relatively constant when an acid or base is added, or when the solution is diluted, are called *buffers*. In general, the kinds of compounds which act as buffers are mixtures of weak acids or bases and their salts, acid salts of polybasic acids, basic salts of polyacidic bases, and amphoteric compounds such as amino acids and peptides. As an example of a buffer we may consider a solution which contains equivalent amounts of acetic acid and sodium acetate. If a strong acid such as hydrochloric is added to this buffer solution, a reaction will occur between the sodium acetate and the hydrochloric acid.

$$CH_3—COONa + HCl \rightarrow CH_3—COOH + NaCl \tag{8}$$

The net effect of adding hydrochloric acid is the formation of an equivalent amount of acetic acid. Inasmuch as acetic acid is only slightly ionized, no great change in hydrogen-ion concentration will take place until most of the sodium acetate has been converted into acetic acid. If

sodium hydroxide is added to an acetate–acetic acid buffer, the sodium hydroxide will react with the acetic acid.

(9) $$CH_3—COOH + NaOH \rightarrow CH_3COONa + H_2O$$

This reaction will convert sodium hydroxide, which is a strong base, into a salt of a weak acid and water. A slight increase in pH will occur, owing to the hydrolysis of the sodium acetate formed. After most of the acetic acid has reacted with sodium hydroxide, the addition of more of the base will result in a rapid increase in the pH value of the solution.

In culturing fungi, it is important to choose buffers which retain the pH of the medium in the desired range. The effective *pH range of buffers* prepared from weak acids and their salts is related to the degree of ionization of the acids. The more highly an acid ionizes, the lower will be the pH range of a buffer prepared from it and one of its salts. The degree of ionization of weak acids is designated by a term called the ionization constant (K_a). Mixtures of weak bases and their salts are also buffers. A few ionization constants of weak acids are acetic, 1.8×10^{-5}; carbonic (first hydrogen), 3.5×10^{-7}; phosphoric (first hydrogen), 1.1×10^{-2}; phosphoric (second hydrogen), 7.5×10^{-8}. Extensive data of this kind may be found in various handbooks of chemistry.

The ionization constants of weak acids may be used to calculate the effective pH range of buffers prepared from these compounds and their salts by means of the following relation:

(10) $$\text{pH} = \text{p}K_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

The symbol pK_a is equivalent to log $(1/K_a)$. When the mole concentrations of the weak acid and its salt are equal, Eq. (10) becomes:

(11) $$\text{pH} = \log \frac{1}{K_a} = \text{p}K_a$$

The pK_a value of a weak acid is thus the pH of a buffer which contains equivalent quantities of a weak acid and one of its soluble salts. The pH of an acetate buffer containing equivalent amounts of acetic acid and an acetate may be calculated using Eq. (11).

(12) $$\text{pH} = \log \frac{1}{1.8 \times 10^{-5}} = \log 1 + \log 10^5 - \log 1.8 = 4.74$$

In an analogous manner it can be shown that the pH of a buffer composed of equivalent amounts of a weak base and one of its salts is related to the ionization constant of the base (K_b) by the following equation:

(13) $$\text{pH} = 14 - \log \frac{1}{K_b} = \text{p}K_b$$

For a derivation of the formulas relating pH and ionization constants

see Umbreit *et al.* (1945). The useful range of a buffer extends one pH unit above and below the pK_a (or pK_b) value. The data in Table 31 giving the pH range of a number of buffer systems of biological interest were calculated using Eq. (11) and (13). The information in Table 31 is useful in selecting buffers which are active in certain pH ranges. Directions for preparing buffer solutions of definite pH are given by Gortner (1949). In practice, media are diluted with these buffers (Lindeberg, 1944).

TABLE 31. THE USEFUL PH RANGE OF A NUMBER OF BUFFER SYSTEMS OF BIOLOGICAL INTEREST CALCULATED BY THE USE OF EQS. (11) AND (13)

Acid or base	Equilibrium reaction	pH range	pK_a or pK_b
Acetic	$CH_3—COOH \rightleftarrows CH_3—COO^-$	3.7– 5.7	4.7
Phosphoric	$H_3PO_4 \rightleftarrows H_2PO_4^-$	1.0– 3.0	2.0
	$H_2PO_4^- \rightleftarrows HPO_4^{=}$	5.7– 7.7	6.7
Carbonic	$H_2CO_3 \rightleftarrows HCO_3^-$	5.5– 7.5	6.5
Fumaric	$HOOC—CH=CH—COOH \rightleftarrows HOOC—CH=CH—COO^-$	2.0– 4.0	3.0
	$HOOC—CH=CH—COO^- \rightleftarrows {}^-OOC—CH=CH—COO^-$	3.5– 5.5	4.5
Malic	$HOOC—CH_2—CHOH—COOH \rightleftarrows HOOC—CH_2—CHOH—COO^-$	2.4– 4.4	3.4
	$HOOC—CH_2—CHOH—COO^- \rightleftarrows {}^-OOC—CH_2—CHOH—COO^-$	4.1– 6.1	5.1
Ammonia	$NH_4OH \rightleftarrows NH_4^+ + OH^-$	8.3–10.3	9.3

A number of other considerations enter into the choice of buffers. They must be nontoxic, but even a buffer composed of an essential nutrient such as phosphate may be inhibitory if used in high concentrations. The use of calcium compounds in media was found to reduce the toxicity of phosphate and citrate buffers to species of *Marasmius* (Lindeberg, 1944). The amount of a buffer required to achieve a given degree of immobilization of pH during growth cannot be specified without considering the fungus involved and the medium used. In experiments where it is desired to maintain the pH of the culture medium essentially constant, the concentration of nutrients, especially the sugar, in the medium should be low, and an organic source of nitrogen should be used in preference to nitrates and ammonium salts. Lindeberg (1944) used *M*/25 phosphate buffer in a medium which contained 10 g. of glucose and 1 g. of asparagine, with satisfactory results.

In general, the usefulness of highly buffered media is restricted to determining pH limits and for special problems. Many fungi would not develop "normally" in media having a fixed pH, especially in certain pH ranges.

If a medium contains several buffer systems, as is frequently the case, each buffer system will play its role over the pH range in which it is active alone. If two buffers with overlapping pH ranges are present, it will require more acid or alkali to effect a change of unit pH than if one buffer were present. The effect of buffer concentration has not been considered in the above discussion. While the pH of a buffer depends solely upon the ratio of the concentrations of the weak acid and its salt, it is obvious that the amount of an acid or base required to change the pH value of a

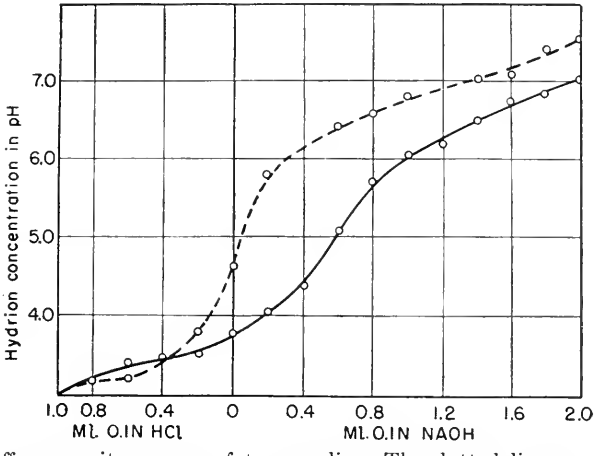


FIG. 21. Buffer-capacity curves of two media. The dotted line was obtained by titrating 20 ml. of glucose-asparagine medium with 0.1*N* hydrochloric acid and 0.1*N* sodium hydroxide. The pH was determined after each addition of acid or base. The solid line was obtained in the same way on the above medium to which 10 mg. of neutralized glutamic acid had been added. (Courtesy of Robbins and Schmitt, *Am. Jour. Botany* **32**: 324, 1945.)

buffer one unit will depend upon the concentrations of the buffer acid and salt present. The *buffer capacity* of a medium is measured by titrating a definite volume of medium (usually 100 ml.) with standard acid and alkali. The pH is measured after each addition of acid or alkali. From the curve drawn from these data the buffer capacity for any range of pH values may be obtained. The curves in Fig. 21 illustrate the buffering capacity of two media (Robbins and Schmitt, 1945). These media differed in the buffers present. The unsymmetrical nature of the curves is due to the presence of overlapping buffers.

The pH of culture media may be controlled within desirable limits, in some instances, by adding calcium carbonate to the medium. Calcium carbonate is essentially insoluble in neutral and alkaline media but acts as a neutralizing agent for acids. The calcium carbonate is used up as acid is produced by a fungus. The degree of neutralization achieved depends upon the amount of calcium carbonate added and whether the

cultures are agitated. See Foster (1949) for a discussion of the use of calcium carbonate in industrial microbiological processes.

For fungi which have an extremely narrow pH range, the special culture flask devised by Cantino (1949) for culturing *Blastocladia pringsheimii* may be used (Fig. 22). A base (or acid) is placed in the side arm and an internal indicator of the desired pH range is added to the medium. A little of the base is tipped into the culture flask as desired. Flasks with two side arms may be used so that either acid or base may be added to the culture medium.

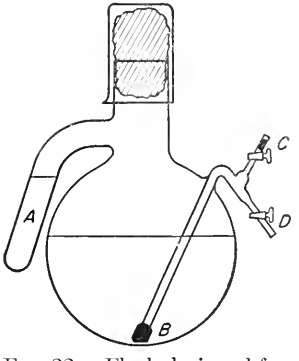


FIG. 22. Flask designed for the study of glucose dissimilation by *Blastocladia*. *A*, the side arm containing NaOH for neutralization; *B*, sintered-glass aerator; *C*, inlet for aeration with different gas mixtures; *D*, the outlet for removal of media. (Courtesy of Cantino, *Am. Jour. Botany* **36**: 100, 1949.)

METHODS OF DETERMINING pH VALUES

Only two general methods of measuring pH values will be discussed. The colorimetric method is simple, inexpensive, and sufficiently accurate for most purposes, but it cannot be used with highly colored or turbid media. The potentiometric method using the glass electrode is more accurate and is often the preferred method.

Colorimetric methods. The use of indicators which change color in response to varying concentrations of hydrogen ion is the basis of this method. Indicators may be considered as weak acids or bases, and as such they act as buffers, but the amounts used are so small they do not affect the accuracy of a determination. For methods of measuring the pH of unbuffered solutions see Snell and Snell (1948). The property of these indicator buffers which distinguishes them from other buffers is that the colors of the salts and free acids or bases (nondissociated) are different. Within the usable pH range, the color of the indicator is a function of the hydrogen-ion concentration of the medium. For example, bromocresol purple (pK_a, 6.3) is yellow in solutions having pH values of 5.2 or less and purple at pH 6.8 or more. Within the pH range 5.2 to 6.8 the color changes from yellow to purple. To determine the pH value of an unknown solution within this range, the indicator is added to equal amounts of standard buffers and the unknown solution, and from the color of the standard buffers of known pH, the pH value of the unknown may be estimated to within 0.1 pH unit. By a suitable choice of indicators the pH range of interest may be covered. A few indicators with their pH ranges are listed in Table 32.

Two methods of color comparison are in general use. The first involves

the use of the familiar comparator block. A slight color or turbidity of the medium may be compensated for by the use of suitable blanks. A porcelain spot plate may be used instead of a comparator block with considerable saving of time and materials, although the accuracy is somewhat less. Drops of the indicator are added to the depressions in the spot plate. A drop of the medium is added to one depression, and drops of standard buffers to the other depressions. The pH of the medium is estimated from the pH of the buffer which yields a color matching that developed in the medium.

TABLE 32. THE pH RANGE AND COLOR CHANGES OF VARIOUS INDICATORS
(Courtesy of Eastman Kodak Company.)

Indicator	pH range	Color change
Bromophenol blue	3.0–4.7	Yellow–blue
Bromocresol green	3.8–5.4	Yellow–blue
Chlorophenol red	4.8–6.8	Yellow–red
Bromocresol purple	5.2–6.8	Yellow–purple
Bromothymol blue	6.0–7.6	Yellow–blue
Phenol red	6.8–8.4	Yellow–red

All colorimetric methods of measuring pH require the use of standard buffers (buffers of known pH) or permanent standards. Buffers may be prepared in the laboratory or purchased from laboratory supply houses. It is convenient to use prepared buffer tablets, which need only to be dissolved in a measured amount of water before use. Potentiometric pH meters also require the use of a standard buffer for calibration. The easiest of these to prepare is a saturated solution of potassium hydrogen tartrate (pH 3.57). The use of this buffer was recommended by Lingane (1947). It is simple to prepare, and temperature affects the pH very little.

From Table 32 it will be noted that the pH range of a single indicator is less than two pH units. Much time can be saved in pH determinations by the use of a *wide-range indicator* to determine the approximate pH before using a single indicator for the final measurement. Wide-range indicators (pH range 2 to 10) may be purchased or prepared by mixing suitable indicators (Snell and Snell, 1948). The pH value of a medium may easily be determined within 0.5 pH unit by the use of a wide-range indicator. Either the comparator block or the spot-plate method may be used. For detailed information about indicators, see Kolthoff and Rosenblum (1937).

Potentiometric methods. The potential difference which develops between certain pairs of electrodes when they are dipped into a solution is a function of the hydrogen-ion concentration. Solutions which give

rise to the same potential difference have the same pH value. Modern pH meters are calibrated in pH units so that direct readings are obtained. Color or turbidity does not affect potentiometric measurement of pH.

The glass electrode in conjunction with the calomel half cell is the most commonly used for liquids of biological interest. The glass electrode consists of a bulb blown from a special glass. The bulb is filled with 0.1*N* hydrochloric acid. A potential difference develops between the inside and the outside of the electrode; the magnitude of this potential difference depends upon the hydrogen-ion concentration of the liquid in which the bulb is dipped. Measuring the potential difference which develops between the glass electrode and the calomel half cell is equivalent to determining the pH value of the unknown solution. Sensitive auxiliary electrical equipment is required to measure this potential difference. For a discussion of the glass electrode, see Dole (1941).

Many suitable pH meters are available. The trend appears to be toward instruments which use alternating current rather than batteries as a source of power. Since the details of operation are somewhat different for the various makes, the directions of the manufacturer should be consulted.

The pH of media should be determined before autoclaving and the reaction adjusted by the addition of acid or alkali if necessary. The pH of a sample of a medium should also be determined after autoclaving and before inoculation. The pH value at this time is known as the *initial pH*. Alkaline media absorb carbon dioxide from the atmosphere, causing a slow decrease in pH. Pritham and Anderson (1937) reported that the pH of uninoculated alkaline media may decrease as much as two units during the course of an experiment. This factor is of particular importance when upper pH limits are being investigated. For methods of adjusting pH, see Suggested Laboratory Exercises.

EFFECTS ON FUNGI

Hydrogen and hydroxyl ions are present in all media and in substrates upon which fungi grow in nature. The pH of the medium exerts a decided effect upon the rate and amount of growth and many other life processes. A medium may have a pH which is favorable for growth and unfavorable for sporulation or other processes. The production of pigments, vitamins, and antibiotics may be influenced by the pH of the medium. As a result of metabolic activity a fungus ordinarily changes the pH of the medium upon which it grows.

pH limits. The upper and lower pH values between which a fungus grows form the pH range of that species. The pH values which inhibit growth vary with the species. Between the limiting pH values there is a pH range which allows optimum growth. An initial pH of 5 to 6 is

satisfactory (not necessarily optimum) for the majority of the fungi. The optimum pH ranges for *Blastocladia pringsheimii*, *Allomyces arbuscula*, and *Blastocladiella simplex* are rather narrow (Emerson and Cantino, 1948) (see Fig. 23). Most of the pH optima reported in the literature are less than 7. Meacham (1918) reported pH 3 to be optimum for *Lenzites saepiaria*, *Fomes roseus*, *Merulius lacrymans*, and *Coniophora cerebella*. Wolpert (1924) found the pH optimum of various Basidiomycetes to be in the neighborhood of 5.5. Johnson (1923) reported that the upper pH limit of *Penicillium variabile* is 10.1 to 11.1, which is con-

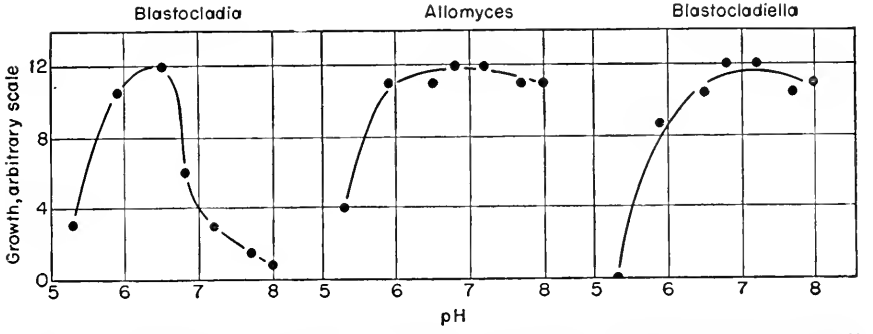


FIG. 23. Relation of pH of the medium to growth of *Blastocladia pringsheimii*, *Allomyces arbuscula*, and *Blastocladiella simplex*. (Courtesy of Emerson and Cantino. *Am. Jour. Botany* **35**: 162, 1948.)

siderably higher than that of most fungi. The lower pH limits reported vary from 5.3 for *B. simplex* (Emerson and Cantino, 1948) to 0.5 for *Acontium velatum* and an unidentified imperfect fungus (Starkey and Waksman, 1943).

The method used to determine the pH limits of a fungus is to inoculate a series of nutrient solutions having pH values spaced 0.2 to 0.4 unit apart. Growth may be observed visually, or the mycelium may be weighed. Such media should be well buffered. The pH limits for a given fungus as determined in different laboratories are frequently at variance. This is not unexpected, since the composition of the medium and the nature of the buffer influence the tolerance of fungi to hydrogen and hydroxyl ions. The behavior of *Marasmius graminum* is revealing (Lindeberg, 1944). Calcium ion was effective in overcoming the toxic effect of an initial pH of 3.3. The weight of *M. graminum* after 12 days was 0.4 mg., but when calcium sulfate was added to the medium, the yield was 8.0 mg. Tamiya (1928) also found calcium ion to protect *Aspergillus oryzae* to some extent against high concentrations of hydrogen ion. The optimum pH for *Gibberella saubinetti* is lower when calcium is present in the medium (Lundegårdh, 1924). Wolpert (1924) also found

the pH range of many fungi to vary on different media and concluded that the widest pH range was obtained on favorable media.

The temperature of incubation may influence the optimum pH as well as the pH range of a fungus. The optimum pH for *Phacidium infestans* is 4.5 at 5°C., 5.0 at 10°C., 5.5 at 15°C. and 6.0 at 20°C. (Pehrson, 1948). The pH range of *Armillaria mellea* on a sucrose-peptone medium was reported to be 2.5 to 7.5 at 15°C., 2.0 to 7.8 at 25°C., and 2.5 to 7.4 at 35°C. (Wolpert, 1924).

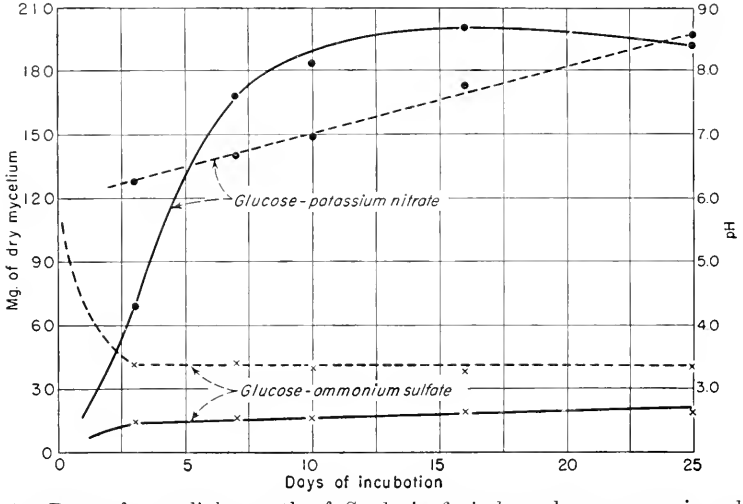


FIG. 24. Rate of mycelial growth of *Sordaria fimicola* and accompanying changes in pH of two media. Media contained biotin but no thiamine. Solid lines indicate growth, and the broken lines represent pH values.

Two pH optima have been reported for a number of fungi. *Rhizopus nigricans,* when grown on potato-glucose liquid medium, has two optimum pH ranges, one on either side of the isoelectric point of the mycelium, which was about pH 5.5 (Robbins, 1924). Scott (1924) reported *Fusarium lycopersici* to have two optimum pH ranges for growth on glucose-nitrate medium: pH 4.5 to 5.3 and 5.8 to 6.8. Mathur *et al.* (1950) obtained evidence that there are two optimum pH ranges for the sporulation of *Colletotrichum lindemuthianum.*

In addition to the use of media having low initial pH values, the lower pH limit may be determined in some instances by choosing a medium in which the fungus produces sufficient acid to inhibit growth completely. This is illustrated by the pH and growth curves of *Sordaria fimicola* in Fig. 24. This fungus was grown upon a glucose–ammonium sulfate medium having initial pH 6.0; after a few days the pH of the culture medium fell to 3.3 and remained there for 5 weeks. More difficulty may be experienced in determining the upper pH limit. If a fungus is able

to make a trace of growth in an alkaline medium, the carbon dioxide produced will lower the pH. Organic acids may also be produced. Carbon dioxide from the air will be absorbed by alkaline media.

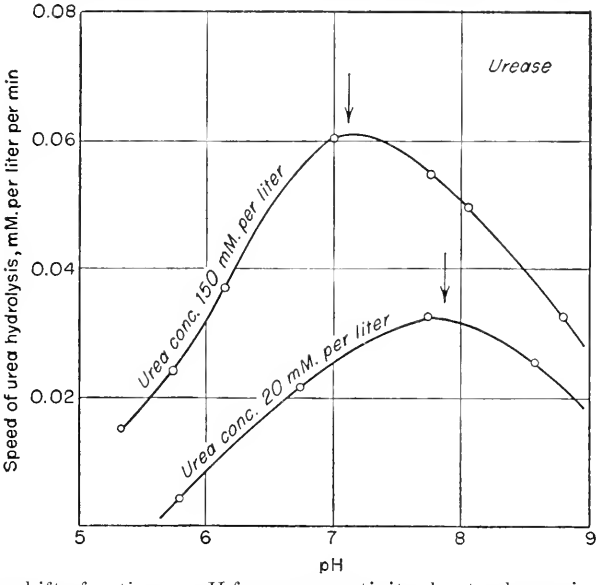


FIG. 25. The shift of optimum pH for urease activity due to change in concentration of urea. (Courtesy of Van Slyke, *Advances in Enzymol.* 2: 41, 1942. Published by permission of Interscience Publishers, Inc.)

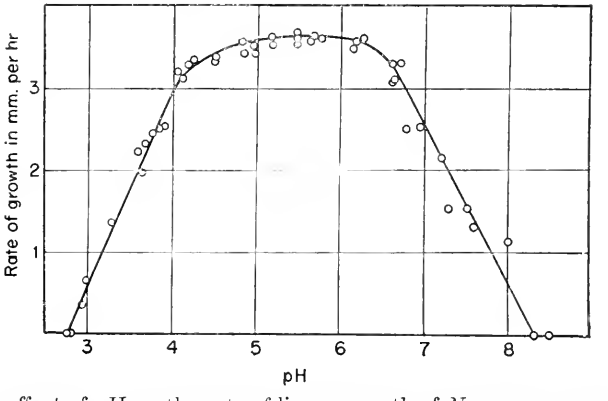


FIG. 26. The effect of pH on the rate of linear growth of *Neurospora crassa*. (Courtesy of Ryan, Beadle, and Tatum, *Am. Jour. Botany* **30**: 790, 1943.)

It was pointed out in Chap. 4 that pH affects the activity of enzymes. In general, there is a striking correlation between the optimum pH range for most enzymes and the optimum pH range for growth. In a survey of the literature Haldane (1930) found all but 9 of 105 enzymes to have optima between pH 4 and 8. Most fungi have pH optima for growth

between these limits. The effects of pH upon the activity of urease (Van Slyke, 1942) and upon the rate of growth of *Neurospora crassa* (Ryan *et al.*, 1943) are shown in Figs. 25 and 26. From the general similarity of these two curves it appears probable that pH affects the rate of growth of fungi, at least in part, by modifying the rate of certain enzymatic reactions.

pH changes in media during growth. Fungi, as a result of their metabolic activities, ordinarily change the pH of the media in which they grow. These changes cannot be studied by making a single deter-

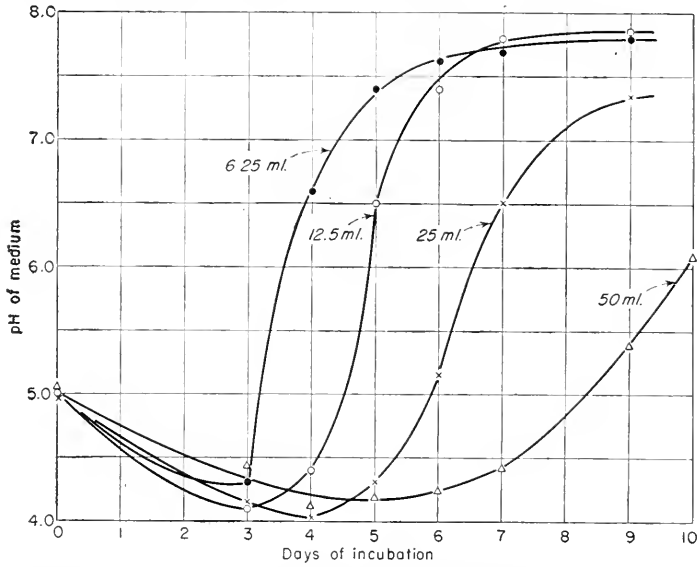


FIG. 27. Changes in pH during incubation of *Sordaria fimicola* in different volumes of liquid glucose–casein hydrolysate medium at 25°C.

mination of pH at any fixed time. Just as it is necessary to study growth as a function of time of incubation (growth curve), it is necessary to determine the pH changes in an inoculated medium day after day to obtain a complete representation of these changes (pH curve). The pH of the medium should be followed in connection with the other functions being studied. Since fungi differ in their metabolic activity and rate of growth, the pH changes produced in the culture medium will differ. The patterns of pH changes for the same fungus will depend upon the composition and concentration of the media used.

As an illustration of the effect of the composition of the medium upon the pH changes, some of our data for *Sordaria fimicola* are given in Fig. 24. The correlation of the pH changes with the rate and amount of growth of this fungus may be obtained by comparing the growth curves obtained at the same time. From Fig. 27 it is evident that the hydrogen-

ion concentration of a nutrient solution may change 10,000-fold during a few days as a result of the metabolic activities of a fungus. These changes in pH are due to changes in the relative amounts of acids and bases formed or withdrawn and to the ionization constants of these compounds. Some of the metabolic processes which result in a change in pH of a nutrient solution are discussed below.

The utilization of cations, such as ammonium ion, for the synthesis of protoplasm or for any other purpose whereby essentially non-ionic compounds are formed, leaves an equivalent number of anions in the nutrient solution. Since solutions are electrically neutral, an equivalent number of both cations and anions must be present. Thus, when an equivalent of ammonium ion is transformed into non-ionic compounds, an equivalent of some other cation or cations will be formed in the nutrient solution. These "new" cations are usually hydrogen ions, which are formed from water. If it is assumed that both cations and anions are adsorbed on the protoplasmic membrane, the process may be thought of as replacement. The production of acid would result from the utilization of other cations as well.

The utilization of nitrate ion or other anion such as phosphate or sulfate for the formation of non-ionized compounds has the effect of increasing the hydroxyl-ion concentration of the medium. We may assume the same type of mechanism as before, except that the anion released to the nutrient solution is the hydroxyl ion.

Fungi produce acids from nonacidic nutrients such as carbohydrates. Among these acids are carbon dioxide and various organic acids such as pyruvic, citric, and succinic acids. Carbon dioxide combines with water to form carbonic acid, which is unstable in the presence of stronger acids and decomposes to set free carbon dioxide. Under alkaline conditions carbonic acid reacts with bases to form bicarbonates. Pyruvic acid accumulates in the nutrient solution in which many fungi are grown, and in some instances the formation of this acid accounts for a considerable part of the early depression of pH. The eventual utilization of pyruvic acid causes the pH of the nutrient solution to rise. Other metabolizable acids behave similarly. Ammonia is, perhaps, the most common basic substance produced by fungi. *Piricularia oryzae* produces ammonia in considerable amounts (Henry and Andersen, 1948). The production of ammonia results from the deamination of amino acids and proteins. The processes discussed above may occur simultaneously. Whether a culture solution becomes more acid or alkaline depends upon the extent of these various processes. In general, the processes which produce acid predominate during early growth, especially when ammonium nitrogen is used.

The importance of the composition of the medium in determining what

changes in pH will take place during growth is illustrated by the work of Dimond and Peltier (1945), who studied the pH changes produced by *Penicillium notatum* as a function of the carbon and nitrogen nutrition. When the initial pH was 6.0 and sodium nitrate was the nitrogen source, the lowest pH values attained on different sugars were glucose, 5.1; sucrose, 4.0; lactose, 3.2; maltose, 4.8; and galactose, 4.8. These were the lowest pH values attained under these conditions. In another experiment a mixture of tryptophane, asparagine, and cystine was used as the nitrogen source. The pH again varied with the sugar used in the medium The lowest pH attained with fructose was 5.3; glucose 3.5; sucrose, 4.0; and an equimolecular amount of fructose and glucose, 3.5. When lactose was used in combination with these amino acids, the pH of the culture medium remained essentially constant at 7.0

Any changes in environmental factors which affect the rate of growth of a fungus may also affect the changes in pH of the culture medium. Robbins and Schmitt (1945) found that the time required for *Phycomyces blakesleeanus* to lower the pH of a glucose-asparagine medium to a given level was a function of temperature of incubation. Growth and the production of acid were more rapid at 26°C than at 20°C. The rate at which the pH of a culture medium is changed by a fungus is also dependent upon the volume of medium used in flasks of the same size. Some of our data which illustrate this for *Sordaria fimicola* are shown in Fig. 27. The time required for these cultures to attain maximum weight and to produce perithecia correlated with the changes in pH.

Effect of acidity on media. The composition of a medium may be changed as a result of changing the pH. The various cations and anions may combine to form insoluble compounds at certain pH values. Magnesium and phosphate ions are compatible in acidic solutions, but as the concentration of hydrogen ion is decreased, these ions combine to form an insoluble compound, the solubility of which becomes less as the pH is increased. Calcium phosphate is likewise less soluble in alakline solutions. Ferric iron may be largely removed from media as either the hydroxide or the phosphate, by making the media alkaline. If an alkaline medium is filtered, certain constituents will be removed to a greater or lesser extent. Lilly and Leonian (1945) found that by making a medium alkaline to pH 8 and filtering, the iron concentration was lowered to such levels that *Rhizobium trifolii* made about one-fifth as much growth as when 250 μg of iron per liter was added to the medium. If a precipitate is not removed by filtration, the situation is different. Any insoluble precipitate is in equilibrium with the dissolved compound, as indicated below.

$$\underset{\text{solid}}{FePO_4} \rightleftarrows \underset{\text{in solution}}{FePO_4} \rightleftarrows \underset{\text{ionized}}{Fe^{+++} + PO_4^{\equiv}}$$

As the ions are utilized, more and more of the precipitate will dissolve. The effect of a change in pH of the solution as a result of the metabolic activities of the fungus must be considered. An acid reaction will hasten solution of the precipitate, while an increase in alkalinity will delay the process. It is possible that the harmful effects sometimes noted in alkaline media may be due, in part, to an induced iron deficiency.

The influence of pH on the solubility of certain ions may be modified by the presence of other compounds, especially those which form complexes. The solubility of iron in alkaline solutions is greatly increased in the presence of hydroxy organic acids such as citric, tartaric, and malic acids. Ammonia and amino acids also form complexes with certain ions, *e.g.*, copper. The presence of any complex-forming compound may modify the availability of the ions with which it forms complexes. The chemical changes in media due to alteration of pH, whether imposed from the outside or caused by the fungus, affect metabolic processes. The pH of a culture medium changes during the growth of a fungus, and these changes may affect the composition of the medium and thus the response of the fungus.

pH and oxygen supply. The solubility of oxygen in water is slight, being less than 10 mg. per liter at 20°C. The rate of diffusion of oxygen into media is dependent upon the composition and the pH. Rahn and Richardson (1941) have described a simple and elegant method of measuring the rate of diffusion of oxygen into agar media. Methylene blue, an organic dye which is colorless when reduced and blue when oxidized, was used as an indicator. When this dye (1/200,000) is autoclaved with media which contain easily oxidized constituents such as glucose, the dye is reduced to the leuco, or colorless, form. As oxygen diffuses into the medium, the reduced dye is oxidized, and the rate at which the blue zone advances into the medium is a measure of the rate of oxygen diffusion. The pH of the medium also affects the ease with which certain constituents are oxidized. Some data of Rahn and Richardson (1941) on the rate of oxygen diffusion into a peptone medium are shown in Fig. 28. The amount of oxygen available to submerged mycelium is greater in acidic than in alkaline media.

Effect of pH on utilization of nutrients. Before any substance (ion or molecule) is utilized, it must first pass through the cell wall and the protoplasmic membrane. The cell wall is nonliving and consists of polysaccharide-like compounds. For a discussion of the nature of the cell wall and literature citations, see Brian (1949). The protoplasmic membrane appears to be composed of proteins and lipoid-protein complexes. Proteins are colloidal amphoteric compounds. An amphoteric compound possesses both acidic and basic properties and may form salts with either acids or bases. The protoplasmic membrane has acidic

properties due to carboxyl and sulfhydryl groups and basic properties by virtue of having amino and other basic groups. The protoplasmic membrane, therefore, should form salt-like compounds with both cations and anions.

Bacteria are considered by McCalla (1940) to act as ion-exchange substances, and fungus spores have been shown to act in the same manner. McCalla investigated ion replacement by saturating cells of *Escherichia*

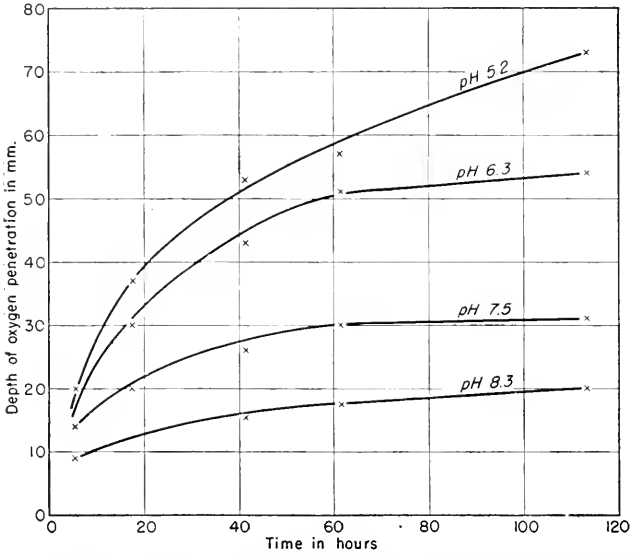


FIG. 28. The effect of hydrogen-ion concentration on the rate of diffusion of oxygen into 1 per cent peptone in phosphate buffer. Leucomethylene blue was used as an indicator. The rate of penetration of oxygen with time was followed by measuring the depth of the blue zone. (Drawn from the data of Rahn and Richardson, *Jour. Bact.* **41**: 240, 1941. By permission of The Williams & Wilkins Company.)

coli with magnesium ion and tested the replacing effects of other cations. Sodium and potassium ions replaced only a little magnesium, while hydrogen and calcium ions were much more effective.

From this viewpoint the relative amounts of the various cations adsorbed from a medium would be a function of the concentration of the ions present and the relative affinity of the membrane proteins for the different cations. The concentrations of the hydrogen and hydroxyl ions in a culture medium change during growth and may act to regulate the adsorption of other ions. The pH of the culture medium may alter the relative adsorption of other ions which are essential to nutrition or which are toxic. At the lower pH limit the protoplasmic membrane may be so thoroughly saturated with hydrogen ions that the essential cations are unable to enter the cell in adequate amounts. The same situation would

exist at the limiting alkaline pH values, except that it is the adsorption of essential anions which would be hindered by hydroxyl ions.

A satisfactory explanation of all the phenomena involved in cell permeability is lacking. It is known that the external pH affects the absorption of various compounds, particularly those which ionize. The mycelium of *Aspergillus niger* takes up acid dyes, such as light green and methyl orange, when the external pH is 3.1 or less. Basic dyes such as methylene blue and neutral red are absorbed only when the external pH is greater than 3.1 (Bünning, 1936). These dyes escaped from the cells only when the external pH was in the same range in which these dyes were absorbed.

Wyss *et al.* (1944) found the utilization of *p*-aminobenzoic acid by a deficient mutant of *Neurospora crassa* to be greatly increased in acidic media. The ionization constant of *p*-aminobenzoic acid is about 2×10^{-5} (pK_a, about 4.8). Therefore, at pH 3.8 about 90 per cent of the metabolite would exist in the form of the free acid, and at pH 5.8 only 10 per cent would be in this form. It was found that about eight times as much of this vitamin was required at pH 6.0 as at pH 4.0 to support the same amount of growth (see Fig. 41). On theoretical grounds, it is probable that the pH of the medium would affect the utilization of other vitamins which are weak acids (biotin, pantothenic and nicotinic acids).

The external pH of the medium has been shown to affect the internal pH of fungus cells. By changing the external pH and by using indicators, Bünning (1936) found the internal pH of *Aspergillus niger* cells could be changed between 4.2 and 5.0 without injuring the cells. Greater changes in internal pH were possible, but injury and death ensued. Armstrong (1929) crushed the fruit bodies of a number of fleshy fungi and measured the pH of the expressed juice. The pH range of these liquids was 5.9 to 6.2. At best these are but average values.

It is well known that the external pH may affect certain processes within the fungus cells. For example, growth of *Sordaria fimicola* in glucose–casein hydrolysate medium was slow when the initial pH of the medium was 4.0, but when the initial pH of the medium was 3.6 to 3.8, normal development did not occur (Lilly and Barnett, 1947). This failure to grow was traced to a thiamine deficiency, for when thiamine was added to the medium (initial pH 3.6 to 3.8), normal growth and perithecial formation took place. It appears possible that the low external pH may have lowered the internal pH to such an extent that the synthesis of thiamine was prevented (this fungus is self-sufficient for thiamine when the pH of the medium is 4 or greater). These effects are shown in Figs. 38 and 40. Additional evidence indicated that these conclusions are correct, for pyruvic acid accumulated in the culture medium when the initial pH was 3.6 to 3.8. On the addition of thiamine this acid disappeared from the culture medium.

SUMMARY

All aqueous solutions contain hydrogen and hydroxyl ions. Hydrogen-ion concentration is most often expressed in terms of Sørensen's scale of pH. The pH scale is logarithmic. Acidity and alkalinity are expressed on the same scale. A pH of 7 indicates equivalent concentrations of hydrogen and hydroxyl ions. Values of less than 7 indicate acidity, and pH values greater than 7 indicate alkalinity. The smaller the pH values, the greater the concentration of hydrogen ions.

Buffers are substances which tend to maintain the pH of a solution constant when either strong acid or strong alkali is added or when the solution is diluted with water. Mixtures of weak acids or bases and their soluble salts, and amphoteric compounds such as amino acids and proteins act as buffers. The pH range over which a given buffer is effective is a function of the ionization constant of the weak acid (K_a) or base (K_b) from which the buffer is made. The effective pH range of a simple buffer is two pH units.

The upper and lower pH values between which a fungus is able to grow is called the pH range. The pH ranges of various species are different. Fungi generally tolerate more acid than alkali. The optimum pH range may be different for growth and sporulation. The pH of a medium in which a fungus is growing may change. High buffer concentration and limited growth may keep the changes in pH at a minimum. To follow the changes in pH of a culture medium, frequent determinations should be made.

Four metabolic processes operate to change the pH of a culture medium: (1) utilization of cations, (2) utilization of anions, (3) formation of acids from neutral metabolites (especially carbohydrates), and (4) formation of bases (especially ammonia) from amino acids and proteins. The net change in pH is the result of the interaction of all of these processes.

REFERENCES

Armstrong, J. I.: Hydrogen-ion phenomena in plants. Hydrion concentration and buffers in the fungi, *Protoplasma* **8**: 222–260, 1929.

Brian, P. W.: Studies on the biological activity of griseofulvin, *Ann. Botany* (N.S.) **13**: 59–77, 1949.

Bünning, E.: Ueber Farbstoff- und Nitrataufnahme bei *Aspergillus niger*, *Flora* **131**: 87–112, 1936.

Cantino, E. C.: The physiology of the aquatic Phycomycete, *Blastocladia Pringsheimii*, with emphasis on its nutrition and metabolism, *Am. Jour. Botany* **36**: 95–112, 1949.

★Dimond, A. E., and G. L. Peltier: Controlling the pH of cultures of *Penicillium notatum* through its carbon and nitrogen nutrition, *Am. Jour. Botany* **32**: 46–50, 1945.

Dole, M.: The Glass Electrode. Methods, Applications, and Theory, John Wiley & Sons, Inc., New York, 1941.

★Emerson, R., and E. C. Cantino: The isolation, growth, and metabolism of *Blastocladia* in pure culture, *Am. Jour. Botany* **35**: 157–171, 1948.

Foster, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

Gortner, R. A.: Outlines of Biochemistry, 3d ed., John Wiley & Sons, Inc., New York, 1949.

Haldane, J. B. S.: Enzymes, Longmans, Roberts and Green, London, 1930.

Henry, B. W., and A. L. Andersen: Sporulation by *Piricularia oryzae*, *Phytopathology* **38**: 265–278, 1948.

Johnson, H. W.: Some relationships between hydrogen ion, hydroxyl ion and salt concentration and the growth of seven soil molds, *Iowa State Coll. Agr. Mech. Arts Research Bull.* 76, 1923.

Kolthoff, I. M., and C. Rosenblum: Acid-base Indicators, The Macmillan Company, New York, 1937.

★Lilly, V. G., and H. L. Barnett: The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*, *Am. Jour. Botany* **34**: 131–138, 1947.

Lilly, V. G., and L. H. Leonian: The interrelationship of iron and certain accessory factors in the growth of *Rhizobium trifolii* strain 205, *Jour. Bact.* **50**: 383–395, 1945.

Lindeberg, G.: Ueber die Physiologie Ligninabbauender Bodenhymenomyzeten, *Symbolae Botan. Upsalienses* **8**(2): 1–183, 1944.

Lingane, J. J.: Saturated potassium hydrogen tartrate solutions as a pH standard, *Anal. Chem.* **19**: 810–811, 1947.

Lundegårdh, H.: Der Einfluss der Wasserstoffionenkonzentration in Gegenwart von Salzen auf das Wachstum von *Gibberella Saubinetti*, *Biochem. Zeit.* **146**: 564–572, 1924.

McCalla, T. M.: Cation adsorption by bacteria, *Jour. Bact.* **40**: 23–32, 1940.

Mathur, R. S., H. L. Barnett, and V. G. Lilly: Sporulation of *Colletotrichum lindemuthianum* in culture, *Phytopathology* **40**: 104–114, 1950.

Meacham, M. R.: Note upon the hydrogen ion concentration necessary to inhibit the growth of four wood-destroying fungi, *Science* **48**: 499–500, 1918.

Pehrson, S. O.: Studies on the growth physiology of *Phacidium infestans* Karst., *Physiologia Plantarum* **1**: 38–56, 1948.

Pritham, G. H., and A. K. Anderson: The carbon metabolism of *Fusarium lycopersici* on glucose, *Jour. Agr. Research* **55**: 937–949, 1937.

Rahn, O., and G. L. Richardson: Oxygen demand and oxygen supply, *Jour. Bact.* **41**: 225–249, 1941.

Robbins, W. J.: Isoelectric points for the mycelium of fungi, *Jour. Gen. Physiol.* **6**: 259–271, 1924.

★Robbins, W. J., and M. B. Schmitt: Factor Z_2 and gametic reproduction by *Phycomyces*, *Am. Jour. Botany* **32**: 320–326, 1945.

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

★Scott, I. T.: The influence of hydrogen-ion concentration on the growth of *Fusarium lycopersici* and on tomato wilt, *Missouri Coll. Agr. Research Bull.* 64, 1924.

Snell, F. D., and C. T. Snell: Colorimetric Methods of Analysis, 3d ed., D. Van Nostrand Company, Inc., New York, 1948.

Starkey, R. L., and S. A. Waksman: Fungi tolerant to extreme acidity and high concentrations of copper sulfate, *Jour. Bact.* **45**: 509–519, 1943.

Tamiya, H.: Studien über die Stoffwechselphysiologie von *Aspergillus oryzae*. II. *Acta Phytochim.* (*Japan*) **4**: 77–213, 1928.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer: Manometric Techniques and

Related Methods for the Study of Tissue Metabolism, Burgess Publishing Co., Minneapolis, 1945.

Van Slyke, D. D.: The kinetics of hydrolytic enzymes and their bearing on methods of measuring enzyme activity, *Advances in Enzymol.* **2**: 33–47, 1942.

Wolpert, F. S.: Studies in the physiology of the fungi. XVII. The growth of certain wood-destroying fungi in relation to the H-ion concentration of the media, *Ann. Missouri Botan. Garden* **11**: 43–97, 1924.

Wyss, O., V. G. Lilly, and L. H. Leonian: The effect of pH on the availability of *p*-aminobenzoic acid to *Neurospora crassa*, *Science*, **99**: 18–19, 1944.

CHAPTER 9

VITAMINS AND GROWTH FACTORS

It is known that, for normal growth and development, animals and man require in their diet minute amounts of certain organic compounds, in addition to those which yield energy or are used for structural purposes. Similarly, certain fungi must obtain from the substrate some of the same substances for growth, reproduction, and other vital functions. Other fungi are able to synthesize these compounds, which are called *growth factors*, or *vitamins*. Both terms have often been applied to the same compounds, although the terms are not always synonymous. Originally, the term vitamin was applied to the accessory factors in animal nutrition, and some workers would restrict its use to animals and man. The term growth factor has a somewhat broader connotation than vitamin. It includes the components and derivatives of some vitamins, as well as other compounds which cannot be classified otherwise at present. The chemical names of the vitamins also may be used.

GENERAL CONSIDERATIONS

A number of vitamins, such as thiamine and biotin, have been shown to perform definite functions in fungi as well as in animals, and there is no reason to assume that the fundamental functions in the two groups of organisms are essentially different. The characteristic features of a growth factor (vitamin) include the following: (1) its organic nature; (2) its activity in minute quantities; (3) its catalytic action; (4) the specificity of its action. It is known that some vitamins are components of enzyme systems, and it may be assumed that all act in this way.

In the fungi the relative effects of the presence of vitamins in the medium usually are measured by the resultant vegetative growth, although vitamins are known to affect reproduction and other processes. Needless to say, studies of vitamin deficiencies must be carried out under carefully controlled conditions, using clean glassware, purified chemicals, and precaution against contamination. Despite all precautions possible, variable results often occur, and tests may need to be repeated several times before the vitamin deficiencies of some fungi can be definitely determined.

SYNTHESIS OF VITAMINS BY FUNGI

Many fungi are able to grow and develop normally on a substrate containing no vitamins. For example, *Aspergillus niger* grows well on a

synthetic medium composed of pure chemicals (glucose, asparagine, salts, and micro elements). *Phycomyces blakesleeanus* makes no growth on this medium unless thiamine is added. We may conclude that *A. niger* either does not need thiamine in its metabolism or is capable of synthesizing from the compounds of the medium all vitamins in sufficient quantities to meet its needs. The growth of *P. blakesleeanus* on the culture filtrate of *A. niger* is proof that thiamine is synthesized by the latter species. Thus, *A. niger* may be called a *self-sufficient* fungus with respect to vitamins. Schopfer (1943) has applied the term *autotrophic with respect to* vitamins to this group of organisms. The detection of self-sufficient fungi in the laboratory is dependent upon their ability to grow on vitamin-free synthetic media containing suitable sources of carbon and nitrogen. A discussion of the economic importance of certain vitamins as metabolic products of fungi is given in Chap. 13.

Some fungi which have been reported to be self-sufficient with respect to vitamins are listed below:

Aspergillus (most species tested)
Basisporium gallarum
Botrytis allii
Cercospora apii
C. beticola
Chaetomium globosum
Cordyceps militaris
Daldinia concentrica
Fusarium (most species tested)
Glomerella cingulata
Helminthosporium gramineum
H. victoriae
Monascus purpurea
Monilinia fructicola (some isolates)
Neocosmopara vasinfecta
Penicillium (most species tested)
Phoma betae
Rhizopus nigricans
Sclerotinia sclerotiorum
Septoria nodorum
Sphaeropsis malorum
Ustilago striiformis

Growth curves of *Chaetomium globosum* are presented in Fig. 29, as an example of a self-sufficient fungus. It is evident that good mycelial growth was made in the vitamin-free medium and that the addition of four vitamins caused no significant increase in the rate of growth at any time.

VITAMIN DEFICIENCIES IN FUNGI

As pointed out above, some fungi do not grow on synthetic media composed of pure chemicals, because they are unable to synthesize certain vitamins. These fungi have been called variously *vitamin-deficient*, *vitaminless*, or *heterotrophic with respect to* one or more specific vitamins. We prefer to use the term vitamin-deficient, following Robbins and Kavanagh (1942). Vitamin deficiencies among the fungi have been detected only for certain members of the water-soluble B-complex group. The most common vitamins involved are thiamine, biotin, inositol, pyridoxine, nicotinic acid, and pantothenic acid. Vitamin deficiencies can be detected accurately only on synthetic media which, other than

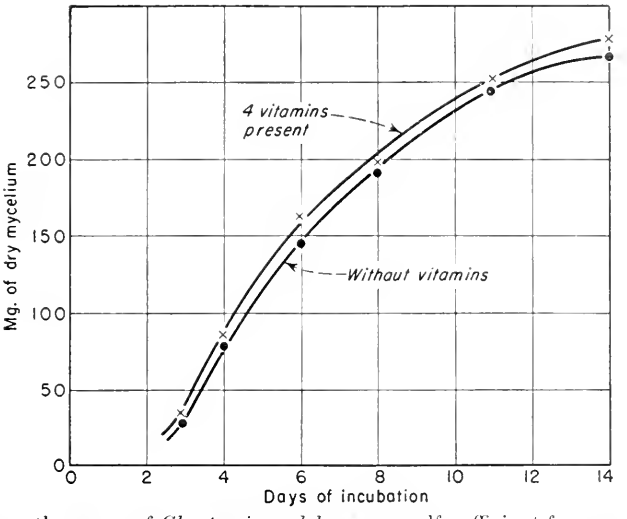


FIG. 29. Growth curves of *Chaetomium globosum*, a self-sufficient fungus, in 25 ml. of liquid glucose–casein hydrolysate medium in the absence of vitamins and when thiamine, biotin, inositol, and pyridoxine were added.

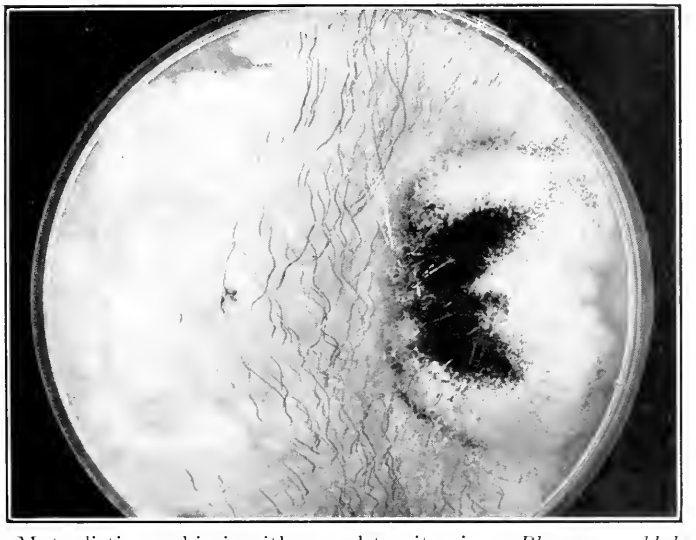


FIG. 30. Mutualistic symbiosis with regard to vitamins. *Phycomyces blakesleeanus*, thiamine-deficient, inoculated on the right and *Sordaria fimicola*, biotin-deficient, inoculated on the left. Both fungi made only slight growth until the two colonies met. Note the peritheeia of *Sordaria* on the right and the sporangiophores of *Phycomyces* on the left. Each fungus excreted into the medium the vitamin which the other could not synthesize.

vitamins, meet all the requirements for normal growth and development of the fungus under study. The effect of one deficient fungus on another is shown in Fig. 30.

Methods of detecting vitamin deficiencies. Tests for vitamin deficiencies of fungi are not difficult to perform, but they do require clean glassware and careful preparation of media. It is convenient to conduct preliminary experiments using only the four vitamins (thiamine, biotin, inositol, pyridoxine) for which fungi are most frequently deficient. A greater number of vitamins may be included in subsequent tests if a fungus does not grow well on any of the media first used. Either agar or liquid media may be used, and the visual measure of growth is satisfactory for the screening tests. A simple and convenient method for preliminary tests for deficiencies in filamentous fungi isolated from nature is by the use of agar media in test tubes, as shown in Fig. 31. Slight growth on agar media without added vitamins may be due to impurities in the medium. A high percentage of the deficiencies will be detected by this method, since deficiencies for only one or two vitamins are common among the filamentous fungi. After the deficiencies have been identified by preliminary experiments, it is then highly desirable to grow a fungus in liquid media, so that the mycelium may be harvested and dry weights determined (see Suggested Laboratory Exercises for directions). The casein hydrolysate-glucose medium, given in Chap. 10, has proved quite satisfactory for accurate vitamin studies. From the dry weights of cultures determined at intervals throughout the growth period of a fungus, growth curves may be plotted. Such curves are necessary for accurate interpretations of the effects of vitamins in the medium.

A somewhat different method is used by Burkholder (1943) for deficiency studies of yeasts, where deficiencies for more than two vitamins are common. This method is illustrated in Fig. 32. A deficiency is detected by the inability to grow in a medium which is complete except for one vitamin. Failure to grow in a medium indicates a deficiency for the vitamin omitted. Liquid media in test tubes are used for yeasts, so that growth may be measured by photoelectric colorimeter.

Total and partial deficiencies. *Phycomyces blakesleeanus* was widely used in the early studies of thiamine. Schopfer established the deficiency for thiamine and determined the requirements for this vitamin. Schopfer's graph (Fig. 33) shows the growth curves of the fungus over a period when different amounts of thiamine were added to the basal culture medium (Schopfer, 1943). The fact that no growth occurred in the medium lacking thiamine is not shown by the graph. An increase in both the rate and the total amount of growth, as the amount of thiamine is increased, is clearly shown between the fifth and seventh days. Thus *P. blakesleeanus* is unable to synthesize thiamine, which it must obtain

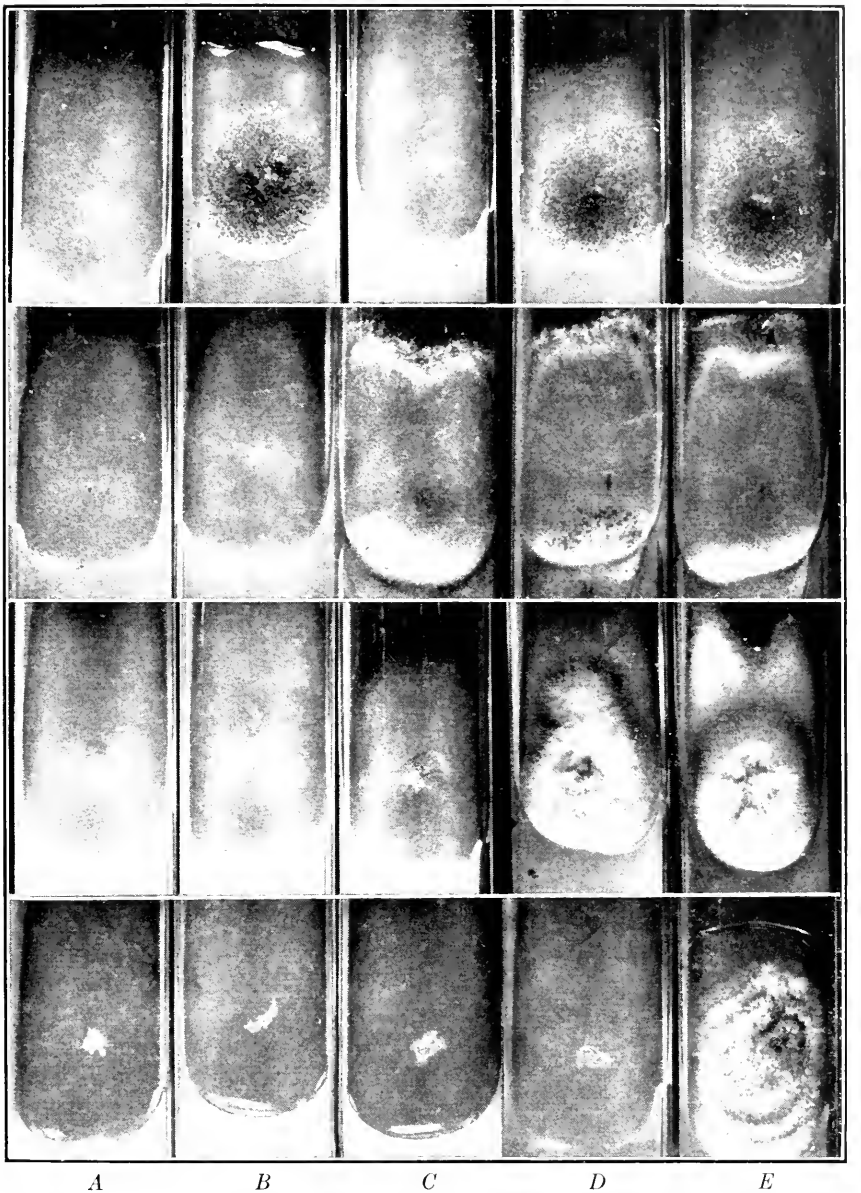


FIG. 31. Method of detecting common vitamin deficiencies of filamentous fungi. Deficiencies are evident by failure to grow on media lacking the necessary vitamin or vitamins. The above media contained: *A*, no vitamins; *B*, thiamine; *C*, biotin; *D*, thiamine and biotin; *E*, thiamine, biotin, inositol, and pyridoxine. The fungi, from top to bottom, are *Ceratostomella fimbriata*, *Sordaria fimicola*, *Pleurage curvicolla*, and *C. ulmi*.

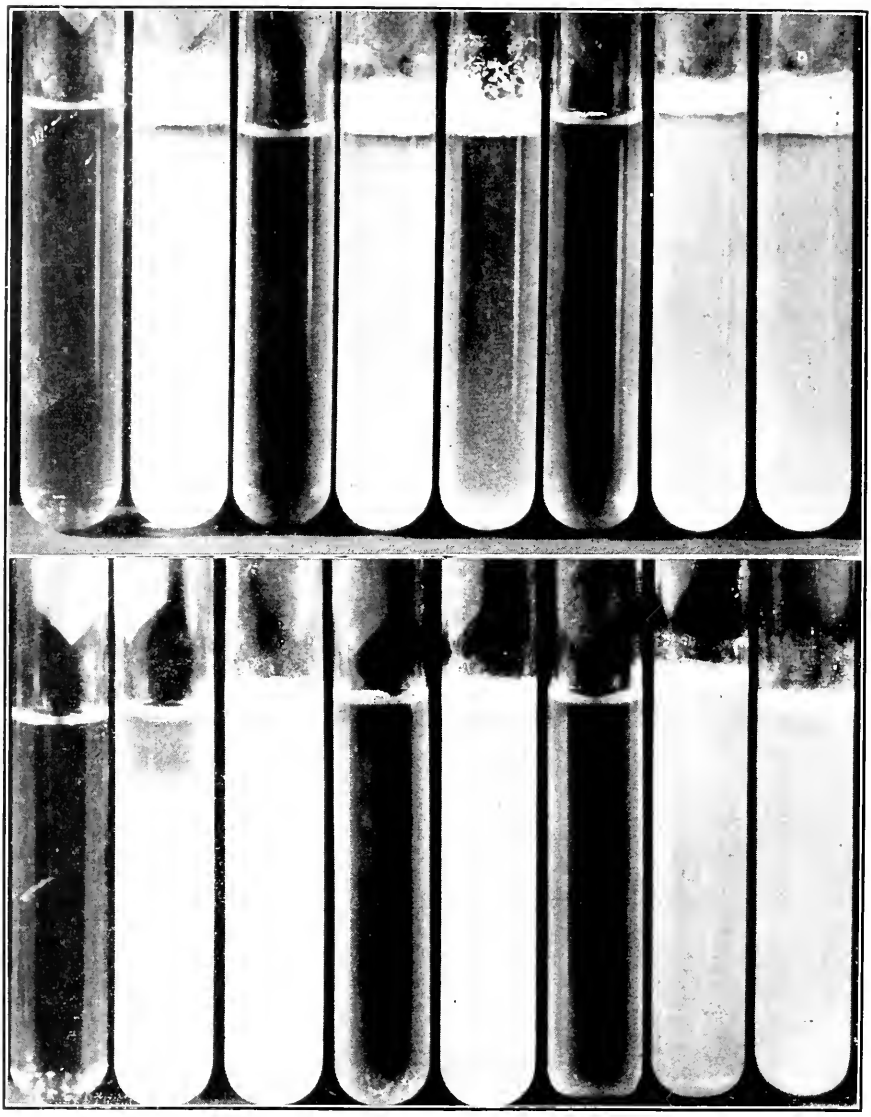


FIG. 32. Method of detecting multiple vitamin deficiencies of yeasts. Failure to grow in the absence of a particular vitamin indicates a deficiency if the culture grew in a medium supplied with a combination of vitamins. Growth of a strain of *Saccharomyces cerevisiae* (above) and *Mycoderma valida* (below) after 5 days at 25°C. From left to right the vitamin supplements were: Tube 1, none; 2, less thiamine; 3, less pantothenic acid; 4, less pyridoxine; 5, less inositol; 6, less biotin; 7, less nicotinic acid; 8, all six vitamins.

from its substrate. Figure 33 emphasizes two important features which must be considered in vitamin studies: (1) the effects of different amounts of the vitamin in the medium, and (2) the response of the fungus over a period of time sufficiently long to allow maximum growth. The three-dimensional graph permits one to plot dry weight against both variables.

The failure of a fungus to make an appreciable amount of growth even after an extended period of incubation on a medium essentially free of a

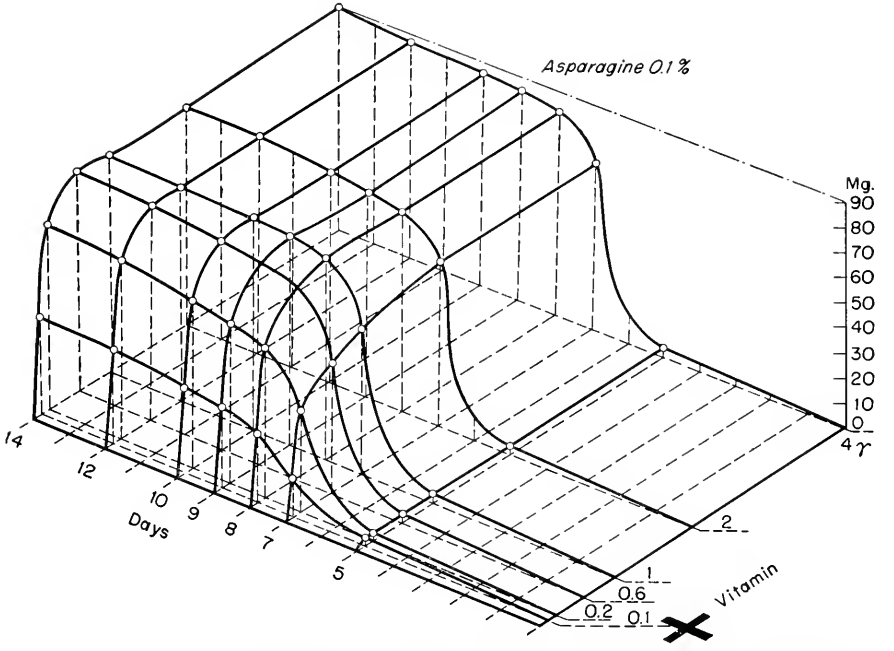


FIG. 33. Three-dimensional graph showing growth of *Phycomyces blakesleeanus* on a synthetic medium as a function of thiamine concentration and time. (Courtesy of Schopfer, *Protoplasma* **28**: 383, 1937; also from the book "Plants and Vitamins," p. 102, 1943. Published by permission of Chronica Botanica Co.)

particular vitamin, like the case illustrated by *P. blakesleeanus* and thiamine, indicates that the deficiency is *total; i.e.*, the synthesis of that vitamin is zero. Vitamin deficiencies of many fungi are only *partial*, as shown by a slower rate of growth in a vitamin-free medium than in the presence of added vitamins. The degree of partial deficiency may vary widely, from slight to nearly total. Partial deficiencies may be easily overlooked by terminating an experiment too soon. An incubation period of 1 or 2 months is often required to distinguish between partial and total deficiencies of some fungi.

An example of partial thiamine deficiency is illustrated by *Lenzites trabea* (Fig. 34). In a medium containing thiamine, maximum weight

was attained in 20 days, while, in medium lacking thiamine, the fungus required approximately 40 days to reach the maximum weight. This is attributed to the slow rate of synthesis of thiamine. Other isolates of *L. trabea* showed varying degrees of partial deficiency (Lilly and Barnett, 1948).

Single and multiple deficiencies. The above discussion has dealt with examples of *single* deficiencies (for a single vitamin). For example, *Sordaria fimicola* is deficient only for biotin (Fig. 31), *Lenzites trabea*,

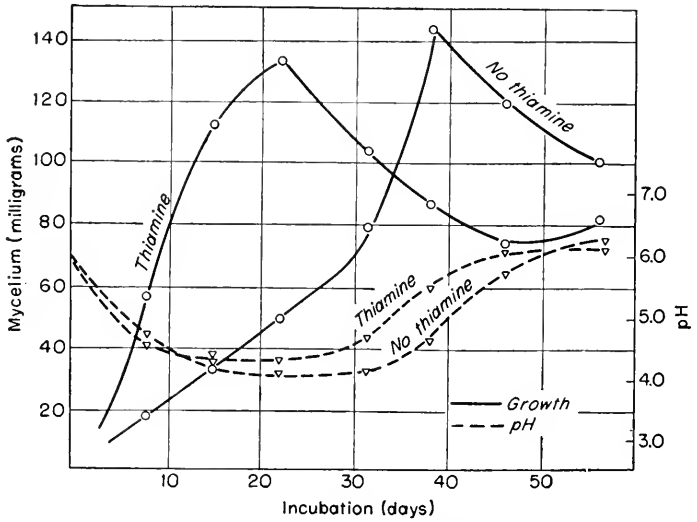


FIG. 34. Growth of a haploid isolate of *Lenzites trabea* and change in pH of liquid glucose–casein hydrolysate medium at 25°C., with and without the addition of thiamine. These curves illustrate a partial deficiency for thiamine. (After Lilly and Barnett, *Jour. Agr. Research* **77**: 290, 1948.)

Ceratostomella fimbriata (Fig. 31), and *Phycomyces blakesleeanus* for thiamine only. On the other hand, some fungi have *multiple* deficiencies (for two or more vitamins). These may be total or partial. An illustration of multiple deficiency is furnished by *Sclerotinia camelliae* (Fig. 35). Little or no growth occurred on vitamin-free medium or that containing either thiamine or biotin alone; the fungus grew well only in media containing both thiamine and biotin. When inositol also was added, growth was consistently better than in the presence of the two vitamins. This indicates a partial deficiency for inositol, in addition to the total, or near total, deficiencies for thiamine and biotin. Pyridoxine, when added to the other three vitamins, had little or no effect on growth under these conditions.

Other examples of multiple vitamin deficiencies are common. *Pleurage curvicolla* (Fig. 31), *Chaetomium convolutum*, *Coemansia interrupta*, and

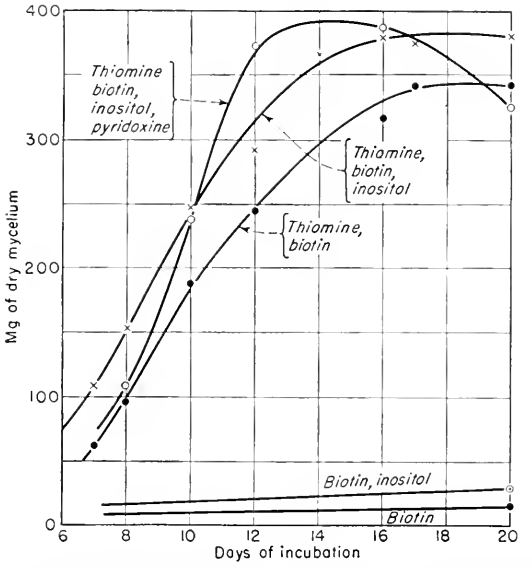


FIG. 35. Growth of *Sclerotinia camelliae* in 25 ml. of liquid glucose–casein hydrolysate medium at 25°C. Note the nearly total deficiency for biotin and the partial deficiency for inositol. Failure to grow in thiamine alone and in the absence of vitamins indicates total deficiency for thiamine.

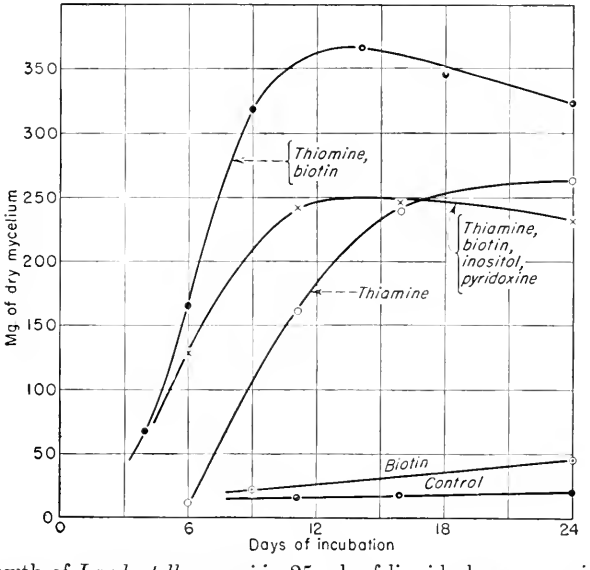


FIG. 36. Growth of *Lambertella pruni* in 25 ml. of liquid glucose–casein hydrolysate medium containing various vitamins. Partial deficiencies for both thiamine and biotin are evident, being greater for thiamine. Note that the addition of inositol and pyridoxine to media containing thiamine and biotin depressed growth.

Ophiobolus graminis are highly or totally deficient for both thiamine and biotin. Partial deficiencies for both thiamine and biotin are illustrated by *Lambertella pruni* (Fig. 36). Slight growth in the control and excellent growth only in media containing both thiamine and biotin identify the deficiencies. Intermediate growth in thiamine alone and in biotin alone shows that the deficiencies are partial. The synthetic capacity is relatively greater for biotin than for thiamine. The deficiencies of *Endothia parasitica* are similar to those of *L. pruni*. *Blastocladia pringsheimii* was

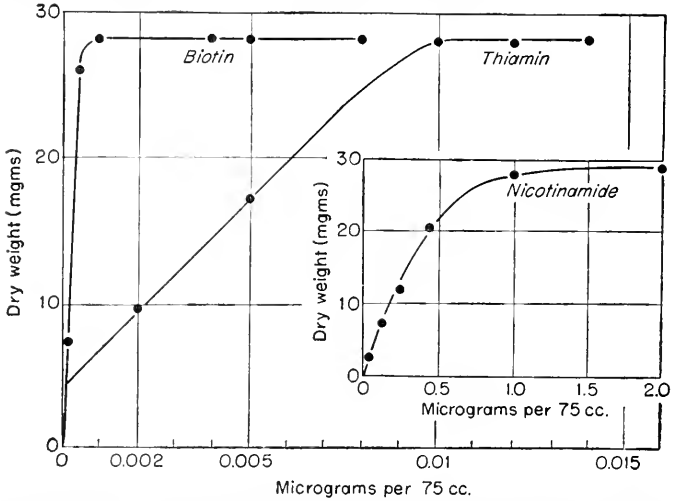


FIG. 37. The effect of concentration of essential vitamins on dry weight of *Blastocladia pringsheimii*. (Courtesy of Cantino, *Am. Jour. Botany* **35**: 241, 1948.)

reported (Cantino, 1948) to be partially deficient for thiamine and biotin and nearly totally deficient for nicotinic acid (Fig. 37). *Cerotostomella ips* No. 255 was shown to be completely deficient for thiamine, biotin, and pyridoxine (Robbins and Ma, 1942*a*).

Multiple vitamin deficiencies are more common among the yeasts than among the filamentous fungi, and some yeasts show deficiencies not known to exist in filamentous fungi isolated from nature. For these reasons the yeasts as a group have received much attention in vitamin investigations. The vitamin requirements of 38 species and strains of yeast were reported by Burkholder (1943), and for 110 additional named species and varieties by Burkholder *et al.* (1944). A summary of the deficiencies reported in these two papers is as follows: biotin, 114; thiamine, 48; pantothenic acid, 44; inositol, 19; nicotinic acid, 19; pyridoxine, 19. No deficiency for riboflavin was found. Several isolates were deficient for three or more vitamins. *Saccharomyces oviformis* was deficient for biotin, pantothenic acid, and pyridoxine, while *S. macedoniensis* Y-91 showed complete or partial deficiencies for thiamine,

pantothenic acid, nicotinic acid, and biotin. *S. ludwigii* Y-974 and *Kloeckera brevis* were totally or partially deficient for six vitamins (thiamine, biotin, inositol, pyridoxine, nicotinic acid, and pantothenic acid). Growth of all of the 38 yeasts reported by Burkholder (1943) was increased by the addition of liver extract to the medium containing the seven vitamins.

The preceding discussion of the effects of added vitamins on the growth of fungi has been based on the assumption that near-optimum amounts of the vitamins were present in the media. However, the optimum

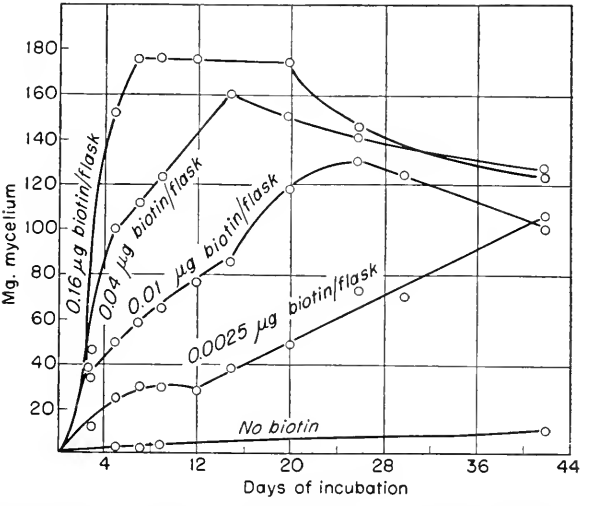


FIG. 38. The effect of concentration of biotin on the rate and amount of growth of *Sordaria fimicola* in 25 ml. liquid glucose–casein hydrolysate medium, initial pH 4.4. Growth in this medium containing biotin but no thiamine is evidence that this fungus can synthesize thiamine under these conditions. (After Lilly and Barnett, *Am. Jour. Botany* **34**: 134, 1947.)

amount of a vitamin may vary with changes in other conditions and may be different for different fungi. We have found that the following amounts per liter of the four commonly needed vitamins are near optimum for many filamentous fungi: thiamine, 100 μg; pyridoxine, 100 μg; biotin, 5 μg; inositol, 5 mg.

The effects of biotin concentration on the growth of *Sordaria fimicola* are illustrated in Fig. 38, which shows a decided increase in growth rate with greater amounts of biotin. Growth was most rapid in a medium containing 6.4 μg biotin per liter (0.16 μg per flask), but a steady slow increase in dry weight is evident in as low as 0.1 μg biotin per liter.

Absolute and conditioned deficiencies. According to Robbins and Kavanagh (1942), the deficiency of a fungus for a specific vitamin may be *absolute* or *conditioned*. *Phycomyces blakesleeanus* and *Ceratostomella*

fimbriata show absolute total deficiencies for thiamine. No environmental condition is known to allow the synthesis of this vitamin by these fungi. In the case of a conditioned deficiency, the synthesis of the vitamin may be influenced by certain environmental conditions, such as temperature, composition, concentration, and pH of the medium.

Pythium butleri failed to grow in a mineral salts–asparagine medium containing 16.4 g. of salts per liter unless thiamine was added (Robbins and Kavanagh, 1938). When the salt concentration was reduced to 1.64 g. per liter, this species grew without the addition of thiamine. A defi-

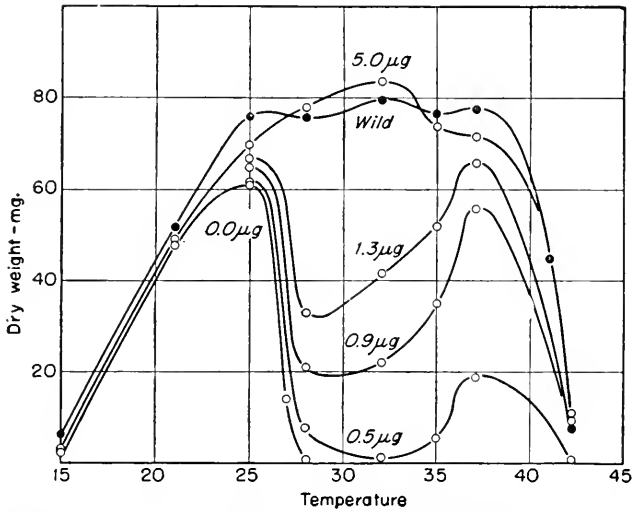


FIG. 39. Growth-temperature relations for wild-type *Neurospora* and a temperature-sensitive mutant deficient for riboflavin. Amounts of riboflavin are indicated on the curves in micrograms per 20 ml. of medium. Below 25°C. growth was good without riboflavin, while no growth occurred above 28°C. without added riboflavin. (Courtesy of Mitchell and Houlahan. *Am. Jour. Botany* **33**: 31, 1946.)

ciency for riboflavin conditioned by temperature was reported by Mitchell and Houlahan (1946) for a mutant of *Neurospora* (Fig. 39). Growth was poor or none at temperatures above 25°C. unless riboflavin was added. Below 25°C. the fungus was able to synthesize riboflavin. The partial deficiency of *Sclerotinia camelliae* for inositol was influenced by temperature, particularly in the above-optimum range (Barnett and Lilly, 1948).

Low pH of the medium resulted in partial thiamine deficiency of *Sordaria fimicola*, while no deficiency for thiamine was apparent at initial pH 4.0 or above (Lilly and Barnett, 1947). Within the range of 3.8 to 3.4, growth was quite slow, but the addition of thiamine overcame the inhibition due to the high acidity (Fig. 40). These results indicate that pH 3.8 or lower inhibits the synthesis of thiamine by *S. fimicola*. In a similar way, the availability of *p*-aminobenzoic acid to a mutant of

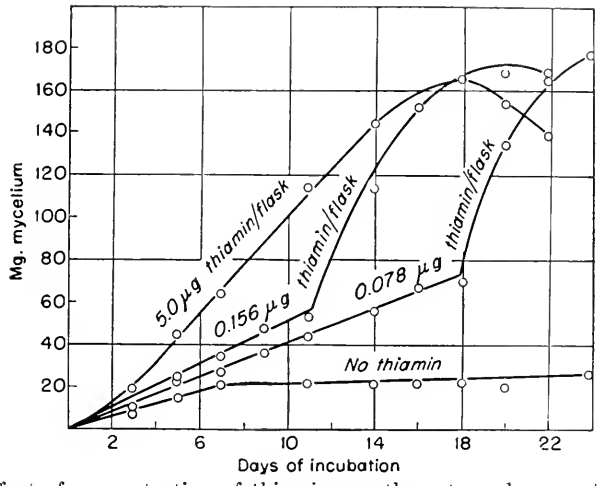


FIG. 40. Effect of concentration of thiamine on the rate and amount of growth of *Sordaria fimicola* in 25 ml. liquid glucose–casein hydrolysate medium, initial pH 3.8. Compare with Fig. 38. (After Lilly and Barnett, *Am. Jour. Botany* **34**: 134, 1947.)

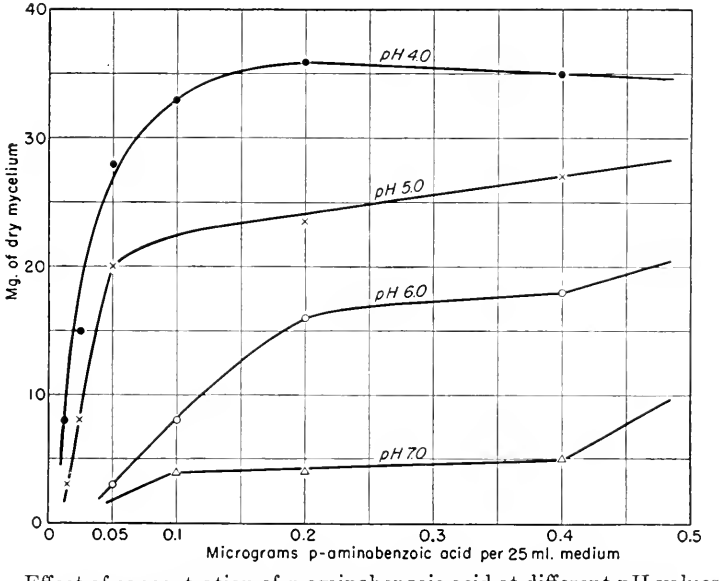


FIG. 41. Effect of concentration of *p*-aminobenzoic acid at different pH values on the growth of a mutant of *Neurospora crassa* deficient for this vitamin. The fungus was grown on liquid glucose–casein hydrolysate medium for 3 days. (Drawn from the data of Wyss, Lilly, and Leonian, *Science* **99**: 18, 1944.)

Neurospora crassa deficient for this vitamin was found to be influenced by the pH of the medium (Wyss *et al.*, 1944) (Fig. 41).

The ability of a mutant of *Neurospora sitophila* to synthesize pyridoxine was shown to be dependent not only on the pH of the medium but also on the source of nitrogen (Stokes *et al.*, 1943). When nitrate, amino, amide, or certain other nitrogen compounds served as the nitrogen source, no growth occurred without the addition of pyridoxine. However, in the presence of ammonium salts, growth occurred at an initial pH range of 5.6 to 7.3, without added pyridoxine. In this pH range, free ammonia is formed. In the absence of free ammonia, the pyridoxine synthesized is unavailable to this mutant (Strauss, 1951).

According to Fromageot and Tschang (1938), the red yeast, *Rhodotorula sanniei*, requires thiamine when the carbon source is glucose, but when redistilled glycerol replaces glucose, thiamine is not needed. It is interesting to speculate whether this fungus is better able to synthesize thiamine in a glycerol medium or whether much less thiamine is required to metabolize glycerol than glucose.

The concentration of the micro essential elements has also been shown to influence the synthesis of vitamins by microorganisms (see Chap. 13 for specific information).

INHIBITORY EFFECTS OF VITAMINS

In certain cases vitamins may have an inhibitory effect on growth, particularly when present in excessive dosages. The interrelated effects of temperature and amount of inositol were described (Barnett and Lilly, 1948) for *Sclerotinia camelliae*. At a temperature below 26°C. the partial deficiency was overcome by adding 5 mg. inositol to the medium. Above 26°C. the fungus was highly sensitive to small changes in temperature and in amounts of inositol in the medium (Fig. 42). The same amount of inositol which stimulated growth at or below 26°C. was strongly inhibitory at 27°C. Increased amounts of inositol caused greater inhibition of growth. Since the maximum temperature for growth is slightly above 27°C., it is believed that, as the temperature approaches this point, the fungus becomes highly sensitive to the increased amounts of inositol in the medium.

Some vitamins are known to have a depressing effect on growth of certain fungi not deficient for these particular vitamins. For example, Fig. 36 shows that *Lambertella pruni* produces more dry weight in the presence of both thiamine and biotin than when inositol and pyridoxine are also added to the medium. Similarly, it is reported (Elliott, 1949) that, for a self-sufficient isolate of *Fusarium avenaceum*, both the rate of growth and maximum amount of mycelium were greater in vitamin-free medium than when vitamins were added. The presence of thiamine also

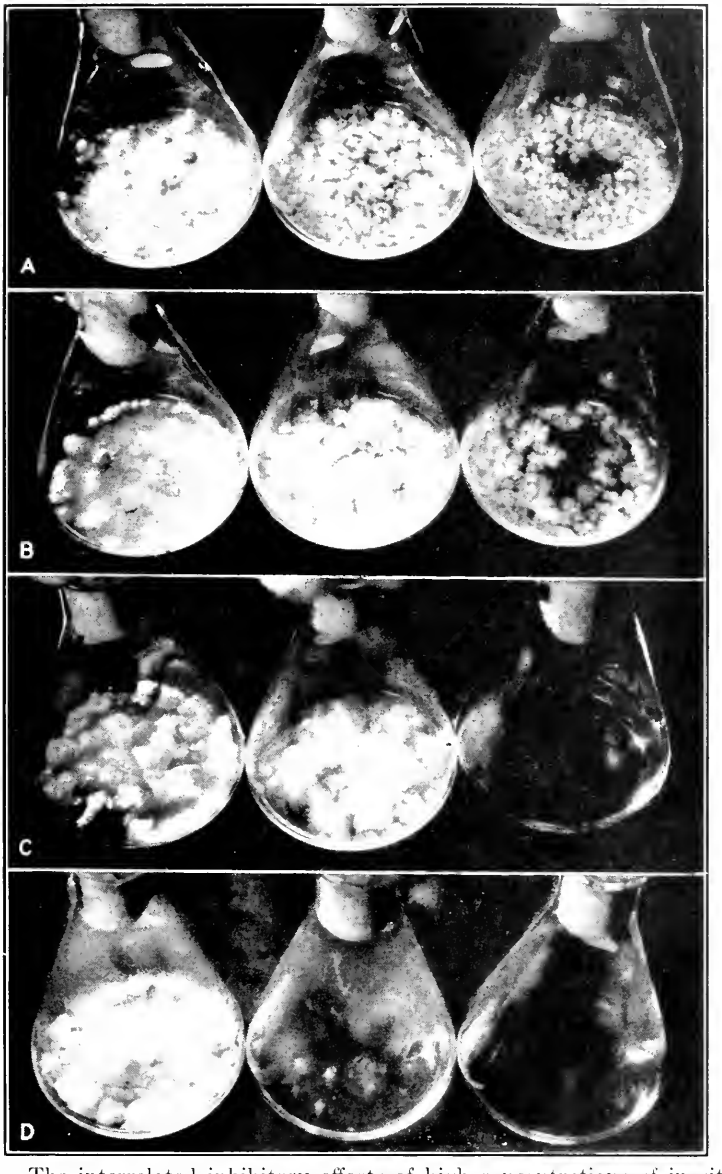


FIG. 42. The interrelated inhibitory effects of high concentrations of inositol and near-maximum temperatures on the growth of *Sclerotinia camelliae*, which is partially deficient for inositol below 26°C. Cultures 19 days old. Thiamine and biotin were added to all media. Temperatures ±0.3°C. Left to right: 26°C., 26.6°C., 27°C. Amounts of inositol added per liter were: *A*, none; *B*, 1 mg.; *C*, 10 mg.; *D*, 100 mg.

has been reported to depress the growth of several fungi, including *Colletotrichum lindemuthianum* (Mathur *et al.*, 1950), *Rhizopus suinus* (Schopfer and Guilloud, 1945), and *Fusarium lini* (Wirth and Nord, 1942). Other cases have been observed in our laboratory. In the case of *Rhizopus suinus*, the addition of inositol overcame the inhibitory effects of thiamine, and we believe it to be effective with certain other fungi. On the basis of these reports, it would seem unwise to add vitamins indiscriminately to media used for the study of fungi which are self-sufficient for these vitamins.

VITAMERS

Certain microorganisms are less specific in their vitamin requirements than are animals, owing apparently to their greater synthetic ability. Some vitamin-deficient fungi may respond well to one of the vitamin moieties, as in the case of thiamine, or to a compound similar to the vitamin. The term *vitamer* was suggested by Burk *et al.* (1944) to denote a compound having vitamin activity but differing in molecular structure from the true vitamin. Usually the structure of a vitamer is closely related to that of the vitamin. More specifically, these compounds are known as thiamine vitamers, biotin vitamers, etc. In general, a vitamer is active for fewer fungi than is the vitamin. Some vitamers are *antivitamins*. This topic is discussed in Chap. 11.

UNIDENTIFIED GROWTH FACTORS

It is quite probable that some fungi will be discovered which are deficient for vitamins or other growth factors which are at present unknown. Fungi which fail to grow in synthetic media to which all the known growth factors have been added offer a challenge and an opportunity to the investigator. Burkholder and Moyer (1943) reported that *Candida albicans* 475 and *Mycoderma vini* 939 did not grow unless liver extract was added to glucose-asparagine medium containing six vitamins. One may speculate that the effect of liver extract was due to some amino acid or to an undetermined growth factor, possibly vitamin B_{12}, which is known to be present in liver. In view of the common experience regarding the stimulating effect of natural substances on growth of fungi, it is evident that much more investigation on this phase of nutrition is needed.

SPECIFIC VITAMINS

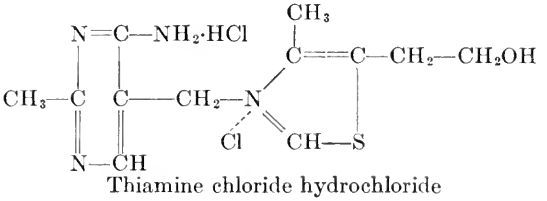
In the first portion of this chapter the general aspects of vitamins and growth factors were considered. Different types of vitamin deficiencies and the methods of detecting deficiencies were discussed. The second portion deals with the specific vitamins, their characteristics and functions.

THIAMINE AND ITS MOIETIES

Thiamine (vitamin B_1, aneurine) was the first vitamin shown to be required by a filamentous fungus. Thiamine deficiency in man is known as beriberi. Certain fungi and other microorganisms resemble man in that they are unable to synthesize this vitamin. It is probably required in the metabolism of all forms of life, and its function, to a large extent, is believed to be the same in all organisms.

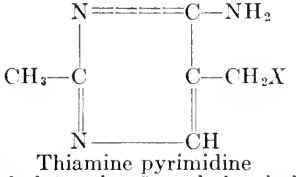
Schopfer (1934) demonstrated that *Phycomyces blakesleeanus* failed to grow in a synthetic medium unless thiamine was added. This was a stimulus for numerous studies on vitamin deficiencies of fungi. The chemical synthesis of thiamine, in 1936, was another important step in vitamin research. The student is referred to Williams and Spies (1938), Rosenberg (1942), and Schopfer (1943) for information on the history, synthesis, and natural occurrence of thiamine.

The structural formula for thiamine is



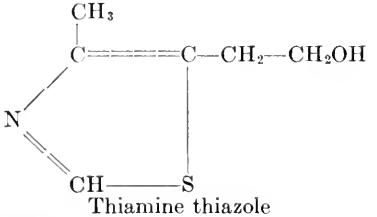
Thiamine chloride hydrochloride

The thiamine molecule contains two ring structures, a substituted pyrimidine and a substituted thiazole. The pyrimidine moiety has the following formula:



Thiamine pyrimidine
2-Methyl-4-amino-5-methylpyrimidine

X in the substituted methyl group on C5 may be hydroxyl, chlorine, bromine, etc. The thiazole moiety has the following formula:



Thiamine thiazole
4-Methyl-5-β-hydroxyethylthiazole

These moieties are referred to in the literature as the thiamine pyrimidine and thiamine thiazole, respectively.

Thiamine is somewhat unstable when exposed to alkali and heat, but at pH 3.5 it is unaffected by autoclaving. Sulfur dioxide and sulfites are destructive at pH 5 to 6. These factors must be taken into consideration, and it is sometimes desirable to sterilize thiamine separately, either by filtration or by autoclaving in an acidified solution. For most investigations, however, it is permissible to autoclave thiamine with the medium. For most fungi 100 μg of thiamine per liter of medium is near optimum for growth and sporulation. However, the optimum varies with the amount of sugar in the medium and with other conditions.

Soon after pure thiamine became available, it was discovered that certain treatments destroyed its activity for animals but did not greatly affect the potency when certain fungi were used as test organisms. The solution to this problem was reached when it became known that thiamine, when autoclaved in the presence of alkali, was broken down into thiamine pyrimidine and thiamine thiazole.

Thiamine-deficient fungi differ in their ability to utilize or synthesize the moieties of thiamine. These fungi may be classified into four groups on this basis: (1) The intact molecule of thiamine is required by some fungi which are unable to synthesize either moiety or to complete the synthesis of thiamine, even when both moieties are supplied. Examples of the group are species of *Phytophthora*. (2) Some other fungi, such as *Phycomyces blakesleeanus*, are capable of utilizing thiamine, or of synthesizing thiamine when furnished with a mixture of the two thiamine moieties. (3) The addition of thiamine or thiamine pyrimidine satisfies the need of those fungi which are able to synthesize the thiazole moiety and combine it with the pyrimidine moiety to make thiamine. Examples are *Parasitella simplex* and *Rhodotorula rubra*. (4) Other fungi are able to synthesize only the thiamine pyrimidine and complete the synthesis of thiamine when furnished with the thiazole moiety. *Mucor ramannianus* and *Stereum frustulosum* are examples.

In the above discussion it was assumed that in every case the intact molecule was the active product and that neither moiety nor the presence of the two had any activity until thiamine was synthesized. Leonian and Lilly (1940) found this hypothesis to be correct. The following fungi were grown in a basal medium to which had been added the minimal growth factor: *Fusarium niveum* (none), *Pythiomorpha gonapodyoides* (pyrimidine), *Mucor ramannianus* (thiazole), *Phycomyces blakesleeanus* (both moieties of thiamine), and *Phytophthora erythroseptica* (thiamine). After growth, the mycelium and the medium were tested for thiamine and its moieties by growing fungi of known thiamine or thiamine-moiety requirements upon media containing the mycelium extract and the medium. Some of these data are collected in Table 33.

TABLE 33. ASSAY FOR THIAMINE AND THIAMINE MOIETIES IN MYCELIUM AND MEDIUM EXTRACTS OF SOME FUNGI AFTER GROWTH ON MEDIA CONTAINING THE MINIMUM GROWTH-FACTOR REQUIREMENTS

Numbers refer to relative growth on the scale of 10. (Leonian and Lilly, *Plant Physiol.* **15**, 1940.)

Fungi tested and minimum vitamin requirements	Test fungi and substance tested for			
	Pythium ascophallon (thiamine)	*Phycomyces blakesleeanus* (both moieties)	*Pythiomorpha gonapodyoides* (pyrimidine)	*Mucor ramannianus* (thiazole)
Fusarium niveum	10*	10	10	10
(none)	0*	2	4	10
Pythiomorpha gonapodyoides	10	10	10	8
(pyrimidine)	1	4	10	8
Mucor ramannianus	1	3	5	8
(thiazole)	0	2	3	7
Phycomyces blakesleeanus	10	10	10	10
(both moieties)	0	8	8	10
Phytophthora erythroseptica	10	8	10	10
(thiamine)	1	6	6	10

* Upper figures refer to extract of mycelium, lower figures to extract of medium.

It is evident that *Fusarium niveum* was able to synthesize thiamine from the basal medium because two test fungi which require thiamine per se grew on extracts prepared from the hyphae. The same type of proof shows that *Pythiomorpha gonapodyoides* synthesized thiamine when thiamine pyrimidine was added to the basal medium. *Mucor ramannianus* synthesized thiamine when thiamine thiazole was added, and *Phycomyces blakesleeanus* synthesized thiamine when both moieties were added.

In all cases the greater portion of thiamine was stored within the mycelium, and only small amounts were present in the medium. The medium extract from three fungi contained no thiamine, although appreciable quantities of the pyrimidine and thiazole moieties were present in all media. This shows that *Phytophthora erythroseptica*, for example, had broken down the thiamine molecule into its moieties, which diffused into the medium and were later utilized by certain fungi, such as *Phycomyces blakesleeanus*. This suggests that in the process of its utilization thiamine is slowly destroyed. The moieties may be recombined by certain organisms but not by those which require the entire thiamine

molecule. Robbins and Kavanagh (1941) showed that *P. blakesleeanus* destroyed the thiazole more rapidly than it did the pyrimidine moiety. Thus, an excess of thiazole in the mixture of the two moieties was more effective than equal quantities. They termed this the *thiazole effect.*

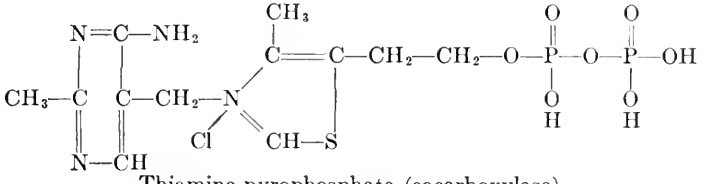
Some thiamine-deficient fungi. A deficiency for thiamine is by far the most common vitamin deficiency among filamentous fungi isolated from nature. Fries (1948) states than over 200 fungi are known to be partially or totally deficient for thiamine. No doubt this is a modest estimate. Deficiency for this vitamin is more common among certain groups of fungi than others. For example, all species of *Phytophthora* studied have been found to require the intact molecule of thiamine. Only a few species of the true Basidiomycetes have been reported to be self-sufficient for thiamine. Many of these fungi show only partial deficiencies, while some are totally deficient. In most cases, however, there seems to be little or no correlation between thiamine deficiency and taxonomic relationship.

Some common filamentous fungi (other than Basidiomycetes) which have been reported to be totally or partially deficient for thiamine or its moieties, with other deficiencies (if any) indicated in parentheses, are as follows: *Blakeslea trispora, Ceratostomella fimbriata, C. ips* (biotin and pyridoxine), *C. montium* (biotin and pyridoxine), *C. pini* (biotin), *Chaetomium convolutum* (biotin), *Choanephora cucurbitarum, Coemansia interrupta* (biotin), *Dendrophoma obscurans, Endothia parasitica* (biotin), *Hypoxylon pruinatum* (biotin), *Lambertella pruni* (biotin), *Lophodermium pinastri* (biotin and inositol), *Melanconium betulinum* (biotin and inositol), *Melanospora destruens* (biotin), *Mucor ramannianus, Nectria coccinia, Ophiobolus graminis* (biotin), *Phycomyces blakesleeanus, Phytophthora* spp., *Piricularia oryzae* (biotin), *Pleurage curvicolla* (biotin), *Podospora curvula* (biotin), *Pythiomorpha gonapodyoides, Pythium arrhenomanes, P. ascophallon, P. butleri, P. oligandrum, Sclerotinia camelliae* (biotin and inositol), *S. minor, Sordaria fimicola,* certain isolates only (biotin), *Thielaviopsis basicola, Valsa pini* (biotin and inositol), and *Xylaria hypoxylon.*

Reports of deficiencies for most of the above-named fungi may be found in the references for this chapter. Some few of these fungi have been studied in our laboratory and have not been previously reported as being deficient for thiamine. For thiamine-deficient yeasts see the reports of Burkholder (1943) and Burkholder *et al.* (1944).

Mode of action. One of the primary uses of thiamine in plants and animals is for the regulation of carbohydrate metabolism. It is also probable that thiamine may be involved in other processes. A vitamin which constitutes a part of an enzyme system is known as a coenzyme. Generally a vitamin must be combined with organic or inorganic com-

pounds (or both) before it combines with the protein portion (apoenzyme) of the enzyme system. The pyrophosphoric ester of thiamine is known as cocarboxylase, or as thiamine pyrophosphate. This compound is the coenzyme of carboxylase.



Thiamine pyrophosphate (cocarboxylase)

This substance is as active as thiamine (mole for mole). Lilly and Leonian (1940) compared the action of thiamine and thiamine pyrophosphate on several thiamine-deficient fungi. No significant differences were found in the maximum weights of mycelium formed in the presence of equivalent quantities of these two growth factors. The rate of early growth was greater with thiamine pyrophosphate than with thiamine for *Phycomyces blakesleeanus* and less for *Mucor ramannianus* and *Phytophthora erythroseptica.*

Pyruvic acid, one of the key intermediate products of carbohydrate metabolism, is transformed into carbon dioxide and acetaldehyde by the action of the enzyme carboxylase. Pyruvic acid accumulates in the culture media of many thiamine-deficient fungi when insufficient thiamine is present. Haag and Dalphin (1940) found that the maximum accumulation in *Phycomyces blakesleeanus* cultures occurred when about one-twentieth of the optimum amount of thiamine was added. Wirth and Nord (1942) studied the effect of added thiamine upon the accumulation of pyruvic acid in cultures of *Fusarium lini,* a self-sufficient fungus with respect to thiamine. Some of the data are presented in Table 34.

The accumulation of pyruvic acid in the culture medium is common, especially during the early period of growth. Pyruvic acid may be detected qualitatively by adding of iodine solution (KI_3) to the culture filtrate and making the solution strongly alkaline with sodium hydroxide. Iodoform is produced instantly without heating. Acetaldehyde, which is very volatile, also reacts with iodine and alkali in the cold to produce iodoform. *Sordaria fimicola, Lenzites trabea,* or other fungi which produce acid during the early stages of growth may be used to demonstrate the production of pyruvic acid.

Specificity. So far as is known, thiamine which occurs in nature has the structure given in the formula. This vitamin has been isolated from only a few substances such as wheat germ and rice polish. The ethyl homologue (ethyl in place of methyl in position 2) of thiamine is slightly more active for certain fungi than ordinary thiamine. Higher homologues

have been reported to be less active or inhibitory. Whether ethyl thiamine occurs in nature is not known. The student is referred to Schopfer (1943) for further information on thiamine specificity.

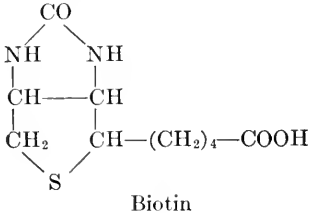
TABLE 34. THE EFFECT OF ADDED THIAMINE UPON THE ACCUMULATION OF PYRUVIC ACID IN THE CULTURE FILTRATE OF *Fusarium lini* GROWN ON GLUCOSE-NITRATE MEDIUM

(Wirth and Nord, *Arch. Biochem.* **1**, 1942. Published by permission of Academic Press, Inc.)

Days of incubation	Glucose fermented, g. per liter		Pyruvic acid accumulated, mg. per liter		Mycelium produced, mg. per 50 ml.	
	Thiamine added, μg per liter					
	0	500	0	500	0	500
2	1.5	2.5	50	Trace	—	—
4	17.3	13.5	1,590	80	132	91
6	34.7	33.9	1,710	260	—	—
8	40.9	40.7	1,550	Trace	259	135

BIOTIN

Biotin (vitamin H) was originally isolated as a growth factor for yeast. It is known to be the factor which prevents raw-egg-white injury to animals and is the respiratory coenzyme (coenzyme R) for species of *Rhizobium*. Biotin is active at greater dilutions than are the other vitamins. Pure biotin methyl ester was first isolated by Kögl and Tönnis (1936) who obtained 1.1 mg. of this substance from 250 kg. of dried duck eggs. The structure of biotin was determined by Du Vigneaud *et al.* (1942*a*) and confirmed by the synthesis of this compound (Harris *et al.*, 1943). The structure of the biotin molecule is as follows:



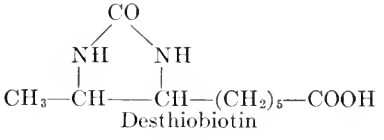
Biotin

Some fungi deficient for biotin. Biotin deficiency appears to be characteristic of most yeasts (Burkholder, 1943; Burkholder and Moyer, 1943; Leonian and Lilly, 1942). Numerous filamentous fungi have been reported to be deficient for biotin, but this number is not so great as that for thiamine. Frequently biotin deficiency accompanies thiamine deficiency.

Among the first investigators to test the action of biotin on filamentous fungi were Kögl and Fries (1937), who showed that *Nematospora gossypii* did not grow in the absence of biotin. As little as 0.4 μg of biotin per liter permitted almost as much growth as did ten times that amount. For most filamentous fungi 5 μg of biotin per liter is adequate. The effects of biotin deficiency on the development of the ascospores of *Sordaria fimicola* are shown in Fig. 68.

Some filamentous fungi reported as being partially or totally deficient for biotin, with other deficiencies (if any) given in parentheses, are as follows: *Chaetomium convolutum* (thiamine), *Coemansia interrupta* (thiamine), *Diplodia macrospora*, *Endothia parasitica* (thiamine), *Hypoxylon pruinatum* (thiamine), *Lambertella pruni* (thiamine), *Melanospora destruens* (thiamine), *Memnoniella echinata*, *Neurospora* spp., *Ophiobolus graminis* (thiamine), *O. oryzinus*, *Ophiostoma catonianum*, *Penicillium digitatum* (thiamine, pyridoxine, pantothenate), *Piricularia oryzae* (thiamine), *Pleurage curvicolla* (thiamine), *Podospora curvula* (thiamine), *Pseudopeziza ribis*, *Rosellinia arcuata*, *Sclerotinia camelliae* (thiamine, inositol), *Sordaria fimicola*, *Sporormia intermedia*, *Stachybotrys atra*, *Thraustotheca clavata*.

Specificity. The biotin molecule is not separable into moieties as is thiamine. One of the first related compounds to be studied was desthiobiotin. As the name indicates, the molecule no longer contains sulfur. The structure of the desthiobiotin molecule is as follows:



Desthiobiotin

The removal of sulfur from the biotin molecule destroyed the tetrahydrothiophene ring and introduced a methyl group. In addition, the acidic chain of desthiobiotin contains one more methylene group than does that of biotin. Stokes and Gunness (1945) tested the growth of some biotin-deficient microorganisms on desthiobiotin and found that this compound was utilized by *Neurospora sitophila* and three strains of *Saccharomyces cerevisiae*, but *Rhizobium trifolii* 209, *Lactobacillus casei*, and *L. arabinosus* 17-5 were unable to utilize desthiobiotin. From further experiments it was concluded that the yeast synthesized biotin, or some other compound which replaced it, from desthiobiotin, rather than utilizing desthiobiotin directly. The source of sulfur in the medium was found to influence the amount of desthiobiotin converted into biotin, with methionine and sodium sulfate being better sources than cystine, sulfanilamide, or thiamine thiazole.

Lilly and Leonian (1944) studied the effect of desthiobiotin on 45

biotin-deficient microorganisms and found that it replaced biotin for some fungi, while it acted as an antibiotin for some few others. Desthiobiotin replaced biotin quantitatively for *Ceratostomella ips*. Goldberg *et al.* (1947) found some homologues of biotin to inhibit growth of yeast 139 and *Lactobacillus casei*. Whether any of these biotin homologues will replace biotin for other microorganisms must await further testing. These preliminary results indicate that the length of the acidic side chain of the biotin molecule is of great importance in biological activity.

Oxybiotin is also known as O-heterobiotin and has the same structure as biotin except that the sulfur in the tetrahydrothiophene ring has been replaced by oxygen. Pilgrim *et al.* (1945) found oxybiotin to be active for *Lactobacillus casei*, *L. arabinosus*, and a strain of *Saccharomyces cerevisiae*. Oxybiotin is apparently used as such and is not converted into biotin by the organism (Axelrod *et al.*, 1947). This is the only instance that has come to our attention where a vitamer is used directly instead of being converted into the vitamin. Rubin *et al.* (1945) had previously reported that oxybiotin was converted into biotin. The cause of this disagreement is unknown.

Pimelic acid is a growth factor for certain strains of the diphtheria bacterium (Mueller, 1937). It is reported (Du Vigneaud *et al.*, 1942) that pimelic acid replaced biotin and was probably the precursor in the synthesis of biotin by a strain of the diphtheria organism. The higher and lower homologs of pimelic acid were ineffective. The formula for pimelic acid is $HOOC—CH_2—CH_2—CH_2—CH_2—CH_2—COOH$. At present there is no evidence that pimelic acid replaces biotin for the fungi. This observation is supported by the findings of Robbins and Ma (1942), who studied 13 biotin-deficient fungi. A favorable effect of the presence of pimelic acid was reported by Eakin and Eakin (1942), who found that *Aspergillus niger* synthesizes much more biotin in the presence of pimelic acid than in its absence. Cysteine and also cystine increase the synthesis of biotin. The lower homologues of pimelic acid (adipic, glutaric, and succinic) were without effect, while the higher homologs (suberic and azelaic) were as effective as pimelic acid. This is interesting, inasmuch as homobiotin and bishomobiotin are reported inactive for yeast growth (Goldberg *et al.*, 1947).

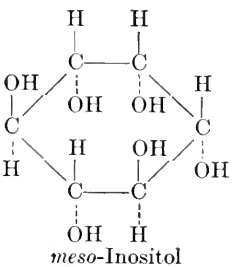
Mode of action. It has been assumed that biotin acts as a coenzyme for various enzyme systems, but definite proof seems to be lacking. Winzler *et al.* (1944) found that, when biotin was added to a biotin-starved yeast, some time elapsed before any effect was noted. The order of response was fermentation, respiration, and growth. The assimilation of ammonia did not take place unless biotin was added.

The presence of aspartic acid in the culture medium has been shown to reduce the amount of biotin required by *Torula cremoris* (Koser *et al.*,

1942) and by *Memnoniella echinata* and *Stachybotrys atra* (Perlman, 1948). There is also evidence (Stokes *et al.*, 1947) that biotin plays a role in the synthesis of aspartic acid by certain bacteria. Thus, it appears probable that one of the functions of biotin is connected with the synthesis of aspartic acid. When aspartic acid is added to the medium, it is unnecessary for the organism to perform this synthesis and the need for biotin is greatly reduced. However, it should be noted that, although the absolute amount of biotin needed is reduced, exogenous biotin is still required by these biotin-deficient organisms. From this it may be deduced that biotin has a multiple role in the cell.

INOSITOL

meso-Inositol (also known as inactive inositol, isoinositol, inosite, or dambose) is widely distributed in both plants and animals. It was first isolated in 1850. It was not until 1928 that Eastcott (1928) showed that it was a growth factor for a strain of yeast. Later, Woolley (1940) recognized it as a vitamin for animals. *meso*-Inositol is a hexahydroxycyclohexane. It has the following configuration:



meso-Inositol

There are seven different cis-trans isomers, which are optically inactive, and a pair of optically active *d* and *l* forms. The available evidence indicates that the stereochemical configuration of *meso*-inositol is specific for vitamin activity. Some of the isomers have only slight activity. Inositol is active only in high concentrations as compared to the other vitamins. The usual amount added is around 5 mg. per liter of medium.

Fungi deficient for inositol. Many strains of yeast are deficient for this vitamin, while others are not. In most cases the deficiency apparently is partial rather than total. Partial deficiencies for various yeasts are reported by Leonian and Lilly (1942), Burkholder (1943), and Burkholder and Moyer (1943). In the last two references total deficiencies for inositol are reported for *Saccharomyces uvarum* Y 969 and *Schizosaccharomyces pombe.*

Kögl and Fries (1937) were apparently the first to investigate the action of inositol on various filamentous fungi. They found that *Nematospora gossypii* was totally deficient and that *Lophodermium pinastri* was

partially deficient for this vitamin. The partial deficiency of *Sclerotinia camelliae* is shown in Fig. 35. Deficiencies for inositol are commonly accompanied by deficiencies for thiamine and biotin. *Trichophyton discoides* is reported as being totally deficient for inositol, pyridoxine, and thiamine (Robbins *et al.*, 1942). Totally deficient mutants of *Neurospora crassa* have been developed. Their use in bioassays for inositol was described by Beadle (1944) and by Leonian and Lilly (1945).

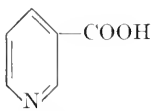
Some filamentous fungi reported to be partially or totally deficient for inositol, with other deficiencies given in parentheses, are as follows: *Colletotrichum lindemuthianum* (certain strains only), *Epichloe typhina* (thiamine), *Lophodermium pinastri* (thiamine, biotin), *Melanconium betulinum* (thiamine, biotin), *Nematospora gossypii* (thiamine, biotin), *Sclerotinia camelliae* (thiamine, biotin), *Trichophyton discoides* (thiamine, pyridoxine), *Valsa pini* (thiamine, biotin).

The effects of temperature upon the synthesis of inositol by *Sclerotinia camelliae* and upon the toxicity of high concentrations of inositol at high temperatures were described by Barnett and Lilly (1948) and are illustrated in Fig. 42.

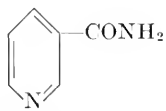
Mode of action. The addition of inositol overcame the inhibition of growth of *Rhizopus suinus* due to excess thiamine (Schopfer and Guilloud, 1945). In part, the inhibition was due to an increased production of alcohol (pyruvate → acetaldehyde → alcohol). Similarly, we have observed in our laboratory the same favorable effect of inositol on growth of certain fungi which are inhibited by the presence of excess thiamine.

NICOTINIC ACID

A deficiency for nicotinic acid, or nicotinic acid amide, leads to pellagra in man and blacktongue in dogs. The structural formulas of these compounds follow:



Nicotinic acid



Nicotinic acid amide

Nicotinic acid was obtained by the oxidation of nicotine in 1867. Knight (1937) and Mueller (1937*a*) recognized that nicotinic acid amide was a growth factor for certain bacteria. So far as is known, the amide is the form utilized by organisms. Some microorganisms can convert nicotinic acid into its amide with ease, others with difficulty; still others are unable to use nicotinic acid but require either nicotinic acid amide or a coenzyme containing the amide.

Fungi deficient for nicotinic acid. Rogosa (1943) tested 114 strains of yeast that ferment lactose and found that all of them required an exoge-

nous supply of nicotinic acid for growth. Rogosa used the technique of serial passage in a medium devoid of nicotinic acid. It is possible to overlook a vitamin deficiency by failure to observe this precaution. Yeasts found to be deficient for this vitamin include *Saccharomyces anamensis* 154, *S. lactis* 131, *S. fragilis* 15, *Zygosaccharomyces lactis* (two strains), *Torula lactosa* 168, *T. sphacrica* 13, *T. cremoris* 2, *Torulopsis kefyr* 149, *Mycotorula lactis* 130. Strains of *Saccharomyces cerevisiae* failed to show deficiency for nicotinic acid (Rogosa, 1943; Leonian and Lilly, 1942; Burkholder, 1943).

Until recently, nicotinic acid deficiency among filamentous fungi isolated from nature was unknown. Cantino (1948) has shown that *Blastocladia pringsheimii* is completely deficient for nicotinamide and partially deficient for thiamine and biotin. Some of Cantino's results are presented in Fig. 37. A second filamentous fungus, a strain of *Microsporum audouini*, is reported as deficient for nicotinic acid (Arêa Leão and Cury, 1949). Mutants deficient for this vitamin have been developed in *Neurospora* by Bonner and Beadle (1946) and in *Penicillium* by Bonner (1946).

Specificity. In so far as the fungi are concerned, nicotinic acid replaces nicotinic acid amide, but few critical studies in this connection have been made. Various studies have been made of the specificity for bacteria of the compounds related to nicotinic acid. Bovarnick (1943) reported that heating asparagine and glutamic acid together produced a compound which replaced nicotinic acid or its amide for various species of bacteria. This author later showed that this substance was nicotinic acid amide. This is an unsuspected way of adding a vitamin to a basal medium.

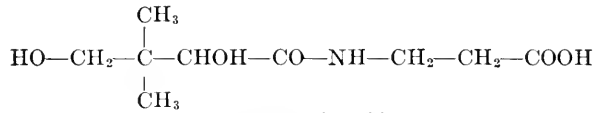
Mode of action. Nicotinic acid amide is a constituent of two or more coenzymes. Codehydrogenase I on hydrolysis yields adenine, nicotinic acid amide, and two molecules of D-ribosephosphoric acid. Codehydrogenase II yields the same products as codehydrogenase I except that three molecules of phosphoric acid, instead of two, are produced. In the literature codehydrogenase I is often referred to as DPN (diphosphopyridine nucleotide) and codehydrogenase II as TPN (triphosphopyridine nucleotide). These coenzymes in combination with specific proteins form enzyme systems which transfer hydrogen (oxidation-reduction). Apparently the amide of nicotinic acid is reversibly oxidized and reduced in the process.

One organism, *Hemophilus parainfluenzae*, requires codehydrogenase I as a growth factor. This organism is unable to form the coenzyme when furnished with the moieties, nicotinic acid amide, adenine, D-ribose, and phosphate. DPN is also known as factor V (Gingrich and Schlenk, 1944). Other bacteria are known which require preformed coenzymes as growth factors. While no fungus isolated from nature has yet been

shown to require such growth factors, it is possible that some do exist. Such requirements may be found among the artificially induced mutants.

PANTOTHENIC ACID

Pantothenic acid was first discovered (Williams *et al.*, 1932) as a growth factor for the Gebrüde Mayer strain of *Saccharomyces cerevisiae*. The isolation, identification, and synthesis of this compound was complete by 1940. It was later shown to be a vitamin for animals. Pantothenic acid consists of two moieties joined together by means of an amide linkage. The chemical formula for this vitamin is given below:



Pantothenic acid

Pantothenic acid may be hydrolyzed to form β-alanine (β-aminopropionic acid), the formula of which is $H_2N—CH_2—CH_2—COOH$, and α,γ-dihydroxy-β,β-dimethylbutyric acid, a substituted butyric acid that forms a lactone by elimination of one molecule of water between the carboxyl and the gamma hydroxyl (pantoyl lactone). Pantothenic acid is thus analogous to thiamine, in that the molecule may be split into two moieties. We might expect to find different pantothenic acid–deficient organisms which require the intact molecule or one or both moieties. It was found that the Gebrüde Mayer strain of *Saccharomyces cerevisae* was stimulated by β-alanine and that this yeast completed the synthesis of pantothenic acid when furnished with β-alanine in the medium (Weinstock *et al.*, 1939). Most yeasts deficient for pantothenic acid are unable to synthesize the β-alanine moiety of this vitamin. In general, this moiety is not used so efficiently as pantothenic acid, and more than an equivalent amount is required to support the same amount of growth. The composition of the medium affects utilization, since, in the presence of sufficient asparagine, β-alanine is not utilized (Atkin *et al.*, 1944).

So far as is known, none of the fungi require pantoyl lactone as a growth factor, but this compound was found (Ryan *et al.*, 1945) to replace pantothenic acid for *Clostridium septicum*. It was shown by microbiological tests that this bacterium completed the synthesis of pantothenic acid.

Fungi deficient for pantothenic acid. Of the 10 strains of *Saccharomyces cerevisiae* tested for vitamin deficiencies by Leonian and Lilly (1942), 9 were highly deficient for this vitamin. Burkholder (1943) found 14 of the 38 species and strains tested to be deficient for pantothenic acid, 9 of these being species of *Saccharomyces*. It appears that deficiency for this vitamin is more common in *Saccharomyces* than in other genera of yeasts. Varying degrees of pantothenic acid deficiency were found in

species of *Zygosaccharomyces* (Lockhead and Landerkin, 1942). β-Alanine could be used in place of pantothenic acid for the deficient species of *Zygosaccharomyces*.

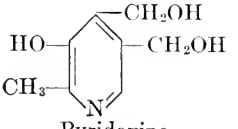
Growth of *Penicillium digitatum* is reported (Wooster and Cheldelin, 1945) to be stimulated by pantothenate, as well as by pyridoxine, biotin, and thiamine. To our knowledge this is the only report of a filamentous fungus isolated from nature being stimulated by the presence of this vitamin. Tatum and Beadle (1945) reported a mutant of *Neurospora* which was deficient for pantothenic acid.

Specificity. As in the case of thiamine and inositol, the structure of pantothenic acid is almost specific for activity. A hydroxypantothenic acid synthesized by Mitchell *et al.* (1940) has a varying ability to replace pantothenic acid for some organisms. The activity of this compound for the Gebrüde Mayer yeast was low as compared with pantothenic acid.

Mode of action. Pantothenic acid was found to favor the accumulation of glycogen by yeasts (Williams *et al.*, 1936), and to increase markedly the rate of carbon dioxide evolution by the pantothenic acid–deficient Gebrüde Mayer yeast (Pratt and Williams, 1939). More recent work (Novelli and Lipmann, 1947) has shown that pantothenic acid is phosphorylated and acts as a coenzyme. This enzyme system performs various oxidations and acetylations in the cell.

PYRIDOXINE

Pyridoxine is also known as adermin or as vitamin B_6. While inositol and pantothenic acid were first investigated as growth factors for microorganisms, pyridoxine was discovered as a result of animal research. This vitamin was isolated independently by five groups of workers in 1938 and was synthesized the next year. The structural formula is given below:



Pyridoxine

Pyridoxine is quite soluble in water and is stable to acid and alkali but is destroyed by light.

Fungi deficient for pyridoxine. Partial or total deficiencies for this vitamin have been reported for various species and strains of yeasts (Eakin and Williams, 1939; Burkholder, 1943). Among these are *Saccharomyces carlsbergensis* var. *mandshuricus* Y-379, *S. chodati* Y-140, *S. oviformis*, *S. ludwigii*, and *Mycoderma valida* Y-7.

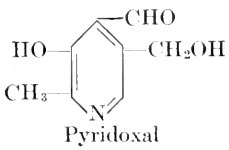
Leonian and Lilly (1942) found that the omission of either thiamine or pyridoxine alone from the medium was without effect on 9 of the 10 strains

of yeast tested. However, the omission of both pyridoxine and thiamine caused a decrease in the growth of two of these strains. Apparently these two yeasts were capable of synthesizing either thiamine or pyridoxine, provided that the other vitamin was present. This is a common effect among fungi partially deficient for two or more vitamins. The presence of one vitamin for which a fungus is partially deficient may enable the fungus to synthesize other vitamins with greater ease.

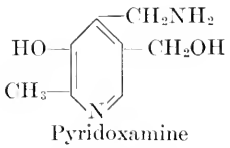
Among the filamentous fungi, deficiency for pyridoxine seems to be characteristic of certain species of *Ceratostomella* and a few other fungi (Robbins and Ma, 1942*a*, 1942*b*). Some species reported to be deficient for pyridoxine, with other deficiencies given in parentheses, are *Ceratostomella ulmi*, *C. ips* (thiamine, biotin), *C. pseudotsugae* (thiamine), *C. piceaperda* 240 (biotin), *C. pini* (thiamine, biotin), *C. montium* (thiamine, biotin), *C. pilifera*, *C. multiannulata* (thiamine), *C. pluriannulata* (thiamine), *C. microspora* (thiamine, biotin), *Ophiostoma catonianum* (thiamine), *Trichophyton discoides* (thiamine, inositol).

Specificity. One of the important uses of vitamin-deficient organisms is for the purpose of vitamin assay. Certain vitamin-deficient fungi and bacteria are used to determine the vitamin content of foodstuffs and other natural products. For such tests to be of any value, it is necessary to know if the organism used responds to substances other than the vitamin itself. Snell *et al.* (1942) found that *Streptococcus faecalis* gave much greater apparent yields of pyridoxine when used for assay than did yeast. It was then discovered (Snell, 1942) that autoclaving pyridoxine with the basal medium for 20 min. increased the activity of pyridoxine forty-one times, and that this change in activity for certain organisms was correlated with oxidation and heating with certain amino acids.

Snell (1944) then postulated that vitamers of pyridoxine were formed by these treatments. When this problem was under investigation, these vitamers of unknown structure were called "pseudopyridoxine," which was later found to consist of either one or both of the following compounds:



Pyridoxal



Pyridoxamine

These two compounds were synthesized by Harris *et al.* (1944) and tested by Snell.

It was concluded that this vitamin consists of three or more closely related compounds. *Saccharomyces carlsbergensis* responds about equally to the three compounds, while the reaction of certain bacteria is much greater to pyridoxal and pyridoxamine than to pyridoxine. *Ceratosto-*

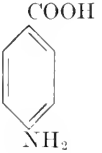
mella ulmi grew at a more rapid rate with pyridoxamine than with pyridoxal or pyridoxine (Snell and Rannefelt, 1945). All three forms of this vitamin occur in natural products. Assays for this vitamin are discussed in Chap. 10.

Mode of action. One of the earliest clues to the action of pyridoxine was discovered by Snell and Guirard (1943) in the interrelationship among glycine, alanine, and pyridoxine and growth of *Streptococcus faecalis* R. They found that alanine could replace pyridoxine for this organism and that glycine caused inhibition which was overcome by the addition of either alanine or pyridoxine. It was also found that β-alanine, serine, and threonine inhibited growth. It is possible that alanine serves as a precursor for pyridoxine in this organism, or that one function of pyridoxine is the synthesis of alanine. At any rate the action of pyridoxine appears to be connected with either amino-acid synthesis or amino-acid utilization, or both. Like other vitamins, pyridoxine (or its conversion products) has been assumed to function in the cell as a part of a coenzyme.

Pyridoxal is phosphorylated before it functions in enzyme systems. In this it is like thiamine and pantothenic acid. Pyridoxal phosphate is said to function as a coenzyme in the transformation of tryptophane into indole by *Escherichia coli* (Wood *et al.*, 1947). We may assume that the function of this vitamin is the same in the fungi as in the bacteria.

p-AMINOBENZOIC ACID

p-Aminobenzoic acid has the following structure:



p-Aminobenzoic acid

Rubo and Gillespie (1940) found *p*-aminobenzoic acid to be a growth factor for nine strains of *Clostridium acetobutylicum*. Most of the interest in this compound centers in its antagonistic action to sulfonamides. A discussion of this subject is presented in Chapter 11.

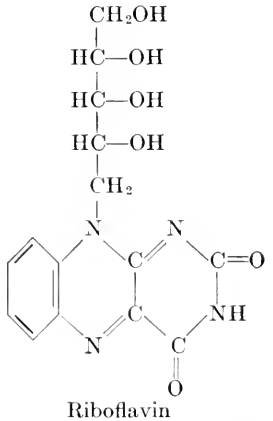
Fungi deficient for *p*-aminobenzoic acid. Robbins and Ma (1944) reported *Rhodotorula aurantica* to be deficient for *p*-aminobenzoic acid and thiamine. Concentrations of as low as 0.03 μg per liter had a positive effect on the growth of this yeast, while maximum growth was attained in the presence of 3 μg per liter. The intensity of the pink color developed by this yeast was a function of the *p*-aminobenzoic acid content of the medium.

In so far as we are aware, no filamentous fungus isolated from nature has been shown to be deficient for *p*-aminobenzoic acid. Tatum and Beadle (1942) described a mutant of *Neurospora* which was unable to synthesize this vitamin. Wyss *et al.* (1944) found that the availability of *p*-aminobenzoic acid to the deficient mutant of *Neurospora crassa* was a function of the pH of the medium (see Fig. 41).

Mode of action. The functions of *p*-aminobenzoic acid are unknown. We may assume, on the basis of the behavior of other vitamins, that it functions as a coenzyme, or as a part of a coenzyme. Recent work indicates that *p*-aminobenzoic acid is a constituent part of folic acid.

RIBOFLAVIN

The structure of riboflavin is given below:



Riboflavin

Many bacteria, especially species of *Lactobacillus* are unable to synthesize riboflavin (Peterson and Peterson, 1945). So far as we are able to determine, none of the fungi isolated from nature have been found to be deficient for riboflavin. This vitamin is synthesized by the fungi. Mitchell and Houlahan (1946) described a mutant of *Neurospora* which required the addition of riboflavin to the medium for growth at temperatures above 28°C. Between 15 and 25°C. the growth rate of the mutant without added riboflavin was equal to that of the wild type. The rate of growth decreased rapidly as the temperature increased from 25 to 28°C.

SUMMARY

It is assumed that all living organisms require a number of vitamins, or growth factors, for normal growth, reproduction, and other vital processes. However, organisms differ widely in their synthetic capacities for the various vitamins. Some fungi are self-sufficient with respect to vitamins, being able to synthesize their vitamins from pure chemicals of a synthetic medium. Others lack the ability to synthesize sufficient

quantities of one or more vitamins and are called vitamin-deficient fungi. The deficiency may be single or multiple, complete or partial. Partial deficiency may vary from nearly complete to nearly self-sufficient and is more pronounced during the early stages of growth.

A single deficiency for thiamine has been more commonly reported among filamentous fungi than any other type. Biotin deficiency is likewise commonly found, often in combination with thiamine deficiency. Deficiencies for inositol and pyridoxine are less common. Two filamentous fungi isolated from nature are reported to be deficient for nicotinic acid. Numerous other deficiencies have been induced in mutants by irradiation. Some yeasts show complete or partial multiple deficiencies for three to six vitamins, while relatively few filamentous fungi are deficient for as many as three vitamins.

Absolute deficiencies are not known to be influenced by the environment, while conditioned deficiencies may be affected either by nutritional factors or by factors of the physical environment. Among these, temperature and the composition and pH of the medium seem to be the most important.

Methods of detecting vitamin deficiencies are exact, and accurate determination depends on the ability or inability of a fungus to grow on a synthetic medium composed of pure chemicals, to which known amounts of the various vitamins to be tested are added. Vegetative growth, measured by dry weight, is apparently the most useful criterion of the utilization of vitamins, although reproduction and other processes are likewise affected.

Compounds having vitamin activity but differing in molecular structure are called vitamers. In general, only compounds of closely related structure have vitamin activity.

The inhibitory effects of vitamins in excess quantities are apparently common. They are usually evident by slight reduction in rate or maximum amount of growth and are more common with self-sufficient fungi than with those deficient for the vitamin in question. Thiamine is more commonly reported as a growth depressor than other vitamins. One instance of severe inhibition due to excess inositol and temperatures near maximum for growth is discussed.

The known effects of vitamins on the growth of fungi emphasize the important fact that growth is a result of a number of interacting factors, among which are the vitamins. A proper balance between the different vitamins and with the other nutritional and environmental factors must exist if maximum rate of growth is to take place.

REFERENCES

ARÊA LEÃO, A. E., and A. CURY: Sôbre a exigência de ácido nicotínico observada em cogumelo filamentoso; (*Microsporum audouini*), *O Hospital (Rio de Janiero)* **35**: 347–351, 1949.

Atkin, L., W. L. Williams, A. S. Schultz, and C. N. Frey: Yeast microbiological methods for determination of vitamins. Pantothenic acid, *Ind. Eng. Chem., Anal. Ed.* **16**: 67–71, 1944.

Axelrod, A. E., B. C. Flinn, and K. Hofmann: The metabolism of oxybiotin in yeast, *Jour. Biol. Chem.* **169**: 195–202, 1947.

Barnett, H. L., and V. G. Lilly: The interrelated effects of vitamins, temperature and pH upon vegetative growth of *Sclerotinia camelliae*, *Am. Jour. Botany* **35**: 297–302, 1948.

Beadle, G. W.: An inositolless mutant strain of *Neurospora* and its use in bioassays, *Jour. Biol. Chem.* **156**: 683–689, 1944.

Bonner, D.: Production of biochemical mutations in *Penicillium*, *Am. Jour. Botany* **33**: 788–791, 1946.

Bonner, D., and G. W. Beadle: Mutant strains of *Neurospora* requiring nicotinamide or related compounds for growth, *Arch. Biochem.* **11**: 319–328, 1946.

Bovarnick, M. R.: Substitution of heated asparagine-glutamate mixture for nicotinamide as a growth factor for *Bacterium dysenteriae* and other microorganisms, *Jour. Biol. Chem.* **148**: 151–161, 1943.

Burk, D., M. L. Hesselback, D. F. MacNeary, and R. J. Winzler: The vitamer concept, *Trans. N.Y. Acad. Sci.* **6**: 275–283, 1944.

★Burkholder, P. R.: Vitamin deficiencies in yeasts, *Am. Jour. Botany* **30**: 206–211, 1943.

Burkholder, P. R., I. McVeigh, and D. Moyer: Studies on some growth factors of yeasts, *Jour. Bact.* **48**: 385–391, 1944.

Burkholder, P. R., and D. Moyer: Vitamin deficiencies of fifty yeasts and molds, *Bull. Torrey Botan. Club* **70**: 372–377, 1943.

Cantino, E. C.: The vitamin nutrition of an isolate of *Blastocladia pringsheimii*, *Am. Jour. Botany* **35**: 238–242, 1948.

du Vigneaud, V., K. Dittmer, E. Hague, and B. Long: The growth-stimulating effect of biotin for the Diphtheria bacillus in the absence of pimelic acid, *Science* **96**: 186–187, 1942.

du Vigneaud, V., K. Hofmann, and D. B. Melville: On the structure of biotin, *Jour. Am. Chem. Soc.* **64**: 188–189, 1942*a*.

Eakin, R. E., and E. A. Eakin: A biosynthesis of biotin, *Science* **96**: 187–188, 1942.

Eakin, R. E., and R. J. Williams: Vitamin B_6 as a yeast nutrilite, *Jour. Am. Chem. Soc.* **61**: 1932, 1939.

Eastcott, E. V.: Wildiers' Bios. The isolation and identification of "Bios 1," *Jour. Phys. Chem.* **32**: 1094–1111, 1928.

Elliott, E. S.: Effect of vitamins on growth of some graminicolous species of *Helminthosporium* and *Fusarium*, *Proc. West Va. Acad. Sci.* **20**: 65–68, 1949.

★Fries, N.: The nutrition of fungi from the aspect of growth factor requirements, *Trans. Brit. Mycol. Soc.* **30**: 118–134, 1948.

Fromageot, C., and J. L. Tschang: Sur la synthèse des pigments carotenoides par *Rhodotorula sanniei*, *Arch. Mikrobiol.* **9**: 434–448, 1938.

Gingrich, W., and F. Schlenk: Codehydrogenase I and other pyridinium compounds as V-factor for *Hemophilus influenzae* and *H. parainfluenzae*, *Jour. Bact.* **47**: 535–550, 1944.

Goldberg, M. W., L. H. Sternbach, S. Kaiser, S. D. Heineman, J. Scheiner, and S. H. Rubin: Antibiotin effect of some biotin homologs, *Arch. Biochem.* **14**: 480–482, 1947.

Haag, E., and C. Dalphin: Une Réaction biochimique de l'aneurine d'une grande sensibilité, *Compt. rend. soc. phys. hist. nat. Genève* **57**: 76–77, 1940.

HARRIS, S. A., D. HEYL, and K. FOLKERS: The structure and synthesis of pyridoxamine and pyridoxal, *Jour. Biol. Chem.* **154**: 315–316, 1944.

HARRIS, S. A., D. E. WOLF, R. MOZINGO, and K. FOLKERS: Synthetic biotin, *Science* **97**: 447–448, 1943.

KNIGHT, B. C. J. G.: The nutrition of *Staphylococcus aureus:* Nicotinic acid and vitamin B_1, *Biochem. Jour.* **31**: 731–737, 1937.

KÖGL, F., and N. FRIES: Ueber den Einfluss von Biotin, Aneurin und Meso-Inosit auf das Wachstum verschiedener Pilzarten, *Zeit. physiol. Chem.* **249**: 93–110, 1937.

KÖGL, F., and B. TÖNNIS: Ueber das Bios-Problem. Darstellung von krystallisiertem Biotin aus Eigelb, *Zeit. physiol. Chem.* **242**: 43–73, 1936.

KOSER, S. A., M. H. WRIGHT, and A. DORFMAN: Aspartic acid as a partial substitute for the growth stimulating effect of biotin on *Torula cremoris*, *Proc. Soc. Exptl. Biol. Med.* **51**: 204–205, 1942.

LEONIAN, L. H., and V. G. LILLY: Auxithals synthesized by some filamentous fungi, *Plant Physiol.* **15**: 515–525, 1940.

LEONIAN, L. H., and V. G. LILLY: The effects of vitamins on ten strains of *Saccharomyces cerevisiae*, *Am. Jour. Botany* **29**: 459–464, 1942.

LEONIAN, L. H., and V. G. LILLY: The comparative value of different test organisms in the microbiological assay of B vitamins, *West Va. Agr. Expt. Sta. Bull.* **319**, 1945.

LILLY, V. G., and H. L. BARNETT: The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*, *Amer. Jour. Botany* **34**: 131–138, 1947.

LILLY, V. G., and H. L. BARNETT: The inheritance of partial thiamine deficiency in *Lenzites trabea*, *Jour. Agr. Research* **77**: 287–300, 1948.

LILLY, V. G., and L. H. LEONIAN: The growth rate of some fungi in the presence of co-carboxylase, and the moieties of thiamin, *Proc. West Va. Acad. Sci.* **14**: 44–49, 1940.

LILLY, V. G., and L. H. LEONIAN: The anti-biotin effect of desthiobiotin, *Science* **99**: 205–206, 1944.

LOCKHEAD, A. G., and G. B. LANDERKIN: Nutrilite requirements of osmophilic yeasts, *Jour. Bact.* **44**: 343–351, 1942.

MATHUR, R. S., H. L. BARNETT, and V. G. LILLY: Sporulation of *Colletotrichum lindemuthianum* in culture, *Phytopathology* **40**: 104–114, 1950.

MITCHELL, H. K., and M. B. HOULAHAN: *Neurospora.* IV. A temperature-sensitive riboflavinless mutant, *Am. Jour. Botany* **33**: 31–35, 1946.

MITCHELL, H. K., E. E. SNELL, and R. J. WILLIAMS: Pantothenic acid. IX. The biological activity of hydroxy-pantothenic acid, *Jour. Am. Chem. Soc.* **62**: 1791–1792, 1940.

MUELLER, J. H.: Studies on cultural requirements of bacteria. X. Pimelic acid as a growth stimulant for *C. diphtheriae*, *Jour. Bact.* **34**: 163–178, 1937.

MUELLER, J. H.: Nicotinic acid as a growth accessory substance for the diphtheria bacillus, *Jour. Bact.* **34**: 439–441, 1937*a*.

NOVELLI, G. D., and F. LIPMANN: Bacterial conversion of pantothenic acid into co-enzyme A (acetylation) and its relation to pyruvic oxidation, *Arch. Biochem.* **14**: 23–27, 1947.

PERLMAN, D.: On the nutrition of *Memnoniella echinata* and *Stachybotrys atra*, *Am. Jour. Botany* **35**: 36–41, 1948.

PETERSON, W. H., and M. S. PETERSON: Relation of bacteria to vitamins and other growth factors, *Bact. Revs.* **9**: 49–109, 1945.

PILGRIM, F. J., A. E. AXELROD, T. WINNICK, and K. HOFMANN: The microbiological activity of an oxygen analog of biotin, *Science* **102**: 35–36, 1945.

PRATT, E. F., and R. J. WILLIAMS: The effects of pantothenic acid on respiratory activity, *Jour. Gen, Physiol.* **22**: 637–647, 1939.

ROBBINS, W. J., and F. KAVANAGH: Thiamin and growth of *Pythium butleri*, *Bull. Torrey Botan. Club* **65**: 453–461, 1938.

ROBBINS, W. J., and F. KAVANAGH: Thiazole effect on Phycomyces, *Proc. Natl. Acad. Sci. U.S.* **27**: 423–427, 1941.

★ROBBINS, W. J., and V. KAVANAGH: Vitamin deficiencies of the filamentous fungi, *Botan Rev.* **8**: 411–471, 1942.

ROBBINS, W. J., and R. MA: Pimelic acid, biotin and certain fungi, *Science* **96**: 406–407, 1942.

★ROBBINS, W. J., and R. MA: Vitamin deficiencies of *Ceratostomella* and related fungi, *Am. Jour. Botany* **29**: 835–843, 1942*a*.

ROBBINS, W. J., and R. MA: Vitamin deficiencies of *Ceratostomella*, *Bull. Torrey Botan. Club* **69**: 184–203, 1942*b*.

ROBBINS, W. J., and R. MA: A *Rhodotorula* deficient for para-aminobenzoic acid, *Science* **100**: 85–86, 1944.

ROBBINS, W. J., J. E. MACKINNON, and R. MA: Vitamin deficiencies of *Trichophyton discoides*, *Bull. Torrey Botan. Club* **69**: 509–521, 1942.

ROGOSA, M.: Nicotinic acid requirements of certain yeasts, *Jour. Bact.* **46**: 435–440, 1943.

ROSENBERG, H. R.: Chemistry and Physiology of the Vitamins, Interscience Publishers, Inc., New York, 1942.

RUBIN, S. H., D. FLOWERS, F. ROSEN, and L. DREKTER: The biological activity of O-heterobiotin, *Arch. Biochem.* **8**: 79–90, 1945.

RUBBO, S. D., and J. M. GILLESPIE: *Para*-aminobenzoic acid as a bacterial growth factor, *Nature* **146**: 838–839, 1940.

RYAN, F. J., R. BALLENTINE, E. STOLONG, M. E. CORSON, and L. K. SCHNEIDER: The biosynthesis of pantothenic acid, *Jour. Am. Chem. Soc.* **67**: 1857–1858, 1945.

SCHOPFER, W. H.: Ueber die Wirkung von reinen kristallisierten Vitaminen auf *Phycomyces*, *Ber. d. deut. botan. Ges.* **52**: 308–311, 1934.

SCHOPFER, W. H.: Recherches sur le métabolisme de l'azote d'un microorganisme acellulaire (*Phycomyces blakesleeanus* Bgf.). Le rôle des facteurs de croissance, *Protoplasma* **28**: 381–434, 1937.

★SCHOPFER, W. H.: Plants and Vitamins, Chronica Botanica Co., Waltham, 1943.

SCHOPFER, W. H., and M. GUILLOUD: Recherches expérimentales sur les facteurs de croissance et le pourvoir de synthèse de *Rhizopus cohnii* Berl. et de Toni (*Rhizopus suinus* Neilson), *Zeit. für Vitaminforsch.* **16**: 181–296, 1945.

SNELL, E. E.: Effect of heat sterilization on growth-promoting activity of pyridoxine for *Streptococcus lactis* R., *Proc. Soc. Exptl. Biol. Med.* **51**: 356–358, 1942.

SNELL, E. E.: The vitamin activities of "pyridoxal" and "pyridoxamine," *Jour. Biol. Chem.* **154**: 313–314, 1944.

SNELL, E. E., and B. M. GUIRARD: Some interrelationships of pyridoxine, alanine and glycine in their effect on certain lactic acid bacteria, *Proc. Natl. Acad. Sci. U.S.* **29**: 66–73, 1943.

SNELL, E. E., B. M. GUIRARD, and R. J. WILLIAMS: Occurrence in natural products of a physiologically active metabolite of pyridoxine, *Jour. Biol. Chem.* **143**: 519–530, 1942.

SNELL, E. E., and A. N. RANNEFELT: The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms, *Jour. Biol. Chem.* **157**: 475–489, 1945.

Stokes, J. L., J. W. Foster, and H. B. Woodward: Synthesis of pyridoxin by a "pyridoxin-less" x-ray mutant of *Neurospora sitophila*, *Arch. Biochem.* **2**: 235–245, 1943.

Stokes, J. L., and M. Gunness: Microbiological activity of synthetic biotin, its optical isomers and related compounds, *Jour. Biol. Chem.* **157**: 121–126, 1945.

Stokes, J. L., A. Larsen, and M. Gunness: Biotin and the synthesis of aspartic acid by microorganisms, *Jour. Biol. Chem.* **167**: 613–614, 1947.

Strauss, B. S.: Studies on the B_6-requiring, pH-sensitive mutants of *Neurospora crassa*, *Arch. Biochem.* **30**: 292–305, 1951.

Tatum, E. L., and G. W. Beadle: Genetic control of biochemical reactions in *Neurospora:* an "aminobenzoic-less" mutant, *Proc. Natl. Acad. Sci. U.S.* **28**: 234–243, 1942.

Tatum, E. L., and G. W. Beadle: Biochemical genetics of *Neurospora*, *Ann. Missouri Botan. Garden* **32**: 125–129, 1945.

Weinstock, H. H., H. K. Mitchell, E. F. Pratt, and R. J. Williams: Pantothenic acid. IV. Formation of *beta*-alanine by cleavage, *Jour. Am. Chem. Soc.* **61**: 1421–1425, 1939.

Williams, R. J., C. M. Lyman, G. H. Goodyear, and J. H. Truesdail: Is the nutrilite for Gebrüde Mayer yeast of universal biological importance?, *Jour. Am. Chem. Soc.* **54**: 3462–3463, 1932.

Williams, R. J., W. A. Mosher, and E. Rohrman: The importance of "pantothenic acid" in fermentation, respiration and glycogen storage, *Biochem. Jour.* **30**: 2036–2039, 1936.

Williams, R. R., and T. D. Spies: Vitamin B_1 (Thiamin) and Its Use in Medicine, The Macmillan Company, New York, 1938.

Winzler, R. J., D. Burk, and V. du Vigneaud: Biotin in fermentation, respiration and nitrogen assimilation by yeast, *Arch. Biochem.* **5**: 25–47, 1944.

Wirth, J. C., and F. F. Nord: Essential steps in the enzymatic breakdown of hexoses and pentoses. Interaction between dehydrogenation and fermentation, *Arch. Biochem.* **1**: 143–163, 1942.

Wood, W. A., I. C. Gunsalus, and W. W. Umbreit: Function of pyridoxal phosphate: Resolution and purification of the tryptophanase enzyme of *Escherichia coli*, *Jour. Biol. Chem.* **170**: 313–321, 1947.

Woolley, D. W.: The nature of the antialopecia factor, *Science* **92**: 384–385, 1940.

Wooster, R. C., and V. H. Cheldelin: Growth requirements of *Penicillium digitatum*, *Arch. Biochem.* **8**: 311–320, 1945.

Wyss, O., V. G. Lilly, and L. H. Leonian: The effect of pH on the availability of *p*-aminobenzoic acid to *Neurospora crassa*, *Science* **99**: 18–19, 1944.

CHAPTER 10

FUNGI AS TEST ORGANISMS

Numerous physiological problems are accessible to investigation through the use of microorganisms. By the proper choice of deficient organisms, it is feasible to detect minute amounts of physiologically active compounds such as the vitamins and amino acids. Knowledge has been gained of the way vitamins and amino acids are synthesized and destroyed by various organisms. The amino-acid composition of proteins and the availability of certain essential elements in soil may be determined by the use of fungi and bacteria. These highly practical studies are based upon a knowledge of the compounds and elements essential for the nutrition of microorganisms. Since these are, in general, the same elements and compounds needed by animals, there is a very close relation between fungus and animal physiology in nutritional problems. Foodstuffs for man and animals are the most common materials analyzed in routine assays.

Some of the advantages which have contributed to the widespread use of microorganisms for assay purposes are simple technique and apparatus, sensitivity, specificity, and the short time required. Perhaps the most important single factor is the small sample needed and the fact that little or no purification or concentration of the active material is required. These advantages are to be compared with chemical methods or the use of animals for obtaining the same information. All analytical methods have advantages and disadvantages. A knowledge of the limitations of any method is essential for valid results.

Most microbiological assays depend upon the proportional response of deficient test organisms to the substances for which they are deficient. This proportional response occurs only for a limited range of concentrations. The usable range of concentration depends upon the substance being assayed, the test organism, and the basal medium. In theory, any organism may be used to assay any substance for which it is deficient, but in practice not all organisms having the same deficiency are equally suitable. For example, *Rhizobium trifolii* 205 is about 100 times as sensitive to biotin as *Sordaria fimicola*.

The following are essential to any quantitative microbiological assay: (1) a suitable test organism; (2) the preparation of a basal medium adequate in all respects, but essentially free from the substance to be assayed; (3) liberation, from the material to be analyzed, of the substance to be

assayed, in a water-soluble condition; (4) a standard sample of the substance to be analyzed; (5) preparation of a range of concentrations of the known and unknown substances in the basal medium; (6) uniform inoculation; (7) incubation under uniform conditions; (8) measuring the response of the test organism; (9) construction of the standard curve from the response of the test organism to known amounts of the substance under test; (10) calculating the content of the substance contained in the sample.

The above discussion assumes the use of pure compounds in obtaining standard curves. The utility of microbiological assay methods is not confined to the assay of known compounds. They are of great utility in studies of methods of isolation of new growth factors and other active compounds. These occur in complex natural products and, before they are isolated, are known only by the physiological effects they produce in living organisms. Given a deficient fungus, or other organism, it is possible to follow the efficiency of the various steps in an isolation procedure. The isolation of many of the water-soluble vitamins has been facilitated by the use of test fungi. The use of a biotin-deficient yeast enabled Kögl and Tönnis (1936) to isolate biotin for the first time as a pure compound.

GENERAL PROCEDURES

The following discussion of the steps involved in microbiological assay may serve also as a guide to the *quantitative* study of the physiology of fungi. Such studies are the surest way to gain knowledge and understanding of the physiology of the fungi.

Selection of test organisms. The first requirement of a test organism is specificity for the compound under assay. A fungus which responds to either or both moieties of thiamine is less suitable than one which requires the intact thiamine molecule. Other considerations may outweigh the advantages of strict specificity, but the response of the test organism to moieties, vitamers, and related compounds must be known.

Other considerations besides specificity enter into the selection of test organisms. Test organisms should be easily maintained in culture, easily handled in the laboratory, and have stable biochemical characteristics. Rapid and uniform growth is desirable. The habit of growth is important. A fungus which forms mucilaginous colonies which adhere to the walls of the flasks is difficult to harvest, and yeasts which clump are difficult to determine by turbidimetric methods.

The basal medium. Except for the compound or element under investigation the basal medium should be complete and balanced. If a test organism is deficient for more than one factor, all the factors except the one under investigation should be present in optimum amounts. Other requirements are easily available sources of carbon and nitrogen and a medium which is adequately buffered in the optimum pH range.

The basal medium should be essentially free from the vitamin or other factor under test. The response of the test organism to the basal medium should be slight; this value is known as the blank, or control. The size of the blank depends upon the residual concentration of the factor in the basal medium and the amount and kind of inoculum used. The degree to which a basal medium should be freed of the substance under test depends upon the sensitivity of the test organism.

The best basal medium for any test organism can be determined only after a prolonged investigation of the nutritional requirements of the organism. This arduous task is too infrequently attempted. Frequently, it is desirable to use some natural material in the medium. A complex medium which supplies several sources of carbon and nitrogen as well as other organic compounds may support more rapid growth than a simple minimal medium.

The sample being analyzed may contain compounds which stimulate or depress growth. Stimulation of growth due to the presence of accessory factors, is perhaps more often encountered than growth depression. The adequacy of the basal medium may be tested by comparing the growth curve obtained on the sample with the standard curve. If the response of the test organism to the sample is due solely to the factor contained in the sample, the two curves will be identical. The presence of inhibiting substances in the sample is detected when the sample curve falls below the standard curve. Stimulating substances are revealed by an upward drift of the sample curve.

If biologically pure compounds were available, the preparation of basal media for assay purposes would be greatly simplified. No general method of purification is useful for all purposes. Riboflavin is destroyed by light, and media can be freed of this vitamin by exposure to strong illumination. Activated charcoal (Norit or Darco) is very useful in adsorbing residual traces of many vitamins. Recrystallization of sugars, asparagine, and mineral salts is helpful in some instances. Casein is extracted with hot alcohol to remove vitamins. The essential micro elements may be removed in the ways discussed in Chap. 5. Frequently reagents made by one manufacturer are purer in certain respects than those of another.

Three basal media which have been used for fungi in microbiological assays are given below.

GLUCOSE-ASPARAGINE

Glucose	30 g.
Asparagine	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
KH_2PO_4	1.5 g.
Distilled water to make	1 liter

This medium was used for thiamine assay using *Phycomyces blakesleeanus* as the test fungus (Schopfer, 1945).

SUCROSE–AMMONIUM TARTRATE–AMMONIUM NITRATE

Sucrose	20 g.
KH_2PO_4	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Ammonium tartrate	5.0 g.
NH_4NO_3	1.0 g.
NaCl	0.1 g.
$CaCl_2$	0.1 g.
B	0.01 mg.
Mo	0.02 mg.
Fe	0.2 mg.
Cu	0.1 mg.
Mn	0.02 mg.
Zn	2.0 mg.
Biotin	5 μg
Distilled water to make	1,000 ml.

This medium was used by Horowitz and Beadle (1943) and by Beadle (1944) for the assay of choline and inositol by biochemical mutants of *Neurospora crassa*.

GLUCOSE–CASEIN HYDROLYSATE

Glucose	25 g.
Casein hydrolysate equivalent to	2 g. casein
$MgSO_4 \cdot 7H_2O$	0.5 g.
KH_2PO_4	1.0 g.
Fumaric acid	1.32 g.
Na_2CO_3	1.12 g.
Fe^{++} as sulfate	0.2 mg.
Zn^{++} as sulfate	0.2 mg.
Mn^{++} as sulfate	0.1 mg.
Distilled water to make	1 liter

This medium was used by Leonian and Lilly (1945) for the assay of certain vitamins. Various deficient yeasts and filamentous fungi were used as test organisms. This medium is suitable for testing fungi for vitamin deficiencies.

Preparing for an assay. In general, the compound being assayed should be brought into aqueous solution before assaying. Many vitamins occur in a "bound" condition and must be liberated before analysis. The procedure used to liberate bound vitamins depends upon the vitamin involved, as well as the nature of the substance being assayed. Snell (1948) has listed tentative methods for the liberation of the various vitamins. In general, acid or enzymatic hydrolysis is used. Proteins are hydrolyzed before amino-acid assay. Acid hydrolysis is destructive

to certain amino acids, especially tryptophane. Alkaline hydrolysis of proteins has been recommended for this amino acid (Greene and Black, 1944).

The concentrations of the standard compound and of the sample for assay should be so chosen that the response of the test organism is roughly linear. Every concentration should be run in duplicate. Control flasks to which neither the standard compound nor the assay sample have been added should form a part of every assay. This provides a means of evaluating the basal medium and should never be omitted.

The type of culture vessel and the volume of the basal medium used will depend upon the test organism. Bacteria are frequently cultured in test tubes. These are also useful for yeasts. Uniform test tubes which can be used in a photoelectric colorimeter allow measurement of turbidity without transfer (Lindegren and Raut, 1947). The filamentous fungi are usually cultured in Erlenmeyer flasks. The volume of medium should be so chosen that the liquid is less than 1 cm. deep. All glassware must be clean. Accuracy in measuring the basal medium and the known and unknown solutions is essential.

Inoculation and incubation. The medium upon which the inoculum is grown should be complete and contain an adequate but not excessive amount of the factor under investigation. Certain fungi, especially the yeasts, cease to be deficient for certain vitamins when continuously cultured upon media free from these factors.

Spore inoculum may be used with advantage with many filamentous fungi. Frequently it is desirable to use germinated spores for inoculum. *Phycomyces blakesleeanus* spores require the Z factors for rapid germination (Robbins, 1940). If the test sample contains these factors and the basal medium does not, early growth will be more rapid in the sample series. It is convenient to germinate the spores of this fungus and others by preparing a spore suspension in dilute peptone solution a few hours before inoculation. These germinated spores grow essentially without interruption and shorten the time of incubation. Fragmented mycelium may also be used to advantage. A uniform amount of inoculum must be used. This is easy to achieve when a suspension of spores or fragmented mycelium is used. Inocula of these types provide a multitude of growing points, which results in uniform growth. Disks of mycelium on agar are, in general, unsatisfactory.

An obvious advantage of using a large amount of inoculum is the shorter time required for an assay. However, there is danger of introducing with a large inoculum enough of the substance under investigation to give abnormally high blanks. Washing the inoculum with sterile distilled water reduces this hazard but increases the work and multiplies the chances of contamination. A very small inoculum results in a longer lag period, and the time required for analysis may be prolonged.

Test organisms during an assay should be cultured under uniform conditions with respect to light and temperature. In general, the filamentous fungi should not be agitated during the period of incubation. Yeasts are frequently grown with continuous or intermittent shaking.

There are two schools of thought concerning the time of incubation for assay. The first recommends a uniform short period of growth and determination of the yield before the organism reaches its maximum development. There is a saving in time in this method, but the influence of accessory factors in the sample may make such results unreliable. A comparison should always be made between the analytical data for a short and a long period of incubation before choosing the length of incubation period. In general, we feel that assays tend to be more reliable when the period of incubation is long enough to allow maximum development of the test organism.

Measuring the response. The methods used for measuring the response of test organisms vary. The growth response of bacteria may be measured either by titrating the acid produced or by determining the turbidity with a suitable photoelectric colorimeter. The growth response of yeasts may be measured as turbidity, or the cells may be weighed. The first procedure is by far the simpler. The growth of filamentous fungi is commonly measured by collecting the mycelium and determining the dry weight (see discussion in Chap. 3).

Calculation of results. A growth curve (acidity, turbidity, or weight) is plotted from the response of the test organism to the different concentrations of the standard substance. The concentration of the substance in the sample is then calculated from the standard curve. It is necessary to use a new standard curve for each series of assays. Unsuspected variations in the basal medium and in technique from day to day make this precaution necessary. In making the calculations, it is assumed that equal amounts of the substance, whether as a pure compound or in the sample, will cause the same amount of response by the test organism. It is customary to report the concentrations of vitamins and micro essential elements in micrograms per gram of original sample.

As an example of the type of calculation involved in an assay, the standard curve (Fig. 43) and protocol of a biotin assay are given below. The substance assayed was air-dry yeast cells. Biotin was liberated from the sample by acid hydrolysis, and the cell extract was neutralized and made up to such volume that 1 ml. of hydrolysate was equivalent to 50 mg. of original yeast cells. The test organism, *Saccharomyces cerevisiae*, Gebrüde Mayer strain, was incubated for 72 hr at 25°C. Twenty-five milliliters of glucose-casein hydrolysate medium was used per 250-ml. flask. The cultures were agitated 10 min. every hour. The data for the response of the test organism to varying amounts of yeast hydrolysate are given in Table 35.

TABLE 35. YIELD OF *Saccharomyces cerevisiae* CELLS PRODUCED WHEN DIFFERENT AMOUNTS OF YEAST HYDROLYSATE WERE ADDED TO 25 MILLILITERS OF A BIOTIN-FREE GLUCOSE–CASEIN HYDROLYSATE MEDIUM

Yeast hydrolysate, ml. per flask	Equivalent weight of sample, mg.	Yield, mg.	
		Flask 1	Flask 2
0.03125	1.5625	9.2	8.9
0.0625	3.125	20.2	19.0
0.125	6.25	32.8	32.8
0.25	12.5	48.0	47.6

The amount of biotin in the original sample may then be calculated. The amount of biotin in 6.25 mg. of the sample produced 32.8 mg. of

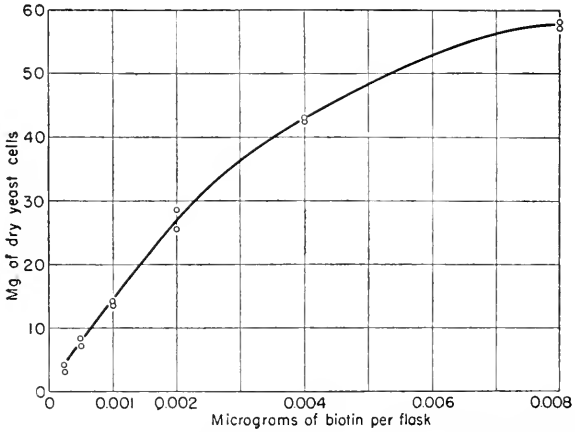


FIG. 43. Standard curve for a biotin assay using *Saccharomyces cerevisiae*, Gebrüde Mayer strain, as the test fungus. Basal medium was glucose–casein hydrolysate, 25 ml. per 250-ml. Erlenmeyer flask. Cultures were incubated at 25°C., agitated 10 min. each hour, and harvested after 72 hr.

dry yeast cells. From the standard curve this is seen to be equivalent to 0.0025 μg of biotin. The biotin content of the sample is therefore equal to 0.0025 × 1,000/6.25, or 0.4 μg of biotin per gram of sample.

VITAMIN ASSAYS

It is beyond the intent of this chapter to include detailed information about techniques in connection with individual assays. The following references are useful for entry into the literature. Schopfer (1945) has considered the philosophy underlying the use of microorganisms for assay. Leonian and Lilly (1945) investigated the use of many test organisms to assay the vitamin content of a single substance. This work showed that widely different assay values for some vitamins are obtained

when different test organisms are used. The review of Snell (1948) represents the critical judgment of an active investigator in this field.

While the filamentous fungi are frequently passed over in favor of bacteria and yeasts, they offer certain advantages when only simple apparatus is available, or where occasional assays are to be made. The test organisms for the specific vitamins listed below are in part those recommended by Snell (1948).

Thiamine. *Phycomyces blakesleeanus.* This fungus responds to the two moities of thiamine. Schopfer (1935, 1945) used a glucose-asparagine medium and used dry weight of mycelium to measure growth. This is an excellent organism to use in gaining experience with a microbiological assay. Schultz *et al.* (1942) used *Saccharomyces cerevisiae* (Fleischmann's baker's yeast) and measured the evolution of carbon dioxide, which was proportional to the thiamine content of the sample.

Pyridoxine. *Saccharomyces carlsbergensis.* Snell (1945*a*) found that this yeast responds about equally to pyridoxine, pyridoxal, and pyridoxamine. Growth may be measured turbidimetrically or by weighing the cells. Differential assays for these three vitamers have been devised.

***p*-Aminobenzoic acid.** *Neurospora crassa* mutant. Various laboratories have used this organism (Tatum *et al.*, 1946). For the effect of pH on utilization of this vitamin see Wyss *et al.* (1944).

Pantothenic acid. *Saccharomyces carlsbergensis.* Most, if not all, yeasts respond to the β-alanine moiety of pantothenic acid. Atkin *et al.* (1944) noted that the incorporation of *l*-asparagine in the basal medium reduced interference due to β-alanine.

Nicotinic acid. *Lactobacillus arabinosus.* This organism responds equally to nicotinic acid and nicotinamide. Growth may be measured either by titrating the acid produced, or turbidimetrically (Krehl *et al.*, 1943). *Zygosaccharomyces marxianus* was used by Leonian and Lilly (1945).

Inositol. *Neurospora crassa* mutant. This mutant was first used by Beadle (1944) to assay inositol. It is an easy organism to handle, and since this mutant forms few conidia, it is not a great source of contamination to a laboratory. Snell (1948) recommends the use of *Saccharomyces carlsbergensis* for inositol assay.

Biotin. *Saccharomyces cerevisiae.* Various strains have been used. Many, if not all, strains respond also to desthiobiotin (Lilly and Leonian, 1944). The existence of many biotin vitamers makes the choice of a test organism difficult. *Neurospora crassa* and *N. sitophila* may also be used. It is probable that some of the divergence of assay values obtained when different test organisms are used is due to biotin complexes. Such a complex, biocytin, has been isolated by Wright *et al.* (1950). The analytical results were unchanged by acid hydrolysis when *Lactobacillus*

casei was used but were increased when *L. arabinosus* was the test organism.

Riboflavin. *Lactobacillus casei.* Fatty acids stimulate growth. Growth may be measured by titrating the acid formed, or turbidimetrically (Roberts and Snell, 1946). It is probable that mutants of *Neurospora* deficient for this vitamin may also be used in assay.

AMINO-ACID ASSAYS

The importance of the amino-acid composition of proteins used in animal nutrition makes any advance in analytical methods of great interest and value. The general techniques for amino-acid determinations by microbiological procedures are the same as for other assays. The first requirement of this type of microbiological assay is a suitable test organism. Few fungi isolated from nature are deficient for amino acids. For this reason bacteria have been extensively used. The following references will give an entry into the literature on the use of bacteria for amino-acid assay: Hutchings and Peterson (1943); Shankman (1943); Dunn *et al.* (1944); Snell (1945); and Horn *et al.* (1950).

Some mutants of *Neurospora* have been found to be deficient for amino acids. Mutants having the following amino-acid deficiencies have been studied: leucine, isoleucine, valine, lysine, methionine, serine, or glycine. Only the mutant deficient for leucine appears to have been much used in microbiological assay (Ryan and Brand, 1944; Brand *et al.*, 1945). The growth of a lysine-deficient mutant was completely inhibited by arginine when the molecular ratio of arginine to lysine was 2 to 1 (Doermann, 1944).

Ryan (1948) has considered the possibility of microbiological assay of amino acids by observing the percentage of germination of conidia from deficient mutants in the presence of different concentrations of the specific amino acid. An assay can be completed within a few hours by this method. Unfortunately the inhibiting action of certain amino acids introduces complications into the proposed method.

Mutations of *Neurospora* and certain other fungi have been induced by chemicals, such as nitrite or nitrous acid, colchicine, nitrogen mustard gas, and hydrogen peroxide, or by irradiation with ultraviolet and X rays. These mutants are frequently characterized by inability to synthesize various metabolites. They differ from the parent wild type in that one or more genes have been inactivated. It is thought that each gene controls a single biochemical reaction. Mutants having the same gross deficiency may differ in the specific gene inactivated.

Horowitz (1947) studied four mutants of *Neurospora* which were unable to synthesize methionine from inorganic sources of nitrogen and sulfur. One of these mutants was able to grow in the presence of cysteine, cysta-

thionine, homocysteine, and methionine. The second was unable to utilize cysteine but was able to utilize the other three compounds. The third isolate utilized either homocysteine or methionine, while the fourth isolate utilized only methionine. From these results the steps in the synthesis of methionine and the genes inactivated may be summarized as follows: $\xrightarrow{\text{gene 4}}$ cysteine $\xrightarrow{\text{gene 3}}$ cystathionine $\xrightarrow{\text{gene 2}}$ homocysteine $\xrightarrow{\text{gene 1}}$ methionine. From similar studies Srb and Horowitz (1944) concluded that *Neurospora* synthesizes arginine as follows: ornithine $\longrightarrow$ citrulline $\longrightarrow$ arginine.

Fungus mutants have proved to be powerful tools for investigating pathways of synthesis and utilization of vitamins, amino acids, and other compounds, and in studies of biochemical mutations. From these studies also comes the realization that each step in the synthesis or utilization of a compound may be controlled or limited by a specific gene. The review papers of Bonner (1946) and Beadle (1945, 1945*a*) should be consulted for further information and literature citations.

ASSAYS FOR ESSENTIAL ELEMENTS

Microorganisms may be used to determine the presence of essential elements. In view of the speed and accuracy of chemical and spectroscopic methods, it might be assumed that microorganisms would be of little value in such applications. The value of microbiological tests would appear to be in applications where availability as well as total amounts are of importance. Problems of this sort frequently arise in connection with mineral deficiencies in soil. It is recognized that the absolute content of an essential element in a soil may not measure the availability of that element for green plants. Microbiological and chemical methods of analysis must be correlated with plant tests before they are of much value.

The possible number of test organisms is unlimited except for the important considerations of sensitivity, ease of handling, and time required to make an assay. In practice, only a few organisms have been used. There exists a wide field for investigations dealing with the correlation between availability to microorganisms and availability to green plants of certain essential elements in soil.

Copper. Mulder (1939–1940) used *Aspergillus niger* to determine copper in soil. The range of concentrations in the standard series was 0.0 to 2.5 μg Cu^{++} per culture; 40 ml. of medium was used in liter flasks. One gram of sterile soil was used as the sample. The method of measuring the response of *A. niger* to copper was very simple, inasmuch as the *number* and *color* of the spores produced were functions of the copper content of the medium. No spores developed on the control medium, but

with increasing concentrations of copper the spores were yellow, yellow-brown, gray-brown, brown, and black.

The color of the spores produced on copper-deficient media by different isolates of *A. niger* varied. Excellent correlation between the copper content of various soils as determined by this method and the incidence of copper deficiency in grain was found. Some of Mulder's results are presented in Table 36.

TABLE 36. THE CORRELATION OF COPPER DEFICIENCY IN WHITE OATS AND THE COPPER CONTENT OF THE SOIL AS DETERMINED BY *Aspergillus niger* METHOD
All soil was from the same field. (Mulder, *Antonie van Leeuwenhoek* **6**, 1940.)

Condition of Oats	Available Copper, μg per G. of Soil
Severely diseased	0.25
Less severely diseased	0.8
Healthy (from a portion of the field not showing the disease)	1.7
Healthy (copper sulfate added to the soil)	2.5

Magnesium. Smit and Mulder (1942) postulated that a microbiological method would show better correlation with magnesium deficiency in green plants than chemical methods. This was confirmed for the Netherlands soils investigated. *Azotobacter chroococcum* and *Aspergillus niger* were used as test organisms. Preference was given to the fungus inasmuch as only 4 to 5 days were required for an assay. A simple technique was used, and visual comparison was sufficiently accurate to diagnose magnesium deficiency in soils.

Potassium. *Aspergillus niger* was used by Niklas and Toursel (1940) to determine available potassium and other elements in soils. These authors weighed the mycelium produced. Rogosa (1944) has shown that *Lactobacillus casei* may be used to determine small amounts of potassium.

TABLE 37. THE EFFECT OF MOLYBDENUM CONTENT OF A GLUCOSE-NITRATE MEDIUM UPON YIELD OF MYCELIUM AND SPORULATION OF *Aspergillus niger*
(Mulder, *Plant and Soil* **1**, 1948.)

μg $Na_2MoO_4 \cdot 2H_2O$, in 50 ml. medium	Mg. mycelium per culture	Sporulation	Appearance of mycelium
0.0	165	0	Entirely mucous
0.0025	294	0	Partially mucous
0.010	558	0	Partially mucous
0.050	868	Normal	Normal

Molybdenum. The amount of this element needed by fungi and green plants is greater when nitrogen is supplied as nitrate than when ammonium nitrogen is furnished. This fact introduces a complication into the microbiological assay of molybdenum in that the sample must be ashed

before analysis. It is probable that amino acids and other nitrogen sources containing reduced nitrogen would also affect the amount of molybdenum needed. Mulder (1948) investigated the use of *Aspergillus niger* as a test organism (Table 37). For further discussion and references to the use of microorganisms in essential-element assay see Vandecaveye (1948).

SUGARS

Yeasts and other microorganisms have been used to separate optical isomers and complex mixtures of sugars. Pasteur (1860) used *Penicillium glaucum* to obtain the "unnatural" isomer of tartaric acid from *dl*-tartaric acid. Fischer and Hertz (1892) used brewer's yeast to ferment D-galactose, while L-galactose in the same medium was not utilized. Auernheimer *et al.* (1948) used the specific fermentative powers of *Hansenula suaveolens* and *Candida guilliermondi* in the separation of L-arabinose and D-xylose obtained from the hydrolysis of straw and corn cobs. *H. suaveolens* does not utilize L-arabinose, while *C. guilliermondi* utilizes both pentoses. *Saccharomyces carlsbergensis* was used to demonstrate the absence of D-glucose in the hydrolysates. These yeasts were used in conjunction with chemical methods of analysis. Appling *et al.* (1947) found *Saccharomyces carlsbergensis* var. *mandschuricus* to ferment D-galactose but not L-galactose. Similarly, *H. suaveolens* utilized D-xylose but not L-xylose.

These citations indicate the usefulness of yeasts and other organisms in the solution of problems difficult to solve by other methods. The value of microorganisms in such applications is due to their specificity.

TESTS FOR CERTAIN METABOLIC PRODUCTS

Fungi excrete into the media in which they grow various physiologically active substances. In the older literature these are referred to as *staling products*. Among the metabolic products are those which may stimulate or inhibit growth and reproduction. The kind and the amount of compounds excreted depend upon the particular fungus involved as well as the composition of the medium. The effect of the metabolic products of one fungus upon another is simply demonstrated when fungi are grown in association. The beneficial effect of one fungus upon another was demonstrated by Kögl and Fries (1937). Neither *Nematospora gossypii* or *Polyporus adustus* grew when inoculated *alone* into a synthetic medium, but when both fungi were inoculated *together* in the same flask, both began to grow rapidly after about a week. *N. gossypii* is deficient for biotin but synthesizes thiamine, while *P. adustus* is deficient for thiamine but synthesizes biotin. Kögl and Fries called this artificial symbiosis. Schopfer and Guilloud (1945) cite other examples in connection with work on strains of *Candida guilliermondi* involving vitamin deficiencies.

By using a series of test organisms of known deficiencies, it is easy to demonstrate that fungi excrete vitamins. It is a common experience to find deficient fungi growing in association with contaminants. The method is simple and consists of inoculating plates of vitamin-free medium with two test fungi (Fig. 44). Not all fungi excrete the same amount of a

FIG. 44. Test demonstrating the excretion of biotin by *Aspergillus rugulosus* (right), when grown with *Sordaria fimicola* (biotin-deficient) on vitamin-free glucose-asparagine medium. *Sordaria* (left) made only slight growth until it approached the colony of *Aspergillus*, where a zone of stimulated growth is evident.

given vitamin. This may be shown by choosing test fungi such as *Sordaria fimicola*, which requires more biotin for fruiting than for growth. Some fungi excrete enough biotin to allow growth of *S. fimicola*, while others excrete enough biotin to allow reproduction also. Other compounds besides the vitamins may be excreted and favor the growth of other organisms. Further instances of the favorable effect of one fungus on the sporulation of another are discussed in Chap. 14.

The metabolic products of one fungus may inhibit the growth of another. This phenomenon may be frequently observed on contaminated plates (Fig. 45). Fleming (1929) discovered the action of penicillin in this way.

Many fungi apparently produce substances which inhibit the germination of their spores. Schopfer (1933) found that spores of *Phycomyces blakesleeanus* would not germinate on agar media upon which this fungus had grown. If such a "staled" plate was autoclaved, the medium would then allow germination and growth of *P. blakesleeanus*. These results

indicate that the spore-inhibiting substance was either volatile or unstable. This inhibitory substance was not identified.

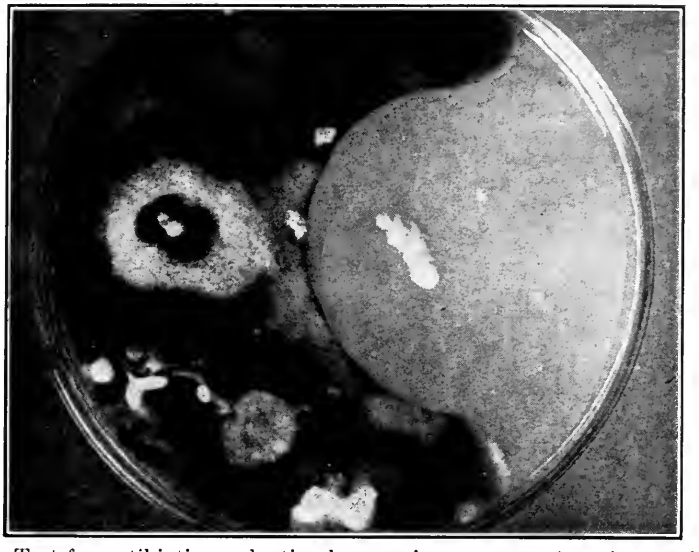


FIG. 45. Test for antibiotic production by growing two organisms in association on the same agar plate. *Helminthosporium sativum* on the left and an unidentified actinomycete on the right.

TESTING FABRIC PROTECTANTS

While the deterioration of cellulosic materials exposed to the weather or in contact with the soil is not solely due to the action of bacteria and fungi, these organisms are the chief agents of destruction. The problem of deterioration of cellulosic materials has received a vast amount of attention, especially in connection with military matériel in humid tropic climates. Work on this problem involves the identification of the responsible microorganisms, laboratory tests, and use of test fungi in evaluating protectants.

The basis of the various methods for determining cellulolytic activity consists in inoculating cotton duck or other test material with the fungi under test. The degree of cellulolytic activity is determined by measuring the decrease in tensile strength of the test specimen. The test medium used is usually an inorganic salt solution having pH 6.8. It is desirable to use a buffered medium inasmuch as cellulase is most active around pH 7. White *et al.* (1948) note that many fungi which are strongly cellulolytic under laboratory conditions cause but little damage in the field. They believe that, under a given set of natural environmental conditions, the actual decay of fibers is caused by a relatively few species of fungi. Among the strongly cellulolytic fungi are *Mem-*

noniella echinata (the variability in strains in laboratory tests is possibly correlated with biotin deficiency), *Chaetomium* spp., especially *C. globosum* (Greathouse and Ames, 1945), *Myrothecium verrucaria* (as strong a cellulose decomposer as yet found in laboratory tests), *Trichoderma viride*, and *Thielavia sepedonium*.

The reduction in tensile strength of cotton duck maintained under specified conditions is used as a measure of the destructive effects of fungi on fabrics. The data in Table 38 are taken from White *et al.* (1948).

TABLE 38. ASSAY OF FUNGI FOR CELLULOLYTIC ACTIVITY BASED UPON LOSS OF TENSILE STRENGTH OF COTTON DUCK
(White *et al.*, *Mycologia* **40**, 1948.)

Species	Strength retained, %			Growth at end of experiment
	6 days	9 days	12 days	
Aspergillus niger PQMD 25a	100	103	105	2
A. terreus PQMD 72f	67	42	32	4
Chaetomium funicolum PQMD 351	0	—	—	4
C. globosum PQMD 32b	18	0	—	4
Fusarium oxysporum Fla C-8	49	36	30	3
Gliomastix convoluta PQMD 4c	—	51	22	4
Myrothecium verrucaria PQMD 70h	0	—	—	4
Thielavia sepedonium PQMD 47g	15	0	—	4
Trichoderma viride PQMD 6a	18	10	8	4
T. viride PQMD 63d	100	99	98	0

The evaluation of protective fungicides for fabrics, paper, and other cellulosic materials consists in comparing the effects of known cellulolytic fungi upon treated and untreated specimens of material. In addition to causing loss of tensile strength, some fungi cause great damage by surface growth (mildew). Abrams (1948) has reviewed the techniques used at the Bureau of Standards for testing mildew- and rotproofing agents. *Aspergillus niger* was used to determine mildew resistance, and the effectiveness of various treatments was evaluated by visual observation. *Chaetomium globosum* and a species of *Penicillium* (USDA 66) were used in rot-resistance tests. Of some 36 compounds tested, copper naphthenate and pyridyl mercury compounds were most effective. The effectiveness of the fungicides varies with the test organisms used. For data on fungicide evaluation the reader is referred to Abrams (1948).

SUMMARY

The use of microorganisms for analytical purposes is based upon specific biochemical characteristics of selected test organisms. Within a certain range of concentration, the response is proportional to the amount of

test substance present in the medium. Among the substances for which quantitative assay procedures have been developed are the vitamins, amino acids, and essential elements. Microorganisms have also been used to discover pathways of biochemical synthesis and degradation, to separate isomers, and for other analytical purposes.

The essential features of a microbiological assay are (1) a suitable test organism, (2) a suitable basal medium essentially free from the substance under test, (3) preparation of the sample, (4) a reference standard (a pure compound where possible), (5) two series of cultures to which a known range of concentrations of the standard and unknown have been added, (6) uniform inoculation, (7) incubation under uniform conditions, (8) measuring the response of the test organisms, (9) construction of a standard curve, and (10) calculating the results.

When microbiological assay procedures are used, it is unnecessary to isolate the compound being assayed from the other constituents present in the sample. The preparation of the sample for assay is usually simple and ordinarily involves hydrolysis. Microbiological procedures usually require a short time to complete. The amount of sample needed is small, which is an important consideration in some problems. Microbiological assays are invaluable, provided that suitable test organisms are available, in devising chemical procedures for the isolation of new vitamins and other physiologically active compounds.

Biochemical mutants of *Neurospora* and other fungi are particularly useful in determining the pathways of synthesis of amino acids and other compounds.

REFERENCES

Abrams, E.: Microbiological deterioration of organic materials, its prevention and methods of test, *Natl. Bur. Standards (U.S.), Misc. Pub.* 188, 1948.

Appling, J. W., E. K. Ratcliff, and L. E. Wise: Chemical and microbiological differentiation of enantiomorphs of galactose and xylose, *Anal. Chem.* **19**: 496–497, 1947.

Atkin, L., W. L. Williams, A. S. Schultz, and C. N. Frey: Yeast microbiological methods for determination of vitamins. Pantothenic acid, *Ind. Eng. Chem., Anal. Ed.* **16**: 67–71, 1944.

Auernheimer, A. H., L. J. Wickerham, and L. E. Schniepp: Quantitative determination of hemicellulose constituents by fermentation, *Anal. Chem.* **20**: 876–877, 1948.

Beadle, G. W.: An inositolless mutant strain of *Neurospora* and its use in bioassays, *Jour. Biol. Chem.* **156**: 683–689, 1944.

Beadle, G. W.: Biochemical genetics, *Chem. Revs.* **37**: 15–96, 1945.

Beadle, G. W.: Genetics and metabolism in *Neurospora*, *Physiol. Revs.* **25**: 643–663, 1945*a*.

Bonner, D.: Biochemical mutations in *Neurospora*, *Cold Spring Harbor Symposia Quant. Biol.* **11**: 14–24, 1946.

Brand, E., F. J. Ryan, and E. M. Diskant: Leucine content of proteins and foodstuffs, *Jour. Am. Chem. Soc.* **67**: 1532–1534, 1945.

Doermann, A. H.: A lysineless mutant of *Neurospora* and its inhibition by arginine, *Arch. Biochem.* **5**: 373–384, 1944.

Dunn, M. S., S. Shankman, M. N. Camien, W. Frankland, and L. B. Rockland: Investigations of amino acids, peptides, and proteins. XVIII. The amino acid requirements of *Leuconostoc mesenteroides* P-60, *Jour. Biol. Chem.* **156**: 703–713, 1944.

Fischer, E., and J. Hertz: Reduction der Schleimsäure, *Ber. d. deut. chem. Ges.* **25**: 1247–1261, 1892.

★Fleming, A.: On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*, *Brit. Jour. Exptl. Path.* **10**: 226–236, 1929.

Greathouse, G. A., and L. M. Ames: Fabric deterioration by thirteen described and three new species of *Chaetomium*, *Mycologia* **37**: 138–155, 1945.

Greene, R. D., and A. Black: The microbiological assay of trytophane in proteins and foods, *Jour. Biol. Chem.* **155**: 1–8, 1944.

Horn, M. J., D. B. Jones, and A. E. Blum: Methods for microbiological and chemical determinations of essential amino acids in proteins and foods, *U.S. Dept. Agr. Misc. Pub.* 696, 1950.

★Horowitz, N. H.: Methionine synthesis in *Neurospora*. The isolation of cystathionine, *Jour. Biol. Chem.* **171**: 255–264, 1947.

Horowitz, N. H., and G. W. Beadle: A microbiological method for the determination of choline by use of a mutant of *Neurospora*, *Jour. Biol. Chem.* **150**: 325–333, 1943.

Horsfall, J. G.: Fungicides and Their Action, Chronica Botanica Co., Waltham, 1945.

Hutchings, B. L., and W. H. Peterson: Amino acid requirements of *Lactobacillus casei*, *Proc. Soc. Exptl. Biol. Med.* **52**: 36–38, 1943.

Kögl, F., and N. Fries: Ueber den Einfluss von Biotin, Aneurin und Meso-Inosit auf das Wachstum verscheidener Pilzarten, *Zeit. physiol. Chem.* **249**: 93–110, 1937.

Kögl, F., and B. Tönnis: Ueber das Bios-Problem. Darstellung von krystallisiertem Biotin aus Eigelb, *Zeit. physiol. Chem.* **242**: 43–73, 1936.

Krehl, W. A., F. M. Strong, and C. A. Elvehjem: Determination of nicotinic acid. Modifications in the microbiological method, *Ind. Eng. Chem., Anal. Ed.*, **15**: 471–475, 1943.

Leonian, L. H., and V. G. Lilly: The comparative value of different test organisms in the microbiological assay of B vitamins, *West Va. Agr. Expt. Sta. Bull.* 319, 1945.

Lilly, V. G., and L. H. Leonian: The anti-biotin effect of desthiobiotin, *Science* **99**: 205–206, 1944.

Lindegren, C. C., and C. Raut: The effect of the medium on apparent vitamin-synthesizing deficiencies of microorganisms, *Ann. Missouri Botan. Garden* **34**: 75–84, 1947.

Mulder, E. G.: On the use of micro-organisms in measuring a deficiency of copper, magnesium and molybdenum in soils, *Antonie van Leeuwenhoek* **6**: 99–109, 1939–1940.

Mulder, E. G.: Importance of molybdenum in the nitrogen metabolism of micro-organisms and higher plants, *Plant and Soil* **1**: 94–119, 1948.

Niklas, H., and O. Toursel: Die Bodenuntersuchung mittels *Aspergillus niger*, *Bodenkunde und Pflanzen ernäkr.* **18**: 79–107, 1940.

Pasteur, L.: Note relative au *Penicillium glaucum* et à la dissymétrie moléculaire des produits organiques naturels, *Compt. rend. acad. sci.* **51**: 298–299, 1860.

ROBBINS, W. J.: Effect of extracts of *Phycomyces* upon its development, *Am. Jour. Botany* **27**: 559–564, 1940.

ROBERTS, E. C., and E. E. SNELL: An improved medium for microbiological assays with *Lactobacillus casei*, *Jour. Biol. Chem.* **163**: 499–509, 1946.

ROGOSA, M.: Microbiological method for the quantitative determination of small quantities of potassium, *Jour. Biol. Chem.* **154**: 307–308, 1944.

★RYAN, F. J.: The germination of conidia from biochemical mutants of *Neurospora*, *Am. Jour. Botany* **35**: 497–503, 1948.

RYAN, F. J., and E. BRAND: A method for the determination of leucine in protein hydrolysates and in foodstuffs by the use of a *Neurospora* mutant, *Jour. Biol. Chem.* **154**: 161–175, 1944.

SCHOPFER, W. H.: Etude d'un cas de stimulation unilatérale et d'un cas d'inhibition chez un micro-organisme, *Compt. rend. soc. phys. hist. nat. Genève.* **50**: 152–154, 1933.

SCHOPFER, W. H.: Recherches sur l'emploi possible d'un test végétal pour la vitamine B_1. Essai d'étalonnage, *Bull. soc. chim. biol.* **17**: 1097–1109, 1935.

SCHOPFER, W. H.: Les Tests microbiologiques pour la détermination des vitamines, *Experientia* **1**: 1–68, 1945.

SCHOPFER, W. H., and M. GUILLOUD: Un cas de stimulation unilatéral chez las microorganismes expliqué par une action vitaminique, *Verhandl. naturforsch. Ges. Basel* **61**: 299–314, 1945.

SCHULTZ, A. S., L. ATKIN, and C. N. FREY: Determination of vitamin B_1 by yeast fermentation method. Improvements related to the use of sulfite cleavage and a new fermentometer, *Ind. Eng. Chem., Anal. Ed.* **14**: 35–39, 1942.

SHANKMAN, S.: Amino acid nutrition of *Lactobacillus arabinosus*, *Jour. Biol. Chem.* **150**: 305–310, 1943.

SMIT, J., and E. G. MULDER: Magnesium deficiency as the cause of injury in cereals, *Mededeelingen van de Landbouwhoogeschool*, Deel 46, Verhandeling 3, 1942.

SNELL, E. E.: The microbiological assay of amino acids, *Advances in Protein Chem.* **2**: 85–118, 1945.

SNELL, E. E.: The vitamin B_6 group. Evidence for the occurrence of pyridoxamine and pyridoxal in natural products, *Jour. Biol. Chem.* **157**: 491–505, 1945*a*.

★SNELL, E. E.: Use of microorganisms for assay of vitamins, *Physiol. Revs.* **28**: 255–282, 1948.

SRB, A. M., and N. H. HOROWITZ: The ornithine cycle in *Neurospora* and its genetic control, *Jour. Biol. Chem.* **154**: 129–139, 1944.

TATUM, E. L., M. G. RITCHEY, E. V. COWDRY, and L. F. WICKS: Vitamin content of mouse epidermis during methylcholanthrene carcinogenesis. I. Biotin, choline, inositol, *p*-aminobenzoic acid and pyridoxine, *Jour. Biol. Chem.* **163**: 675–682, 1946.

VANDECAVEYE, S. C.: Biological methods of determining nutrients in soil *in* Diagnostic Techniques for Soils and Crops (edited by H. B. Kitchen), The American Potash Institute, Washington, 1948.

★WHITE, W. L., R. T. DARBY, G. M. STECHERT, and K. SANDERSON: Assay of cellulolytic activity of molds isolated from fabrics and related items exposed in the tropics, *Mycologia* **40**: 34–84, 1948.

WRIGHT, L. D., E. L. CRESSON, H. R. SKEGGS, T. R. WOOD, R. L. PECK, D. E. WOLF, and K. FOLKERS: Biocytin, a naturally-occuring complex of biotin, *Jour. Am. Chem. Soc.* **72**: 1048, 1950.

WYSS, O., V. G. LILLY, and L. H. LEONIAN: The effect of pH on the availability of *p*-aminobenzoic acid to *Neurospora crassa*, *Science* **99**: 18–19, 1944.

CHAPTER 11

METABOLITE ANTAGONISTS

This chapter and the one following will deal with chemical compounds which inhibit, injure, or kill fungi. Much can be learned about "normal" physiological processes by studying the factors which interfere with them. The ideas to be discussed here are applicable to the entire field of physiology, and some of our illustrative material will deal with organisms other than fungi. The reviews of Woolley (1944), Welch (1945), Wright (1947), McIlwain (1947), and Roblin (1946, 1949) are extensive and well documented and should be consulted for additional references.

Metabolites are chemical substances which are essential for the functioning and maintenance of living cells. Metabolites may be synthesized by the organism or obtained from the medium, *e.g.*, vitamins, amino acids, etc.

An antimetabolite, or antagonist, is a compound which interferes with the utilization of a normal metabolite. Wright (1947) has classified antagonists (more specifically antivitamins) on the basis of their mode of action: (1) those which act by virtue of destroying or inactivating a metabolite; (2) those which combine irreversibly with enzymes (noncompetitive inhibition); and (3) those which combine with enzymes but which may be displaced by increased concentration of the normal metabolite (competitive inhibition).

Noncompetitive enzyme inhibition is so called because an increase in the concentration of the normal coenzyme or metabolite molecules does not reverse the inhibition. Inhibitors of this type act by combining with some atom or molecular group of either a coenzyme or an apoenzyme. Among inhibitors of this type we may list the heavy metals, various organic mercury and arsenic compounds, iodoacetate, and quinones, which inactivate enzymes by combining with free sulfhydryl groups (see Singer, 1945, and McElroy, 1947, for references). Among the inhibitors which act on the iron-porphyrin enzymes are cyanide, azide, hydrogen sulfide, and carbon monoxide. Most of the discussion to follow will deal with competitive antagonists.

Metabolite antagonists are analogues of normal metabolites, but not all analogues of a metabolite are necessarily antagonists. These "foreign" molecules, because of their close resemblance to normal metabolites, combine with enzymes in the same manner as normal metabolites. How-

ever, these foreign molecules are not transformed by the enzyme to which they are bound. If the antagonist is an analogue of a coenzyme, it presumably forms a pseudoholoenzyme which is unable to function. The close structural relation between a metabolite (*p*-aminobenzoic acid) and its antagonist (sulfanilamide) is shown in Fig. 46.

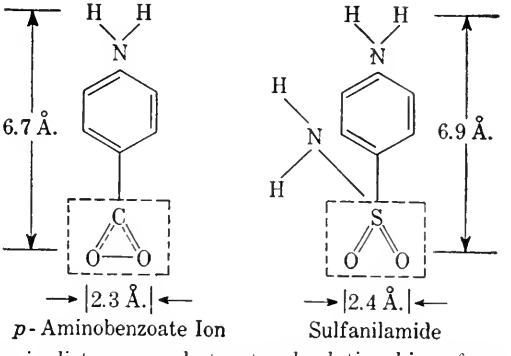


FIG. 46. Interatomic distances and structural relationships of *p*-aminobenzoate ion and sulfanilamide. (Courtesy of Roblin, *Chem. Eng. News* **27**: 3624, 1949. Published by permission of American Chemical Society.)

In spite of the large number of compounds which have been tested for antagonism, it is not possible to specify exactly what changes in metabolite molecules are required to produce antagonists. A single modification of a metabolite molecule is more likely to produce an antagonist than two or more changes in structure. This is to be expected, for an antagonist must closely resemble the corresponding metabolite. Replacing a carboxyl group with a sulfonic-acid group has been effective in many instances.

The specific action of enzymes has been likened to the relation of a lock and its key. Unless an enzyme and a substrate molecule are related in this fashion, no reaction will take place. A modern diagrammatic representation of the lock-and-key simile is shown in Fig. 47. The mechanism of competitive inhibition may be visualized by referring to this figure. Metabolite antagonists may be thought of as "wrong" keys, which jam the lock mechanism. As long as a false key is in the lock, it prevents the true key from entering and opening the lock.

Compounds which resemble coenzymes in structure compete for the active surface of apoenzymes. Because of similarity in structure, an apoenzyme–foreign molecule complex, or pseudoholoenzyme is formed. Such a pseudoenzyme is unable to function. The reversal of enzyme inhibition in such instances is caused by the addition of more coenzyme molecules. The argument is the same when substrate analogues are involved. For example, 3-fluorophenylalanine inhibits the utilization

of phenylalanine (a normal metabolite) by *Neurospora crassa* (Mitchell and Neimann, 1947).

The effect of an antagonist will depend upon the concentration of the normal metabolite present in the medium and cells and upon the organism. In general, enzymes have a greater affinity for metabolites than for antimetabolites. Since both metabolite and antagonist compete for the same enzyme, the amount of inhibition will depend upon the *relative concentrations* rather than upon the absolute amounts of these compounds present. The amount of an inhibitor required to reduce the

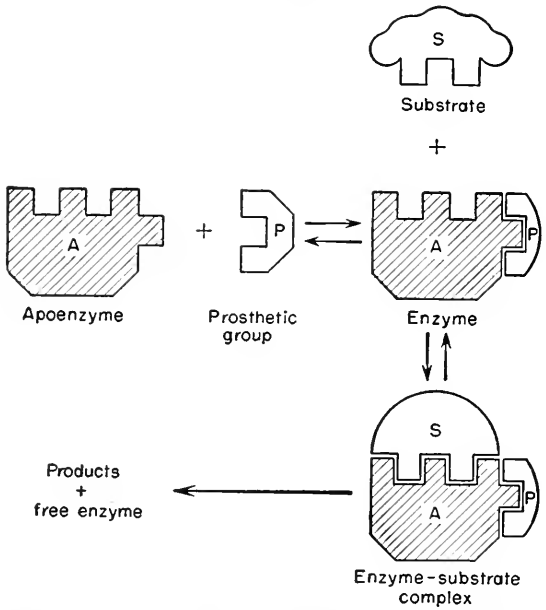


FIG. 47. A diagrammatic illustration of Fischer's simile that an enzyme and its substrate are related as are a lock and its key. (Courtesy of McElroy, *Quart. Rev. Biol.* **22**: 26, 1947. Published by permission of The Williams & Wilkins Company.)

amount of growth to one-half will depend upon the ratio of inhibitor and metabolite present. In simple instances, at least, this ratio is equal to a constant and is called the *inhibition constant*, or *index*. The amounts of sulfadiazine and *p*-aminobenzoic acid required to reduce the amount of growth of *Streptococcus faecalis* R to one-half the normal value gave an inhibition index of 333 (Lampen and Jones, 1946).

The inhibition index is valid only for the particular conditions used in an experiment and for the particular strains of the organism used. In the case of self-sufficient organisms the use of an amount of inhibitor less than that required for total inhibition will only decrease the rate of growth, and thus the inhibition index will change with the time of incuba-

tion. This is due to the synthesis of the metabolite by the organism. Sulfanilamide inhibits the growth of *Aspergillus niger*, but the fungus overcomes this inhibition as the time of incubation is prolonged (Hartelius, 1946). The concentration of a metabolite in the control cultures should be less than the amount which allows maximum growth, because of the nonlinear response of an organism to the metabolite at high concentrations.

The composition of the medium is an important consideration in any investigation of metabolite antagonism. If adequate amounts of a natural metabolite are present, the action of an inhibitor may be overlooked. Synthetic media should be used. The composition of the medium used may also affect the action of an inhibitor in another way. If metabolite *A* is transformed into metabolite *B* by an organism, the presence of metabolite *B* in sufficient amount for optimum growth may be expected to nullify any amount of an antagonist for metabolite *A*. An antagonist of metabolite *B*, however, would exhibit normal competitive inhibition. Shive and Macow (1946) have pointed out that, by the use of a suitable series of inhibitors, it is possible to follow the transformations of a given metabolite step by step. These authors designate this use of metabolite antagonists as *inhibition analysis*. Rydon (1948) found *Bacterium typhosum* to synthesize tryptophane by the following steps: anthranilic acid → indole → tryptophane. The 2- and 4-methylanthranilic acids were potent inhibitors against anthranilic acid but not against indole or tryptophane. Certain analogues of indole and tryptophane were inhibitors of these metabolites.

In discussing metabolite antagonists in a general way, it should be borne in mind that these compounds may inhibit only certain organisms, or a particular organism only under certain conditions. For example, desthiobiotin is a biotin antagonist for *Ceratostomella pini* and *Lactobacillus casei*, while this compound replaces biotin for many strains of *Saccharomyces cerevisiae* (Lilly and Leonian, 1944). Woolley (1944, 1946) is of the opinion that the established facts of inhibition and reversal are more important than the hypotheses which are adopted to explain these phenomena. However, the concept of competitive metabolite antagonism has been very useful in correlating a vast amount of experimental work in apparently unrelated fields.

ANTIVITAMINS

Antivitamins are known for all the water-soluble vitamins which have been synthesized and for at least one of the fat-soluble vitamins (vitamin K).

p-Aminobenzoic acid antagonists. When the sulfonamides were introduced into medicine, it was quickly found that serum and other natural

products antagonized the inhibitive action of sulfanilamide on the growth of certain bacteria. Rubbo and Gillespie (1940) discovered that *p*-aminobenzoic acid was a growth factor for certain bacteria. Woods (1940) found that *p*-aminobenzoic acid in low concentration overcame sulfanilamide inhibition. A general theory was proposed by Fildes (1940) to explain the antagonism between metabolites and compounds having closely related structures.

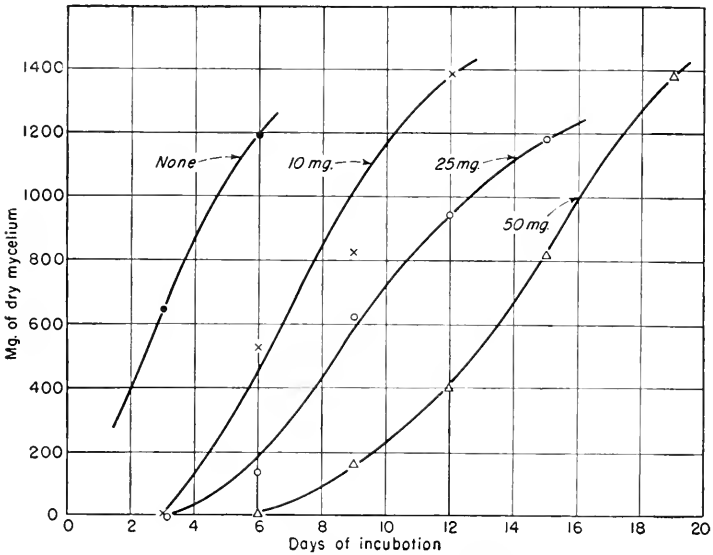


FIG. 48. The effect of various concentrations of sulfanilamide (amounts per flask) upon the time of spore germination and upon the rate and amount of growth of *Aspergillus niger* in flasks containing 55 ml. of sucrose–ammonium sulfate medium at 32°C. (Drawn from the data of Hartelius, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.* **24:** 181, 1946.)

Sulfanilamide was first considered to be antagonized by *p*-aminobenzoic acid, rather than the reverse. This was due to the discovery of the therapeutic value of sulfanilamide before it was known that *p*-aminobenzoic acid was a vitamin. The structural relation between these compounds has already been noted. The literature dealing with the sulfonamides is abundant, but most of it relates to bacteria and medicine. Relatively few papers have been published on the effects of these compounds on the growth of fungi.

Hartelius (1946) investigated the effect of sulfanilamide upon the growth of *Aspergillus niger* and found that the amount of inhibition was dependent upon the amount of inoculum used, the concentration of sulfanilamide in the medium, and the time of incubation. The curves in Fig. 48 illustrate the effect of time of incubation on inhibition, a factor which is too often overlooked in experiments of this kind. The curves

in Fig. 48 indicate that *A. niger* synthesizes either *p*-aminobenzoic acid or some other compound which reverses the inhibitory action of sulfanilamide. When *p*-aminobenzoic acid was added to the medium, sulfanilamide no longer inhibited the growth of *A. niger* (Hartelius and Roholt, 1946). Other fungi have been shown to react like *A. niger* when cultured in media containing sulfanilamide (Fourneau *et al.*, 1936).

It has been assumed that self-sufficient fungi require the same vitamins as the deficient species. The synthesis of a vitamin may suggest its need but does not demonstrate it. Antivitamins (or other antimetabolites) provide a way of demonstrating the need of self-sufficient fungi for the vitamins they synthesize. Thus *A. niger* requires *p*-aminobenzoic acid just as *Rhodotorula aurantica* does, but this need can be demonstrated only in the presence of a specific reversible inhibitor such as sulfanilamide. This technique offers a possible way of discovering new vitamins and other metabolites. If a compound inhibits growth, it is worth while to search for compounds which overcome this inhibition reversibly.

For most purposes sulfanilamide has been replaced by other sulfonamides. However, sulfanilamide appears to be the most active sulfonamide against fungi. For a review of the clinical aspects of the sulfonamides in mycoses and for literature citations, see Wolf (1947).

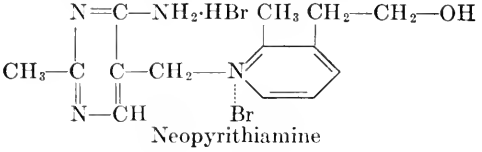
Stoddard (1947) has reported the sulfonamides to be of some value in controlling the X disease of peach (a virus). Addition of *p*-aminobenzoic acid lessened the effectiveness of the treatment.

It is recognized that the simple Woods-Fildes theory of competitive inhibition is inadequate to explain completely the mechanism of sulfonamide therapy. In vivo the environment is much more complex than in simple laboratory media. For further information and references to the literature, see Sevag *et al.* (1945) and Mudd (1945).

Thiamine antagonists. Thiamine may be inactivated by an enzyme, thiaminase, which is found in fish viscera (Sealock *et al.*, 1943) and probably occurs in other organisms. Foxes which are fed raw fish may develop a thiamine-deficiency disease (Chastek paralysis). The mode of inactivation was further investigated by Krampitz and Woolley (1944), who found that thiamine was destroyed by a process of enzymatic hydrolysis whereby the thiazole and pyrimidine moieties were formed. *Mucor ramannianus* (thiazole-deficient) and *Endomyces vernalis* (pyrimidine-deficient) were used as test organisms in the preliminary work. Another thiamine antagonist of unknown nature has been reported to occur in bracken fern (Weswig *et al.*, 1946).

Pyrithiamine, an analogue of thiamine, has been used in studies of competitive thiamine inhibition. Unfortunately, the exact structure of this compound is not known. In papers published before 1949 it was assumed that pyrithiamine had the structure now assigned to neopyri-

thiamine (Wilson and Harris, 1949). Pyrithiamine appears to differ from neopyrithiamine in the amount of pyrimidine moiety it contains. The formula for neopyrithiamine is given below.



Neopyrithiamine

Robbins (1941) found low concentrations of pyrithiamine to replace thiamine for *Pythiomorpha gonapodyoides* (pyrimidine-deficient), while high concentrations inhibited growth. Pyrithiamine did not replace thiamine for *Phycomyces blakesleeanus* (requires both moieties) or *Phytophthora cinnamomi* (requires intact thiamine). The inhibition of growth of various fungi and bacteria caused by pyrithiamine was overcome by increasing the thiamine content of the medium (Woolley and White, 1943). The inhibition index is given in Table 39. The efficiency of pyrithiamine as a thiamine antagonist is related to the specific vitamin requirements of the organisms tested. The inhibition index was low for those species which require intact thiamine, intermediate for those which require either or both moieties, and high for self-sufficient species.

Table 39. The Efficiency of Pyrithiamine as an Inhibitor of Fungus and Bacterial Growth

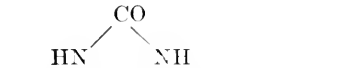
(Woolley and White, *Jour. Exptl. Med.* **78,** 1943.)

Organism	Inhibition index $\frac{\text{pyrithiamine}}{\text{thiamine}}$	Thiamine requirement
Ceratastomella fimbriata	7	Intact thiamine
C. penicillata	10	Intact thiamine
Phytophthora cinnamomi	12	Intact thaimine
Chalaropsis thielavioides	11	Intact thaimine
Endomyces vernalis	130	Pyrimidine
Mucor ramannianus	800	Thiazole
Saccharomyces cerevisiae	800	Both moieties
Neurospora crassa	400,000	None
Lactobacillus arabinosus	40,000	None
L. casei	5,000,000	None

Pyrithiamine was found to inhibit sporulation of *Ceratostomella fimbriata, Choanephora cucurbitarum,* and *Chaetomium convolutum* (Lilly and Barnett, 1948). This inhibition was overcome by thiamine. Pyrithiamine was reported to be a more efficient antagonist for diphosphothiamine than for thiamine when *Penicillium digitatum* was used as a test organism (Sarett and Cheldelin, 1944).

Pyrithiamine causes a thiamine deficiency disease in mice, which may be cured or prevented by the administration of sufficient thiamine (Woolley and White, 1943). Neopyrithiamine is reported to be four times as active as pyrithiamine for the rat (Wilson and Harris, 1949).

Biotin antagonists. Many biotin vitamers are known which are highly specific. The efficiency of an antibiotin in some instances may depend upon whether biotin or one of its vitamers is the competing metabolite. The formulas of two of the compounds are given below. Compare with the formulas of biotin and desthiobiotin given in Chap. 9.



Sulfonic-acid analogue of desthiobiotin

Imidazolidonecaproic acid

Desthiobiotin and imidazolidonecaproic acid differ only by a methyl group. Desthiobiotin was found to act as a biotin vitamer for *Saccha, romyces cerevisiae* and other yeasts (Dittmer *et al.*, 1944; Lilly and Leonian-1944), while imidazolidonecaproic acid is an antibiotin for *S. cerevisiae* (Dittmer and Du Vigneaud, 1944). Both compounds are antibiotins for *Lactobacillus casei*. The sulfonic-acid analogue of desthiobiotin was shown by Duschinsky and Rubin (1948) to be more active against desthiobiotin and oxybiotin than against biotin for *S. cerevisiae*. The replacement of a carboxyl group by a sulfonic-acid group appears to be a rather general method of changing a metabolite into an antagonist. Further examples of this will be cited in connection with pantothenic and amino-acid antagonists.

Egg white contains a specific protein which combines with biotin and thus renders this vitamin inactive. This inactivity is due to the molecular size of the avidin-biotin complex, which prevents its absorption by organisms. Raw egg white may be used to produce experimental biotin deficiency in animals. Avidin is no longer active after heating, and bound biotin is released by this treatment. This specific protein has been used to separate biotin vitamers into two groups, for avidin combines only with those compounds which have an intact urea ring structure. The papers of Eakin *et al.* (1941) and Burk and Winzler (1943) may be consulted for further details.

Pantothenic acid antagonists. Yeasts are the only fungi which have been reported to be deficient for pantothenic acid, and in most instances β-alanine replaces the intact vitamin molecule. One of the commonly studied pantothenic acid antagonists is the compound called pantoyltaurine. The formulas for pantothenic acid and pantoyltaurine are given below

$HO—CH_2—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—COOH$
Pantothenic acid

$HO—CH_2—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—SO_3H$
Pantoyltaurine

Pantoyltaurine is the sulfonic-acid analogue of pantothenic acid. Snell (1941) studied the competitive inhibition of yeast growth by pantoyltaurine and found that this compound was effective when pantothenic acid was the metabolite supplied in the medium but that pantoyltaurine did not compete with β-alanine. The data in Table 40 illustrate this difference.

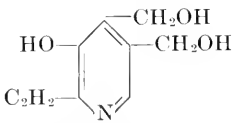
TABLE 40. THE EFFECT OF PANTOYLTAURINE ON THE GROWTH OF *Saccharomyces cerevisiae* IN THE PRESENCE OF PANTOTHENIC ACID AND β-ALANINE

Inoculum used, 0.02 mg., time of incubation, 16 hr. (Snell, *Jour. Biol. Chem.* **141**, 1941.)

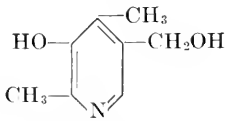
Calcium pantothenate, μg/10 ml.	Sodium salt of pantoyltaurine, μg/10 ml.	Moist cells, mg/10 ml.	β-Alanine, μg/10 ml.	Sodium salt of pantoyltaurine, μg/10 ml.	Moist cells, mg./10 ml.
0.0	0	0.3	0.0	0	0.03
0.5	0	6.6	0.3	0	2.8
0.5	1,000	2.9	0.5	0	5.5
0.5	5,000	0.4	0.3	1,000	3.0
0.5	10,000	0.3	0.3	10,000	3.0
30.0	10,000	6.6	0.5	5,000	5.5
			0.5	10,000	5.7

The synthesis of pantothenic acid via β-alanine by *Escherichia coli* is inhibited by cysteic acid (sulfonic-acid analogue of aspartic acid). This inhibition is reversed by β-alanine or pantothenic acid (Ravel and Shive, 1946). For further information concerning other pantothenic acid and other antagonists, the review of Roblin (1946) should be consulted.

Pyridoxine antagonists. Some of the pyridoxine analogues studied by Robbins and Ma (1942) inhibited the growth of *Ceratostomella ulmi*. This inhibition was reversed by additional pyridoxine. The substitution of an ethyl group for the methyl group of pyridoxine produced an antagonist for *C. ulmi*, but ethyl pyridoxine was as active for excised tomato roots as pyridoxine itself. The above authors suggest that ethyl pyridoxine might be a chemotherapeutic agent for the Dutch elm disease. The formulas of ethyl pyridoxine and desoxypyridoxine are given below:



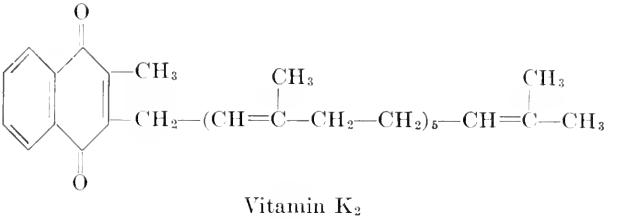
Ethyl pyridoxine



Desoxypyridoxine

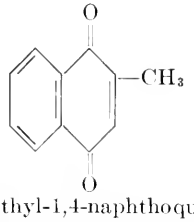
Martin *et al.* (1948) found desoxypyridoxine to be slightly more effective against pyridoxal than pyridoxine, when *Saccharomyces cerevisiae* was used.

Vitamin K antagonists. There are at least two naturally occurring compounds which have vitamin K activity. Certain synthetic analogues are used in medicine to replace the natural vitamins. All these compounds are substituted 1,4-naphthoquinones. The structural formula for vitamin K_2 is given below:

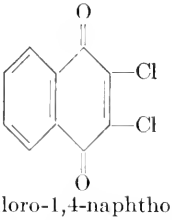


Vitamin K_2

Horsfall (1945) has reported 2-methyl-1,4-naphthoquinone to be a weak fungicide, although this compound replaces natural vitamin K in medicine. On the other hand, 2,3-dichloro-1,4-naphthoquinone (Phygon) is a potent fungicide (Ter Horst and Felix, 1943).



2-Methyl-1,4-naphthoquinone



2,3-Dichloro-1,4-naphthoquinone

Phygon may act as a fungicide by virtue of combination of the quinone with free amine or sulfhydryl groups. This mechanism probably inactivates certain enzymes noncompetitively. On the other hand, Phygon is structurally related to vitamin K, and a competitive type of inhibition should also be possible. Woolley (1945) investigated the inhibitory effect of 2,3-dichloro-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone on the growth of *Saccharomyces cerevisiae* and *Endomyces vernalis*. The first compound was more toxic than the second. In less than toxic concentrations, the second compound partially overcame the toxicity of the first. The amount of 2,3-dichloro-1,4-naphthoquinone required to inhibit yeast (half maximum growth) was 1.7 μg per liter, while 230 μg of 2-methyl-1,4-naphthoquinone were required to produce the same amount of inhibition. Some of Woolley's data are presented in Table 41. Many potent antimalarial drugs are 1,4-naphthoquinone derivatives (Fieser *et al.*, 1948).

It has been assumed in our discussion of the effects of antagonists on

organisms that antimetabolites are active by virtue of interfering with various enzymatic processes. It is also interesting to note that competitive inhibition has been demonstrated with isolated enzyme systems. Schopfer and Grob (1949) found the action of urease to be inhibited by 2-chloro-1,4-naphthoquinone. Most of the activity was restored by the addition of 2-methyl-1,4-naphthoquinone (vitamin K_3).

TABLE 41. THE REVERSAL OF INHIBITION CAUSED BY 2,3-DICHLORO-1,4-NAPHTHOQUINONE BY 2-METHYL-1,4-NAPHTHOQUINONE

Test fungus, *Saccharomyces cerevisiae*. Concentration of 2,3-dichloro-1,4-naphthoquinone, 0.005 μg/ml. (Woolley, *Proc. Soc. Exptl. Biol. Med.* **60**, 1945. Published by permission of the Society for Experimental Biology and Medicine.)

2-methyl-1,4-naphthoquinone, μg/ml.	Turbidity (100 = no growth)
0.0	93
0.04	60
0.02	68
0.01	77
0.005	85

Other vitamin antagonists. The sulfonic-acid analogue of nicotinic acid inhibits the growth of certain bacteria (McIlwain, 1940). Apparently this analogue has not been tested in nicotinic acid–deficient fungi. Woolley (1946*a*) has reported maize to contain a "pellagragenic" agent which may tentatively be considered as a naturally occurring anti-nicotinic-acid factor.

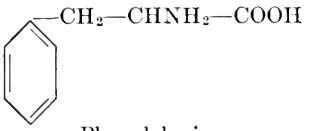
Among the recently developed insecticides, γ-hexachlorocyclohexane is of considerable value. Kirkwood and Phillips (1946) have shown that the growth of *Saccharomyces cerevisiae* is inhibited by this compound, and that the inhibition is overcome by *meso*-inositol. The other isomers of hexachlorocyclohexane were not very effective inhibitors of yeast growth; neither are they of much value as insecticides. These observations point to competitive inhibition as a possible mechanism of insecticidal action of this compound.

AMINO-ACID ANTAGONISTS

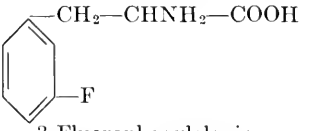
Organisms must either synthesize or obtain from exogenous sources the different amino acids they require for the synthesis of protein. Antimetabolites which antagonize the synthesis or utilization of essential amino acids would have a profound effect upon growth or other functions of organisms. The role of amino acids is not confined to the synthesis of proteins but extends to the synthesis of other essential metabolites. An amino-acid antagonist may act in two ways, (1) by inhibiting protein synthesis and (2) by inhibiting the synthesis of essential metabolites which are derived from amino acids, either directly or indirectly. If an amino acid functions in more than one way, the action of an amino-

acid antagonist may be overcome, at least in part, by the action of secondary metabolites as well as the primary metabolite. The toxic effect of 3-acetylpyridine on rats is reversed by either nicotinic acid amide or tryptophane (Woolley, 1945*a*).

Analogues. Mitchell and Niemann (1947) found that the halogenated derivatives of phenylalanine and tyrosine competitively inhibit growth of the wild strain of *Neurospora crassa* (Table 42). The most effective of these inhibitors was 3-fluoro-DL-phenylalanine. The structural formulas for this analogue and the natural metabolite are shown below:



Phenylalanine



3-Fluorophenylalanine

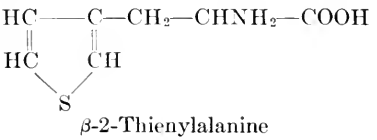
TABLE 42. INHIBITION OF GROWTH OF *Neurospora crassa* BY SOME HALOGENATED ALPHA-AMINO ACIDS

Basal medium contained 30 mg. of DL-phenylalanine or 20 mg. L-tyrosine per liter depending upon the antagonist tested. (Mitchell and Niemann, *Jour. Am. Chem. Soc.* **69**, 1947. Published by permission of the American Chemical Society.)

Compound	Mg./ml. for 50% inhibition	Moles inhibitor / Moles amino acid
3-Fluoro-DL-phenylalanine	0.04	1.2
3-Fluoro-DL-tyrosine	0.23	10.5
3-Fluoro-L-tyrosine	0.15	6.8
3-Fluoro-D-tyrosine	0.41	18.5

The other halogen derivitives (chloro, bromo, and iodo) were less effective inhibitors. 3-Fluorophenylalanine was shown to be an effective inhibitor for various other fungi and bacteria.

The effect of β-2-thienylalanine on the growth of a strain *Saccharomyces cerevisiae* and certain bacteria has been studied by Ferger and Du Vigneaud (1948). The formula for this thiophene analogue of phenylalanine is given below:



β-2-Thienylalanine

Only the L isomer is active in competing with phenylalanine. The replacement of divalent sulfur (—S—) by a vinylene group (—CH=CH—), or vice versa, often leads to the production of an antimetabolite. As another example, the effect of replacing sulfur in cysteine by radicals

containing the vinylene group may be cited. Dittmer *et al.* (1948) found methallylglycine, allylglycine, and crotylglycine to inhibit the growth of *Saccharomyces cerevisiae* and *Escherichia coli*. The effects of these three antimetabolites on the growth of yeast are shown in Fig. 49.

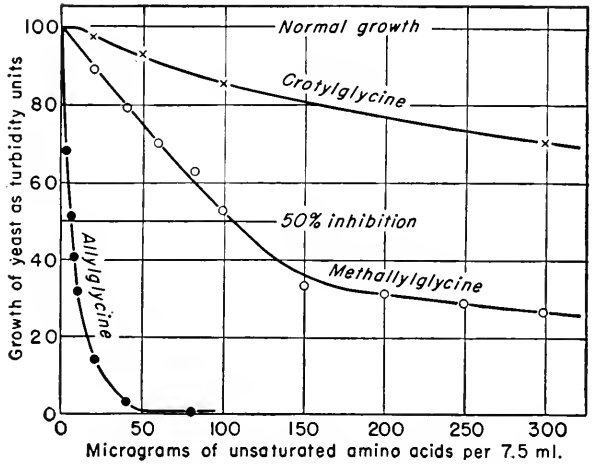
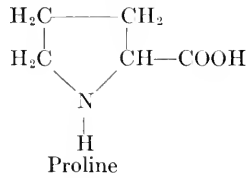
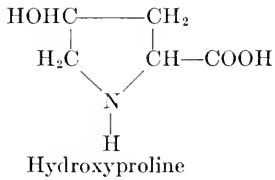


FIG. 49. The inhibition of growth of *Saccharomyces cerevisiae*, strain 139, by DL-allylglycine, DL-methallylglycine, and DL-crotylglycine. (Courtesy of Dittmer, Goering, Goodman, and Cristol, *Jour. Am. Chem. Soc.* **70**: 2501, 1948. Published by permission of the American Chemical Society.)

Natural amino acids. Antagonism among the amino acids is not limited to competitive inhibition between naturally occurring amino acids and their analogues. Robbins and McVeigh (1946) found hydroxyproline to inhibit the growth of several dermatophytes: *Trichophyton mentagrophytes*, *T. gypseum* (granular form), *T. purpureum*, *Epidermophyton floccolosum*, and *Microsporum canis*. This inhibition was overcome by proline. The relationship of these two naturally occurring amino acids is shown below:



Proline



Hydroxyproline

Low concentrations of hydroxyproline stimulated growth of *Trichophyton purpureum*, while higher concentrations inhibited growth. Addition of hydroxyproline to a glucose-asparagine medium increased the growth of *Polyporus squamosus*. Hydroxyproline was without effect on the growth of 19 other species of fungi. Whether amino-acid antagonisms may limit the nitrogen utilization of natural mixtures of these

compounds is unknown, but the possibility of inhibition should be kept in mind when only a few amino acids are used in a medium. The effect of any single compound upon a fungus may be modified by the other constituents of the medium.

Hartelius (1946*a*) found glutamic and aspartic acids, glutamine, and asparagine to inhibit the growth of a strain of yeast when suboptimumal amounts of β-alanine were used in the medium. These amino acids did not inhibit growth when pantothenic acid was used. In fact, these compounds stimulated growth under these conditions. The inhibitory effect in the presence of β-alanine was overcome by increasing the concentration of this provitamin. To obtain maximum growth in the presence of 50 mg. of glutamic acid per flask (55 ml.), twenty times as much β-alanine was required as when glutamic acid was omitted from the medium. Hartelius attributed this effect to the combination of β-alanine and glutamic acid to form an inactive dipeptide.

Among the naturally occurring amino acids, L-canavanine is found free in jack beans. Canavanine is an analogue of arginine; the formulas are shown below.

$$H_2N{-}C(=NH){-}NH{-}CH_2{-}CH_2{-}CH_2{-}CH(NH_2){-}COOH$$

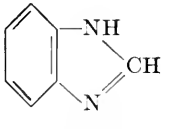
Arginine

$$H_2N{-}C(=NH){-}NH{-}O{-}CH_2{-}CH_2{-}CH(NH_2){-}COOH$$

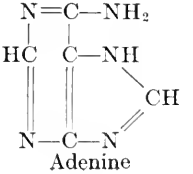
Canavanine

Horowitz and Srb (1948) studied the effect of canavanine on three wild-type strains of *Neurospora* and found one strain to be inhibited completely by concentrations greater than 1.25 mg. per liter; another strain was only partially inhibited, while the third strain was tolerant. Genetic analysis indicated that tolerance and susceptibility segregated by alternative forms of a single gene. L-Arginine was effective in overcoming canavanine toxicity, while L-lysine was less effective. Three molecules of arginine overcame one molecule of canavanine in the strain of *Neurospora* most sensitive to this inhibitor. A similar competitive inhibition between canavanine and arginine in various bacteria has also been observed (Volcani and Snell, 1948).

Other metabolite antagonists. Woolley (1944*a*) found benzimidazole to inhibit the growth of *Saccharomyces cerevisiae* and *Endomyces vernalis*. This inhibition was overcome by adenine and guanine. The structural relationship between benzimidazole and adenine is shown below:



Benzimidazole



Adenine

DEVELOPMENT OF FASTNESS

An organism which has become tolerant, or resistant, to an inhibitor (analogue, drug, antibiotic, etc.) after exposure is said to be fast, or more specifically pyrithiamine-fast, sulfanilamide-fast, or penicillin-fast, as the case may be. Fastness is a very common phenomenon, although it appears to have been but little studied in fungi. It is an important factor which limits the use of many antibiotics and the sulfonamides in medicine. This phase of fungus physiology deserves more attention than it has received. It is conceivable that the prolonged use of a single fungicide to control a fungus pathogen could lead to the development, or selection, of a strain which would be relatively tolerant to the effect of the fungicide. Such findings do not appear to have been reported from field studies, but this possibility should be kept in mind.

Fungi do become fast to various antagonists. Woolley (1944*b*), by repeatedly subculturing *Endomyces vernalis* in a medium containing pyrithiamine, developed a strain which withstood twenty-five times the concentration of pyrithiamine which served to reduce the growth of the parent strain to half the maximum. In this instance, fastness was correlated with the ability of the pyrithiamine-fast strain to cleave the inhibitor molecule into its cyclic moieties. Thus, the development of pyrithiamine fastness may be ascribed to the formation of an adaptive enzyme which destroyed the antagonist. *Escherichia coli*, which is not inhibited by pyrithiamine, also hydrolyzed this compound. These results indicate that adaptive enzymes may play a role in the development of fastness.

In addition to resistance or fastness which develops in organisms cultured in the presence of an inhibitor, it has been found recently that various bacteria not only develop resistance but may develop strains which are actually *dependent* upon the presence of the "inhibitor" before they can grow. Yegian *et al.* (1949) have found that culturing *Mycobacterium tuberculosis* in the presence of streptomycin gave rise to strains which were fast to this antibiotic and also produced strains which cannot grow unless streptomycin is present in the medium.

SUMMARY

The normal utilization of a metabolite may be prevented or inhibited in three ways: (1) destruction or removal in an unavailable combination of a metabolite; the enzymatic hydrolysis of thiamine and the combination of biotin with avidin are representative examples of this mode of inactivation; (2) the noncompetitive inhibition of various enzymes by such compounds as iodoacetate, cyanide, and azide; (3) competitive inhibition due to metabolite antagonists. This type of inhibition is

overcome by increasing the concentration of the normal metabolite. Antagonists are known which inhibit the functioning of vitamins, amino acids, and other metabolites.

It is postulated that a metabolite and its antagonists compete for the active surface of specific enzymes. The ratio of inhibitor to metabolite required to reduce growth to one-half its normal value is called the inhibition index. Effective inhibitors have small inhibition indexes. The same compound may act as an antagonist for some fungi and as a metabolite for others; *e.g.*, desthiobiotin. The medium used for investigating inhibition is important, for the presence of a normal metabolite, or a secondary metabolite derived from it, may prevent inhibition. A given compound may be considered as an antagonist, but it is only an antagonist for certain species, and then only under certain conditions. Organisms may acquire a tolerance or resistance to inhibitory agents and become fast. In extreme instances they become dependent upon the inhibitor, which then acts as a kind of growth factor.

The competitive nature of many inhibitions is firmly established. In most instances there is a close structural relation between a metabolite and its antagonists. The theories which have been advanced to explain these phenomena have been useful in correlating the results of research and for increasing our insight into metabolic processes.

REFERENCES

Burk, D., and R. J. Winzler: Heat-labile, avidin-uncombinable, species-specific and other vitamers of biotin, *Science* **97**: 57–60, 1943.

Dittmer, K., and V. du Vigneaud: Antibiotins, *Science* **100**: 129–131, 1944.

Dittmer, K., H. L. Goering, I. Goodman, and S. J. Cristol: The inhibition of microbiological growth by allylglycine, methallylglycine and crotylglycine, *Jour. Am. Chem. Soc.* **70**: 2499–2501, 1948.

Dittmer, K., D. B. Melville, and V. du Vigneaud: The possible synthesis of biotin from desthiobiotin by yeast and the antibiotin effect of desthiobiotin for *L. casei*, *Science* **99**: 203–204, 1944.

Duschinsky, R., and S. H. Rubin: The synthesis and biological activity of 4-methyl-5-(ε-sulfoamyl)-2-imidazolidone, a sulfonic acid analog of desthiobiotin, *Jour. Am. Chem. Soc.* **70**: 2546–2547, 1948.

Eakin, R. E., E. E. Snell, and R. J. Williams: The concentration and isolation of avidin, the injury-producing protein in raw egg white, *Jour. Biol. Chem.* **140**: 535–543, 1941.

Ferger, M. F., and V. du Vigneaud: The microbial growth inhibition produced by optical isomers of β,2-thienyl-alanine, *Jour. Biol. Chem.* **174**: 241–246, 1948.

Fieser, L. F., *et al.*: Naphthoquinone antimalarials. I. General survey, *Jour. Am. Chem. Soc.* **70**: 3151–3155, 1948.

Fildes, P.: A rational approach to research in chemotherapy, *Lancet* **1**: 955–957, 1940.

Fourneau, E., J. Tréfouël, J. Tréfouël. F. Nitti, and B Bovet: Action du *para*-aminophenylsulfamide sur les moisisseures, *Compt. rend. soc. biol.* **122**: 652–654, 1936.

Hartelius, V.: Antivitamin effect of sulfanilamide on *Aspergillus niger*, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.* **24**: 178–184, 1946.

Hartelius, V.: Glutamic acid, aspartic acid, asparagine and glutamine as anti-growth substances for β-alanine, *Compt. rend. trav. lab. Carlsberg. Sér. physiol.* **24**: 185–222, 1946*a*.

Hartelius, V., and K. Roholt: Effect of marfanil on *Streptobacterium planatarum*, *Saccharomyces cerevisiae* and *Aspergillus niger*, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.* **24**: 163–171, 1946.

Horowitz, N. H., and A. M. Srb: Growth inhibition of *Neurospora* by canavanine and its reversal, *Jour. Biol. Chem.* **174**: 371–378, 1948.

Horsfall, J. G.: Fungicides and Their Action, Chronica Botanica Co., Waltham, 1945.

Kirkwood, S., and P. H. Phillips: The anti-inositol effect of γ-hexachlorocyclohexane, *Jour. Biol. Chem.* **163**: 251–254, 1946.

Krampitz, L. O., and D. W. Woolley: The manner of inactivation of thiamine by fish tissue, *Jour. Biol. Chem.* **152**: 9–17, 1944.

Lampen, J. O., and M. J. Jones: The antagonism of sulfonamide inhibition of certain lactobacilli and entrococci by pteroylglutamic acid and related compounds, *Jour. Biol. Chem.* **166**: 435–448, 1946.

Lilly, V. G., and H. L. Barnett: The effect of pyrithamin upon sporulation of three thiamin-deficient fungi, *Am. Jour. Botany* **35**: 801, 1948.

Lilly, V. G., and H. L. Leonian: The anti-biotin effect of desthiobiotin, *Science* **99**: 205–206, 1944.

Martin, G. J., S. Avakian, and J. Moss: Studies of pyridoxine displacement, *Jour. Biol. Chem.* **174**: 495–500, 1948.

★McElroy, W. D.: The mechanism of inhibition of cellular activity by narcotics, *Quart. Rev. Biol.* **22**: 25–58, 1947.

McIlwain, H.: Pyridine-3-sulphonic acid and its amide as inhibitors of bacterial growth, *Brit. Jour. Exptl. Path.* **21**: 136–147, 1940.

McIlwain, H.: Interrelations in microorganisms between growth and metabolism of vitamin-like substances, *Advances in Enzymol.* **7**: 409–460, 1947.

Mitchell, H. K., and C. Niemann: The competitive inhibition of the metabolism of α-amino acids by their halogenated analogs, *Jour. Am. Chem. Soc.* **69**: 1232, 1947.

Mudd, S.: Can chemotherapy be extended to include intra-cellular disease agents?, *Jour. Bact.* **49**: 527–537, 1945.

Ravel, J. M., and W. Shive: Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. IV. Prevention of pantothenic acid synthesis by cysteic acid, *Jour. Biol. Chem.* **166**: 407–415, 1946.

Robbins, W. J.: The pyridine analog of thiamin and the growth of fungi, *Proc. Natl. Acad. Sci. U.S.* **27**: 419–422, 1941.

Robbins, W. J., and R. Ma: Specificity of pyridoxine for *Ceratostomella ulmi*, *Bull. Torrey Botan. Club* **69**: 342–352, 1942.

Robbins, W. J., and I. McVeigh: Effect of hydroxyproline on *Trichophyton mentagrophytes* and other fungi, *Am. Jour. Botany* **33**: 638–647, 1946.

Roblin, R. O., Jr.: Metabolite antagonists, *Chem. Revs.* **38**: 255–377, 1946.

★Roblin, R. O., Jr.: Metabolite antagonists. The relation between structure and biological activity, *Chem. Eng. News* **27**: 3624–3629, 1949.

Rubbo, S. D., and J. M. Gillespie: *Para*-aminobenzoic acid as a bacterial growth factor, *Nature* **146**: 838–839, 1940.

Rydon, H. N.: Anthranilic acid as an intermediate in the biosynthesis of tryptophane by *Bact. typhosum*, *Brit. Jour. Exptl. Path.* **29**: 48–57, 1948.

Sarett, H. P., and V. H. Cheldelin: Inhibition of utilization of thiamine and diphosphothiamine for the growth of microorganisms, *Jour. Biol. Chem.* **156**: 91–100, 1944.

Schopfer, W. H., and E. C. Grob: Ueber den Einfluss von 1,4-Naphtochinonderivaten mit Vitamin-K- oder Antivitamin-K-Character auf die Urease, *Helv. Chim. Acta* **32**: 829–838, 1949.

Sealock, R. R., A. H. Livermore, and C. A. Evans: Thiamine inactivation by the fresh-fish or Chastek-paralysis factor, *Jour. Am. Chem. Soc.* **65**: 935–940, 1943.

Sevag, M. G., R. A. Richardson, and J. Henry: Studies on the action of sulfonamides on the respiration and growth of bacteria. IIB. Effect of serum on the inhibition of respiration and growth of *Pneumococcus*, Type I, and *Staphylococcus aureus* by sulfonamides and *p*-aminobenzoic acid, *Jour. Bact.* **49**: 139–147, 1945.

★Shive, W., and J. Macow: Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. I. Some transformations involving aspartic acid, *Jour. Biol. Chem.* **162**: 451–462, 1946.

Singer, T. P.: Enzyme inhibitors and the active groups of proteins, *Brewers Digest* **20**: 85–88 104–106, 1945.

Snell, E. E.: Growth inhibition by N-(α,γ-dihydroxy-β,β-dimethylbutyryl)taurine and its reversal by pantothenic acid, *Jour. Biol. Chem.* **141**: 121–128, 1941.

Stoddard, E. M.: The x disease of peach and its chemotherapy, *Conn. Agr. Expt. Sta. Bull.* 506, 1947.

Ter Horst, W. P., and E. L. Felix: 2,3-Dichloro-1,4-naphthoquinone, a potent fungicide, *Ind. Eng. Chem.* **35**: 1255–1259, 1943.

Volcani, B. E., and E. E. Snell: The effect of canavanine, arginine and related compounds on the growth of bacteria, *Jour. Biol. Chem.* **174**: 893–902, 1948.

Welch, A. D.: Interference with biological processes through the use of analogs of essential metabolites, *Physiol. Revs.* **25**: 687–715, 1945.

Weswig, P. H., A. M. Freed, and J. R. Haag: Antithiamine activity of plant materials, *Jour. Biol. Chem.* **165**: 737–738, 1946.

Wilson, A. N., and S. A. Harris: Synthesis and properties of neopyrithiamine salts, *Jour. Amer. Chem. Soc.* **71**: 2231–2333, 1949.

Wolf, F. T.: The action of sulfonamides and antibiotic agents on the pathogenic fungi *in* Biology of Pathogenic Fungi (edited by W. J. Nickerson), Chronica Botanica Co., Waltham, 1947.

Woods, D. D.: The relation of *p*-aminobenzoic acid to the mechanism of the action of sulphanilamide, *Brit. Jour. Exptl. Path.* **21**: 74–90, 1940.

Woolley, D. W.: Some new aspects of the relationship of chemical structure to biological activity, *Science* **100**: 579–583, 1944.

Woolley, D. W.: Some biological effects produced by benzimidazole and their reversal by purines, *Jour. Biol. Chem.* **152**: 225–232, 1944*a*.

Woolley, D. W.: Development of resistance to pyrithiamine in yeast and some observations on its nature, *Proc. Soc. Exptl. Biol. Med.* **55**: 179–180, 1944*b*.

★Woolley, D. W.: Observations on the antimicrobial action of 2,3-dichloro-1,4-naphthoquinone and its reversal by vitamins K, *Proc. Soc. Exptl. Biol. Med.* **60**: 225–228, 1945.

Woolley, D. W.: Production of nicotinic acid deficiency with 3-acetylpyridine, the ketone analogue of nicotinic acid, *Jour. Biol. Chem.* **157**: 455–459, 1945*a*.

Woolley, D. W.: Some aspects of biochemical antagonism *in* Currents in Biochemical Research (edited by D. E. Green), Interscience Publishers, Inc., New York, 1946.

Woolley, D. W.: The occurrence of a "pellagragenic" agent in corn, *Jour. Biol. Chem.* **163**: 773–774, 1946*a*.

Woolley, D. W., and A. G. C. White: Selective reversible inhibition of microbial growth with pyrithiamine, *Jour. Exptl. Med.* **78**: 489–497, 1943.

★Wright, L. D.: The significance of anti-vitamins in nutrition, *Jour. Am. Dietet. Assoc.* **23**: 289–298, 1947.

Yegian, D., V. Budd, and R. J. Vanderlinde: Streptomycin-dependent bacilli: A simple method for isolation, *Jour. Bact.* **58**: 257–259, 1949.

CHAPTER 12

THE ACTION OF FUNGICIDES

The never-ending warfare which man must wage against parasitic fungi in order to protect his crops has been ably chronicled by Large (1940). The saprophytic species which decay wood and other cellulosic materials cause great economic loss, although these species perform a necessary and indispensable role in maintaining the carbon cycle in nature. It is to man's interest and profit that the deterioration of textiles and lumber be prevented or delayed and that his crops be protected from pathogenic fungi. This is done by the use of fungicides, which either kill or inhibit the action of fungi.

By definition, an agent which kills fungi is a *fungicide*. A *fungistatic agent* merely causes inhibition. The same agent is commonly capable of producing both actions. A discussion of the terms fungicidal and fungistatic is given by McCallan and Wellman (1942). These authors point out that the fungistatic activity of an agent is broader than its fungicidal activity.

Both physical and chemical agents may be fungicidal and fungistatic. Of the physical agents, heat and ultraviolet radiation are probably most commonly used, while many chemical compounds are "toxic" to fungi. Whether an agent is fungicidal or fungistatic is primarily a matter of degree of intensity and duration of exposure. We may assume that these agents, whether chemical or physical, act directly upon certain specific enzymes or enzyme systems. If the action is less severe and may be reversed, the result is fungistasis, while if it is irreversible, the action is fungicidal. Since most of the agents employed by man are chemical compounds, much of the following discussion will be limited to the mechanism of action of these compounds.

Chemical fungicides may be applied as *eradicants* or as *protectants*. A protectant is applied to the plant or other material before the inoculum arrives at the infection court and often functions only after the fungus spore germinates. An eradicant kills the fungus already present on or in the substrate material.

The lethal action of a chemical depends upon both the concentration of the active compound or ion and the time of exposure. Species of fungi exhibit great variation in ability to resist the action of certain fungicides. Many fungi are killed by exposure to a few parts per million

of cupric ion, while a few species have been reported to grow in a saturated solution of copper sulfate (Starkey and Waksman, 1943). *There is no useful universal fungicide.*

The intelligent choice of a fungicide depends upon a number of factors, the major ones being the species of fungus to be controlled and the nature of the material to be protected. The solubility of the fungicide is of great importance. For most efficient preservation of wood or protection as a spray, a fungicide must have a low solubility in order that the protection may extend over a long period of time. For surface sterilization a highly soluble fungicide is used. When a fungicide is to be used on a living plant (or other organism), the relative sensitivity of the host and of the fungus to the fungicide must be considered. Host sensitivity limits the use of many potent fungicides. A useful fungicide must be more toxic to the fungus than to the host. For example, copper fungicides are quite toxic to cabbage, cucumber, and pea seed, while beet, eggplant, pepper, and spinach seed are relatively tolerant to copper.

Although there is an enormous accumulation of literature on fungicides, their composition, application, limitations, and economic value (see Frear, 1948, and Horsfall, 1945), relatively little has been published on the mechanism of fungicidal action. This is a practical as well as an academic question, for the intelligent use of known fungicides and the search for new and better ones are based upon a knowledge of how they act.

In the past the most important inorganic fungicides have contained compounds of copper, mercury, or sulfur. In the future, however, excellent fungicides may be made from other toxic elements. For example, cadmium is of potential interest, but the present supply is limited. In controlling fungi and other pests, there is always the danger that they will become tolerant, or fast, to a given toxicant. This means that the more susceptible individuals are killed and that a greater amount of a given fungicide is required to control the more tolerant population which is then built up. It is desirable from several viewpoints to have satisfactory reserve fungicides in the armory of the plant pathologist.

COPPER

The first copper salts to be used as fungicides were the sulfate and acetate (Prévost, 1807). These salts are soluble, and even in low concentration they are too toxic for many uses. Since all the copper is available at once, these salts are toxic to plants, especially to young parts. These soluble salts have a further disadvantage when used as a spray, for a heavy dew or rain will easily wash them off. However, these salts, especially copper sulfate, were successfully used for treating seed grain to destroy surface contaminants. This treatment was devised by Prévost to control bunt.

The next advance in copper fungicides was not until 1885, when Millardet published the formula for making the famous fungicide Bordeaux mixture. Millardet recommended that 8 kg. of copper sulfate pentahydrate (bluestone) be dissolved in 100 liters of water. This solution was then mixed with 15 kg. of quicklime slaked in 30 liters of water. The chemistry of Bordeaux mixture is more complicated than was assumed at first. Instead of cupric hydroxide, a series of basic sulfates are formed, the composition being dependent upon the ratio of copper sulfate and calcium hydroxide used (Frear, 1948). Bordeaux mixture is a copper compound or compounds of low solubility. According to Goldsworthy and Green (1936), Bordeaux mixture in equilibrium with water yields a solution containing about 4 p.p.m. of copper. However, McCallan and Wilcoxon (1936) found that well-washed 4–4–50 Bordeaux mixture was soluble only to the extent of furnishing 1 p.p.m. of copper. After this material was thoroughly dried, as in a spray film, the solubility in terms of copper decreased to 0.2 to 0.3 p.p.m. McCallan and Wilcoxon have reported a comparison between the amounts of Bordeaux mixture and copper sulfate required to inhibit the germination of 90 per cent of the spores of a few species. These data are given in Table 43.

TABLE 43. THE RELATIVE EFFICIENCY OF BORDEAUX MIXTURE AND COPPER SULFATE IN INHIBITING SPORE GERMINATION

(McCallan and Wilcoxon, *Contribs. Boyce Thompson Inst.* 6, 1936.)

Species	Cu, mg./liter, for LD 90	
	Bordeaux mixture	Copper sulfate
Uromyces caryophyllinus	180	1.74
Sclerotinia fructicola	120	1.20
Botrytis paconiae	390	2.23
Glomerella cingulata	500	1.40
Alternaria solani	2,400	6.72

If Bordeaux mixture or other copper spray or dust of low solubility furnishes less than 1 p.p.m. of copper to the solutions with which it is in equilibrium, it is obvious that the concentration of copper is too low for any great amount of toxicity. We must also take into account the rate of solubility of the "insoluble" copper compounds, for if the rate of solution is slow, the maximum concentration may not be attained in time to prevent infection. The only hypothesis which would account for the lethal action of copper compounds of such low solubility would be that of cumulative action. A germinating spore in a saturated solution of the copper compound in equilibrium with the solid copper compound would remove copper from the solution. This process would cause more

of the copper compound to dissolve until the spore was no longer able to take more copper from the solution. This theory is attractive because of its simplicity, but there seems to be no very good evidence for it (McCallan, 1929).

In practice, Bordeaux mixture and other "insoluble" copper sprays act as if they were more soluble than is indicated by chemical tests. However, in practice the fungicide is exposed to the action of the atmosphere, the host plant, and the fungus spores. This is a more complicated situation than that found in the chemical determination of solubility. Barker and Gimingham (1911) found that intact leaves increased the soluble copper from Bordeaux mixture to some extent but were of the opinion that the host plant had only a slight influence on the solubility of such sprays. However, if the leaves were injured, they were quite effective in bringing copper into solution. The possibility that the spores exert a solvent action on "insoluble" copper compounds has long been considered by plant pathologists. The spores of at least some species do exert a solvent action on Bordeaux mixture. McCallan and Wilcoxon (1936) showed that the amount of copper brought into solution by the soluble materials washed from or excreted by 100 million spores of some species varied as follows: *Uromyces caryophyllinus*, 1.01 mg.; *Sclerotinia fructicola*, 0.76 mg.; *Neurospora sitophila*, 0.12 mg.; *Botrytis paeoniae*, 0.10 mg.; *Glomerella cingulata*, 0.046 mg.; *Aspergillus niger*, 0.023 mg.; and *Alternaria solani*, 0.013 mg. Enough spores of *Neurospora sitophila* were collected so that the nature of the soluble materials from the spores could be identified chemically. Malic acid was isolated and identified. The presence of amino acids also was detected. Both malic acid (or malates) and various amino acids dissolve "insoluble" copper compounds under neutral or alkaline conditions with the formation of soluble complex copper compounds. McCallan and Wilcoxon showed that sodium cuprimalate and a copper-glycine compound were about as toxic as copper sulfate. On the other hand, Goldsworthy and Green (1936) were of the opinion that spore secretions played a minor role in increasing the solubility of Bordeaux mixture, but the evidence of McCallan and Wilcoxon seems quite conclusive.

Basic copper carbonate (malachite, $Cu(OH)_2 \cdot CuCO_3$) and cuprous oxide (Cu_2O) are used in treating seeds. Since the seed covered with these materials is planted in soil which contains a variety of protein degradation products, it is easy to understand how these substances are made sufficiently soluble to be fungicidal. Marten and Leach (1944) studied the effect of various nitrogenous compounds upon the solubility of cuprous oxide. Gelatin and peptone were less efficient in dissolving cuprous oxide than were glycine, aspartic acid, asparagine, or cystine. Ammonium hydroxide was also a solvent for cuprous oxide. With all

these "solvents" the solutions were blue in color, which indicates that the copper was oxidized to the cupric state. Marten and Leach investigated the toxicity of the copper-glycine compound to *Pythium debaryanum*. It was noted that an excess of glycine protected the fungus from the action of the copper. Some 200 times as much copper was required to inhibit growth when glycine was present in the medium as when it was absent. Thus, it seems that whether a given amount of copper is toxic or not depends upon the nature and amount of certain constituents in the medium or substrate.

One may ask, By what mechanism does the copper ion cause fungistasis, or how does the copper kill? The common explanation of the toxic action of the heavy metals (copper, mercury, and silver) is based upon the property of these ions of precipitating or denaturing proteins. Enzymes are proteins, and it would be expected that the heavy metals would inactivate these catalysts. However, not all enzymes are equally inactivated by low concentrations of heavy-metal ions. The enzymes which require free sulfhydryl groups for activity appear to be especially susceptible to inactivation by ions of heavy metals. It is probable that copper causes fungistasis by combining with the sulfhydryl groups of certain enzymes. At this stage, the action of copper is reversible. Goldsworthy and Green (1936) found that spores of *Sclerotinia fructicola* which had been treated with insufficient copper to kill made normal growth when sown on copper-free medium. As long as an inhibition is reversible, the process is one of fungistasis. Death of the spore results when irreversible changes occur.

There is reason to believe that the injurious effect of copper fungicides upon the host plant is due to the same mechanism that operates in fungus spores. Foster (1947) attributed the sensitivity to copper of certain seeds to their content of sulfhydryl enzymes.

MERCURY

While a number of inorganic salts of mercury have been used as antiseptics, only two have had wide application as fungicides. Mercuric chloride (corrosive sublimate, bichloride of mercury, $HgCl_2$) is a soluble, highly poisonous compound. It is commonly used for surface sterilization in a concentration of 1/1,000. Mercuric chloride is occasionally used as a special-purpose fungicide.

Mercurous chloride (calomel, HgCl or Hg_2Cl_2) is essentially insoluble in water, sufficiently so to be used in medicine. Calomel slowly decomposes into mercury and mercuric chloride. This decomposition is accelerated by sunlight, which may account for the successful use of calomel to control dollar spot, brown patch, and other turf diseases.

The organic mercury compounds have won wide acceptance in the

treating of seed to control the attack of fungi which cause damping-off and of certain seed-borne pathogens. The organic mercurials are free from many of the objections inherent in the inorganic compounds of mercury. In general, they combine less avidly with proteins, are more selective in their action, and are far less toxic to animal life. As used for seed protection, they are commonly diluted with an inert carrier. Most if not all such organic mercury compounds are sold under trade names, but the active components are required by law to be stated on the label. Among these organic mercury compounds are ethylmercury phosphate (Semesan Jr. and New Improved Ceresan), ethylmercury chloride (Ceresan) and hydroxymercurichlorophenol (Semesan).

The organic mercury compounds used as sprays and for treating seeds are in general related to mercuric chloride in the following way:

$C_2H_5—Hg—Cl$ — Ethylmercury chloride

Cl—Hg—Cl — Mercuric chloride

The ethyl group has replaced a chlorine atom in mercuric chloride. The type formula for compounds like this may be written as R—Hg—X, where R may be any alkyl or aryl (or other) group and X represents any anion, I^-, Cl^-, OH^-, NO_3^-, $PO_4^{\equiv}$. The anion greatly modifies the solubility of the compound in water. In general, this type of organic mercury compound is volatile, and this property may be assumed to aid in penetration. Other organic mercury fungicides are derivatives of alkyl and aryl mercuric hydroxides. These compounds can react with organic acids to form salts. The relation of these compounds to mercuric hydroxide is shown below:

$C_6H_5—Hg—OH$ — Phenylmercury hydroxide

HO—Hg—OH — Mercuric hydroxide

Compounds of this type are used to protect cellulose and leather products.

Parker-Rhodes (1942) investigated the toxicity of the following mercury compounds to *Macrosporium sarcinaeforme* and *Botrytis allii:* mercuric acetate, mercuric chloride, methylmercury nitrate, and tolylmercuric nitrate. All these compounds were toxic to *M. sarcinaeforme*, and all except methylmercury nitrate were toxic to *B. allii*. Perhaps methylmercury nitrate is not soluble enough in fat for the spores of this fungus to absorb a toxic amount of the compound. Dillon-Weston and Booer (1935) found that vapor of ethylmercury iodide was toxic to *Tilletia* spores in the laboratory but afforded no control in the field.

The soluble inorganic mercury salts are protein precipitants, and this property may explain in part their mode of action when used in high concentrations. These salts are frequently fungistatic or bacteriostatic, since the very firmness of the union between the mercuric ion and the cell membrane may form a barrier to further penetration. The first action

of mercuric ion is to cause stasis, which may be reversed by treating the cells with reagents which have a high affinity for mercury. McCalla (1940) demonstrated that cells of *Escherichia coli* which had been treated with mercuric chloride could be revived by hydrogen sulfide. If stasis due to mercury is not overcome within a certain time, irreversible changes occur and death of the cells results.

Organic mercury compounds are not protein precipitants, and this is one of their advantages as disinfectants and fungicides. Fildes (1940) ascribed the action of mercury compounds to combination of mercuric ion with the sulfhydryl group of essential metabolites and enzymes. Others have shown that organic mercury compounds act similarly. According to this view, enzyme inhibition is the basis of the action of mercury compounds. Fildes found that the action of mercury was antagonized by compounds which contained free sulfhydryl groups (thioacetate, cysteine, glutathione). Neither cystine (—S—S—) nor methionine (—S—) was effective in overcoming mercury toxicity.

Organic mercury compounds appear to act by the same mechanism as the mercuric ion. *p*-Chloromercuribenzoate was found to inhibit the action of various sulfhydryl enzymes which take part in carbohydrate metabolism, *e.g.* succinic acid oxidase, yeast carboxylase, malate oxidase, and ketoglutarate oxidase. This organic mercury compound also inhibited the action of *d*-amino acid oxidase, transaminase, *l*-glutamate oxidase, and other enzymes (Barron and Singer, 1945; Singer and Barron, 1945). In many instances the inhibitory action of *p*-chloromercuribenzoate on these enzymes could be reversed by glutathione, cysteine, or hydrogen sulfide.

Cook *et al.* (1946) found phenylmercuric nitrate to depress the respiration of *Saccharomyces cerevisiae*. This depression in rate of respiration was overcome by various compounds having a free —SH group; *e.g.*, cysteine and homocysteine, while cystine and methionine were without effect. The work of these investigators and of others makes it highly probable that mercury compounds are toxic because they inactivate certain essential enzyme systems. The enzyme inhibitions discussed above are examples of noncompetitive inhibition. These inhibitions are reversible, as in the case of competitive inhibition, but the reversing agents are nonspecific, or not limited to a single metabolite.

SULFUR

Of the nonmetallic elements, sulfur and certain of its compounds are widely used as protective and eradicant fungicides. The toxicity of the nonmetallic elements is dependent upon the state of oxidation. In many instances, compounds in the higher states of oxidation are the least toxic. For example, sulfur in the form of the free element (S) and of sulfide

($S^=$) forms excellent fungicides, while sulfites ($SO_3^=$) are only slightly toxic and sulfates ($SO_4^=$) are nontoxic.

According to Large (1940), elemental sulfur has been used to control powdery mildew for slightly over 100 years. The effectiveness of sulfur increases as the particle size diminishes. Finely divided sulfur adheres to plant surfaces much better than larger particles. In addition, the distance between particles tends to be decreased when fine particles are used, and the infection court is thereby better protected. The odds are increased that a fungus spore falling upon a treated leaf will be within the range of action of a particle of sulfur. An example of the effect of particle size of sulfur on toxicity is given in Table 44. The greater toxicity of

Table 44. The Relation between the Particle Size and Toxicity of a Sulfur Dust to the Conidia of *Sclerotinia americana*
(Wilcoxon and McCallan, *Phytopathology* **20**, 1930.)

Treatment	Mean diameter of sulfur particles, μ	Germination, %
Control	—	97.6
Ground roll sulfur	285	62.8
Ground roll sulfur	142	47.2
Ground roll sulfur	60	29.1
Ground roll sulfur	33	20.7

the finely divided sulfur is due to the fact that sulfur enters the spore in the form of vapor. The amount of vapor formed from a given amount of sulfur in a given time depends upon the area of the exposed surface, as well as upon temperature. Therefore, the fineness of the sulfur particles governs the effective concentration of sulfur vapor and its effectiveness as a fungicide.

McCallan (1946) estimated the yearly consumption of sulfur in the United States alone to be 142 million pounds. Of this amount 110 million pounds is used as sulfur dust, 5 million pounds as wettable sulfur, and 27 million pounds as lime-sulfur. Approximately 62 per cent of this is used primarily for the control of apple scab alone.

Since elemental sulfur is insoluble, its action upon fungi cannot be attributed to the sulfur in this form. Two general theories have been proposed to explain the action of sulfur. One theory holds that the action is due to oxidized sulfur, such as SO_2 or SO_3 (which form sulfurous and sulfuric acid, respectively, with water) or pentathionic acid, $H_2S_5O_6$. According to the second theory, the reduced form of sulfur, H_2S, is the active toxic agent. Both these theories are supported by published experimental evidence. All these compounds are toxic to fungi under certain conditions, if in high enough concentrations. However, to

account satisfactorily for the toxic properties of sulfur, it must be demonstrated that the toxic agent is produced under the conditions which prevail in the field in quantities sufficient to account for the observed effects. Any evaluation of these hypotheses must take into account all the variables involved. The caution of Wilcoxon and McCallan (1930) is pertinent:

> In making comparisons of the toxicity of chemical substances to fungus spores, there are two requisites for obtaining accurate results which, though quite obvious, have not always received the consideration they deserve. (*a*) The substance whose toxicity is to be measured must be available in a pure state and of known concentration, and (*b*) the technique employed must be capable of distinguishing between the toxicity of the substances it is desired to compare.

It is agreed that elemental sulfur is not the toxic agent and that sulfur is transformed into the toxic agent. There are three possible agencies for such transformations: the atmosphere, the plant on which the sulfur is dusted, and the fungus spores or mycelium. Sulfur acts at a distance, and since sulfur is volatile at room temperature, this property offers an explanation. Sulfur vapor is a gas, and in this state it should be more easily transformed into the toxicant.

Sulfur is slowly oxidized by the oxygen of the atmosphere to form sulfur dioxide, but the rate at which this reaction occurs at ordinary temperatures makes it impossible for this reaction to account for all the toxic properties of sulfur, even though sulfur dioxide is toxic to fungus spores (McCallan and Weedon, 1940).

Young (1922) set forth the hypothesis that pentathionic acid is the toxic agent formed from sulfur. It is agreed, even by those who do not support Young's hypothesis, that this acid is formed on the surface of sulfur dust. A considerable number of papers were published during the next decade which gave support to this view (Liming, 1932). Wilcoxon and McCallan (1930) investigated this theory thoroughly and concluded that pure pentathionic acid had no toxic properties for the spores of *Sclerotinia americana, Botrytis* sp., *Macrosporium sarcinaeforme*, and *Uromyces caryophyllinus*. If sufficient pentathionic acid was used to reduce the pH to about 4, spore germination was inhibited. Solutions of sulfuric acid having the same pH were equally toxic. Neutral salts of both acids were nontoxic. Roach and Glynne (1928) likewise found pentathionic and sulfuric acids to have the same toxicity when tested against the winter sporangia of *Synchytrium endobioticum*. Wilcoxon and McCallan (1930) performed a decisive experiment when they washed one lot of sulfur dust with alkali to remove pentathionic acid and compared this pentathionate-free dust with the original sample, which contained a trace of this acid. No difference in toxicity of the washed and control samples of this sulfur dust was found.

There is now general agreement that hydrogen sulfide is the common toxic compound produced from sulfur. Not only is hydrogen sulfide toxic to fungus spores, but the mechanism for its production is also present. It is known that hydrogen sulfide is produced from sulfur both by the treated plant and by the fungus spores.

McCallan and Wilcoxon (1931) made qualitative tests for the ability of the spores of 17 species of fungi to produce hydrogen sulfide from sulfur. All produced this substance, but in varying amounts and at varying rates. They showed that the spores need not be in direct contact with solid sulfur to produce hydrogen sulfide. Figure 50 illustrates the method used by these investigators to demonstrate this phenomenon.

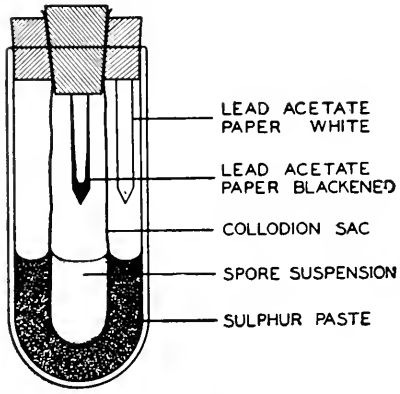


FIG. 50. The production of hydrogen sulfide by *Sclerotinia* spores separated from sulfur by a collodion membrane. Note that the production of hydrogen sulfide takes place on the spore side of the membrane and not on the sulfur side. (Courtesy of McCallan and Wilcoxon, *Contribs. Boyce Thompson Inst.* **3**: 26, 1931.)

These authors investigated the toxicity of hydrogen sulfide to the spores of eight species of fungi. These experiments were performed in a flowing stream of air which contained known amounts of hydrogen sulfide, and the concentration in the water droplet in which the spores were suspended was calculated from Henry's law. These precautions are necessary because hydrogen sulfide is unstable. Neglect of this fact by earlier investigators led to an underestimation of the toxicity of hydrogen sulfide. These results of McCallan and Wilcoxon are presented in Fig. 51. From these curves it is seen that spores of *Venturia inaequalis*, *Uromyces caryophyllinus*, and *Puccinia antirrhini* are inhibited by very low concentrations of hydrogen sulfide, while the spores of *Botrytis* sp. and *Glomerella cingulata* are scarcely affected by ten times as much hydrogen sulfide. By increasing the hydrogen sulfide concentration to 60 p.p.m., complete inhibition of germination of the spores of these two species was obtained. The spores of these eight fungi were shown to produce varying amounts of hydrogen sulfide per unit weight of spores. Whether hydrogen sulfide produced by spores would prove toxic would therefore depend upon the ability of the particular spores to produce hydrogen sulfide and the sensitivity of the spores to this substance. The correlation is shown in Table 45.

The actions of sulfur and hydrogen sulfide are parallel, and it may be concluded that sulfur is toxic to the spores of certain species by virtue

of absorption of sulfur vapor and its reduction to hydrogen sulfide within the spore. Thus, the spores of susceptible species destroy themselves. It is not thought that the hydrogen sulfide evolved from leaves or other spores is absorbed in lethal quantities under natural conditions.

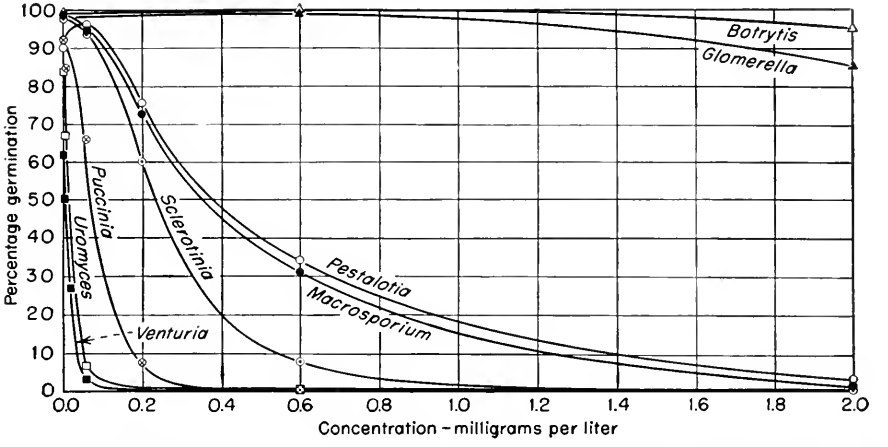


FIG. 51. Toxicity of hydrogen sulfide to urediospores of *Uromyces caryophyllinus* and *Puccinia antirrhini* and to conidia of *Venturia inaequalis, Sclerotinia americana, Macrosporium sarcinaeforme, Pestalotia stellata, Glomerella cingulata,* and *Botrytis* sp. (Courtesy of McCallan and Wilcoxon, *Contribs. Boyce Thompson Inst.* **3**: 31, 1931.)

Liquid lime-sulfur is a common spray material and is prepared by boiling sulfur and calcium hydroxide together. The chief active ingredient is calcium polysulfide. After deposition on leaves the calcium polysulfide is quickly decomposed, yielding sulfur and calcium sulfide,

TABLE 45. COMPARISON BETWEEN THE TOXICITY AND THE PRODUCTION OF HYDROGEN SULFIDE, EXPRESSED IN UNITS EQUAL TO THE AMOUNTS OF HYDROGEN SULFIDE REQUIRED TO REDUCE GERMINATION 50 PER CENT
(McCallan and Wilcoxon, *Contribs. Boyce Thompson Inst.* **3**, 1931.)

Species	Mg. H_2S required to reduce germination of 1,000,000 spores 50%	Mg. H_2S produced by 1,000,000 spores in 12 hr.	Production of H_2S expressed in units equal to the amount of H_2S required to reduce germination 50%
Venturia inaequalis	0.001	0.002	2.0
Uromyces caryophyllinus	0.002	0.019	9.5
Puccinia antirrhini	0.006	0.13	2.2
Sclerotinia americana	0.013	0.039	3.0
Macrosporium sarcinaeforme	0.043	0.013	0.30
Pestalotia stellata	0.049	0.001	0.02
Glomerella cingulata	0.532	0.027	0.05
Botrytis sp	0.665	0.002	0.003

which in turn may decompose by hydrolysis to yield hydrogen sulfide and calcium hydroxide. At the same time some of the calcium polysulfide is oxidized to calcium thiosulfate and sulfur. See Frear (1948) for a discussion of the chemistry involved.

It is known that lime-sulfur exerts an eradicant action on some fungi, including *Venturia inaequalis*, when first applied. After a few days this spray exerts only a protective action like that of elemental sulfur, which probably depends on the elemental sulfur set free by the decomposition of various constituents comprising lime-sulfur. The eradicant action, then, depends upon either the calcium polysulfide or calcium sulfide. We may consider that sulfide ion ($S^=$) is the toxic agent. The alkalinity of the spray may aid in penetration into the mycelium already present.

Lime-sulfur solution may be treated with ferrous sulfate or aluminum sulfate in the spray tank to produce colloidal sulfur and hydrogen sulfide. Aluminum sulfate, $Al_2(SO_4)_3$, hydrolyzes to form aluminum hydroxide and sulfuric acid. A lime-sulfur spray so treated has only a protective action. It has lost its eradicant value. We may assume, therefore, that the decomposition of lime-sulfur in spray tanks when treated with acid (aluminum sulfate) and the decomposition on the leaf follow a somewhat similar pattern. This scheme of producing colloidal sulfur has the drawback that the added iron, when ferrous sulfate is used, is toxic to vegetation, and dangerous amounts of hydrogen sulfide are evolved.

We may assume that hydrogen sulfide exerts its toxic action on fungus spores by inactivating certain enzymes. Hydrogen sulfide is known to inactivate many enzymes, including catalase, cytochrome oxidase, dopa oxidase, lactase, and others. Generally, hydrogen sulfide and cyanide inhibit the same enzymes. It is thought that these metalloenzymes are inhibited by sulfide or cyanide because these agents react with iron or copper to form highly insoluble or little-ionized compounds or complexes.

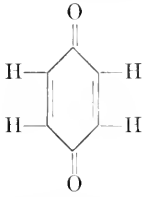
ORGANIC FUNGICIDES

The newer fungicides, with few exceptions, are either organic or organometallic compounds. The organic mercury compounds were considered with the inorganic compounds of mercury, since the mechanism of action appears to be the same in both types of compounds. Many of the organic fungicides exhibit greater specificity than the inorganic fungicides. The possibilities of modification in the structure of organic compounds are almost unlimited. The study of organic fungicides, therefore, offers the opportunity of correlating structure with type and intensity of fungicidal action.

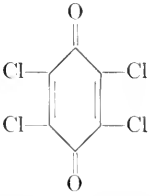
Aldehydes. The first organic fungicide to attain wide acceptance was formaldehyde. At one time this compound was used for the surface sterilization of grain and potato tubers, but at present formaldehyde is

little used. Formaldehyde reacts with free amino groups, and it is probable that its fungicidal action depends upon this property. Some other aldehydes also have fungicidal properties (Uppal, 1926).

Quinones. While there are two series of quinones (*ortho*, or 1,2, and *para*, or 1,4), we shall consider only the 1,4-quinones as fungicides. The simplest quinone is *p*-benzoquinone. Quinones are cyclic compounds which possess a characteristic pair of double bonds. Such a configuration of double bonds is called *quinoid* and is possessed by many dyes, some of which are fungicides. If a considerable series of toxic compounds possess a common functional group or groups, it may be assumed that these groups are involved in fungicidal activity. According to Horsfall (1945), 1,4-benzoquinone has a slight toxicity to fungi. The four hydrogens in 1,4-benzoquinone can be replaced by chlorine to form chloranil (Spergon), which greatly increases the fungicidal properties. The structural formulas for these compounds are given below:



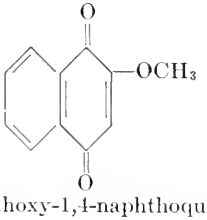
1,4-Benzoquinone



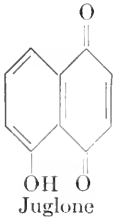
Chloranil (Spergon)

Spergon has been used as a seed protectant.

Substituted naphthoquinones are more important fungicides than the benzoquinones. Among these, 2,3-dichloro-1,4-naphthoquinone (Phygon) is reported to be five to eight times as effective as Spergon (Ter Horst and Felix, 1943). Some of the naphthoquinones synthesized by plants are fungicides. Juglone, 5-hydroxy-1,4-naphthoquinone, is found in walnut hulls and is secreted by walnut roots. The isomeric 2-hydroxy-1,4-naphthoquinone (lawsone) is found in henna leaves. Juglone is reported to be as toxic to fungus spores as Bordeaux mixture. Juglone controls black spot of roses as well as sulfur does (Gries, 1943, 1943*a*). It is also toxic to many plants. Little *et al.* (1948) isolated 2-methoxy-1,4-naphthoquinone from *Impatiens balsamina*. This compound was an active fungicide which exhibited no phytotoxicity toward tomato and bean plants. The formulas of two naphthoquinone fungicides are given below:



2-Methoxy-1,4-naphthoquinone



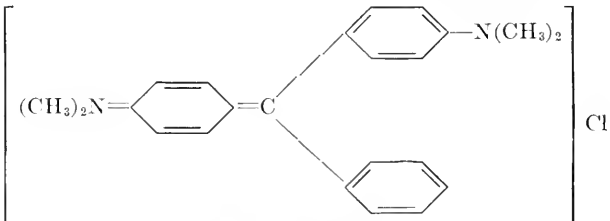
Juglone

The fungicidal action of substituted quinones may be due in part to their property of reacting with free amino groups of proteins (Theis, 1945). Substituted naphthoquinones as antagonists of vitamin K were discussed in Chap. 11. Most of the available evidence indicates that the principal mechanism of quinone toxicity lies in its noncompetitive inhibition of sulfhydryl enzymes.

It has been suggested that the mechanism of inhibition is dependent upon the structure of the substituted naphthoquinones. Colwell and McCall (1946) found the fungistatic and fungicidal concentrations of 2-methyl-1,4-naphthoquinones to be the same when *Aspergillus niger* and an unidentified fungus were used as test organisms. Addition of sodium thioglycolate or cysteine antagonized the toxic action of this naphthoquinone. These authors postulate that only naphthoquinones unsubstituted in position 3 react with sulfhydryl groups, for 2-methyl-3-methoxy-1,4-naphthoquinone was not antagonized by thioglycolate or cysteine. The reaction between certain naphthoquinones and sulfhydryl-containing compounds can be demonstrated in vitro.

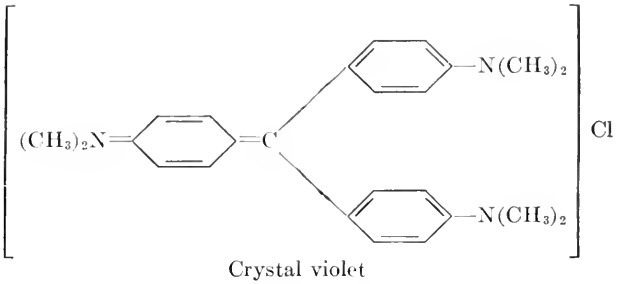
The amounts of various substituted 1,4-naphthoquinones required to cause a 50 per cent inhibition of isolated yeast carboxylase and similar reduction in the germination of *Monilinia fructicola* spores were roughly parallel (Foote *et al.*, 1949). Carboxylase is a sulfhydryl enzyme. It is probable that other sulfhydryl enzymes are also inhibited by naphthoquinones. For further information on the mechanism of quinone inhibition, see Geiger (1946).

Dyes. Various dyes are fungistatic compounds. Malachite green and crystal violet are used to control various fungus infections of the skin. Both these dyes have a benzoquinoid structure, as is shown below:



Malachite green

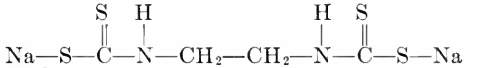
Leonian (1930) made a study of the toxicity of malachite green to many species and strains of *Phytophthora* and found only three species (*P. hydrophila*, *P. melongenae*, and *P.* sp.) able to grow in the presence of 1 p.p.m. of malachite green. Other species were more sensitive to this dye. *P. colocasiae* and *P. richardiae* failed to grow in nutrient solutions containing 1 part of malachite green in 16 million parts of medium. Leonian (1932) investigated the growth-inhibiting properties of malachite



Crystal violet

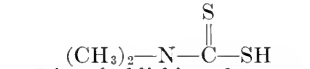
green and crystal violet upon 26 species and isolates of *Trichophyton*. Malachite green proved greatly superior to crystal violet. Over half the isolates tested failed to grow in the presence of 1 part of malachite green to 50,000 parts of medium, and many failed to grow in the presence of 1 p.p.m. of this dye. Crystal violet allowed some growth in all isolates tested at a concentration of 1 part in 50,000 parts of medium. Placing the inoculum in direct contact with the medium containing the dye was more lethal than placing the agar inoculum plug with the mycelium upon the surface of the test medium. Some other dyes such as methylene blue are also toxic to fungi. Both malachite green and methylene blue inhibit carboxylase (Horsfall, 1945).

Dithiocarbamates and related compounds. Barratt and Horsfall (1947) have reported extensive investigations on the homologues and analogues of disodium ethylenebisdithiocarbamate (Nabam). In general, these compounds are formed when primary and secondary amines react with carbon disulfide. The formula for Nabam is given below:



Disodium ethylenebisdithiocarbamate (Nabam)

The zinc (Ziram) and ferric (Ferbam) salts of dimethyldithiocarbamate are effective fungicides for the control of certain fungus pathogens. The formula for dimethyldithiocarbamate is given below:



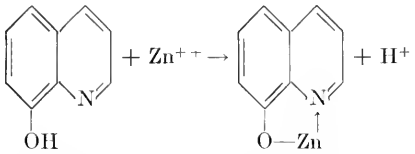
Dimethyldithiocarbamate

The oxidation product of dimethyldithiocarbamate is tetramethylthiuram disulfide (Thiram), which has some value as a seed protectant.

The dithiocarbamate fungicides, such as Nabam, yield hydrogen sulfide on hydrolysis. This reaction takes place spontaneously in the presence of moisture. The mechanism of hydrogen sulfide toxicity has already been discussed. The second mechanism which has been proposed involves the formation of insoluble mercaptides of certain essential metals.

In addition, Nabam on decomposition yields an unidentified toxic gaseous compound, which is neither hydrogen sulfide nor sulfur dioxide (Rich and Horsfall, 1950).

Specific organic reagents for metals. The essential nature of certain micro elements for fungus growth and the role of these elements in enzymes were discussed in Chaps. 4 and 5. The chemistry of these specific organic reagents is treated by Yoe and Sarver (1941). These reagents form insoluble or slightly ionized compounds with metals. Zentmeyer (1944) tested various organic analytical reagents and found 8-hydroxyquinoline (Oxine) and ammonium nitrosophenylhydroxylamine (Cupferron) to be fungistatic. 8-Hydroxyquinoline inhibited the growth of *Fusarium oxysporum* var. *lycopersici*, *Ceratostomella ulmi*, and a species of *Penicillium*. The effectiveness of 8-hydroxyquinoline in forming chelate salts increases as the pH values increase. Below pH 3.5 complex formation does not take place with zinc, copper, iron, and manganese. Zinc ion reacts with 8-hydroxyquinoline as shown below:



8-Hydroxyquinoline Zinc complex of 8-hydroxyquinoline

The fungistatic effect of 8-hydroxyquinoline on *Fusarium oxysporum* var. *lycopersici* and *Ceratostomella ulmi* was overcome by increasing the zinc content of the medium. In the presence of 8-hydroxyquinoline there was competition between this compound and one or more enzyme systems for the zinc present in the medium. Whether or not an organic compound such as 8-hydroxyquinoline will act as a fungistatic agent depends upon the concentration of the reagent, the amount of fungus mycelium, and the concentration of the metallic ion for which the two systems compete. One would expect that such fungicides, in common with all others, would be more effective when the mass of the fungus is small.

Other organic fungicides. Many other types of organic compounds are fungicides, and an intensive search for new ones is in progress. Brief mention of some of these developments is made below. Geiger (1948) reports various unsaturated ketones to be active against *Aspergillus niger*, *Trichoderma köningii*, *Cryptococcus neoformans*, and *Trichophyton mentagrophytes*. The mode of action resembles that of the naphthoquinones in that sulfhydryl enzymes, including succinic acid dehydrogenase, triose phosphate dehydrogenase, and urease, are inhibited. The fungistatic activity of ethylenic and acetylenic compounds has been

tested on *Fusarium graminearum*, *Penicillium digitatum*, and *Botrytis allii* (McGowan *et al.*, 1948). The fungicidal action of substituted pyrazoles was tested on spores of *Alternaria oleracea* and *Sclerotinia americana* in the laboratory, and for the control of apple scab, cedar-apple rust, and late blight of potato and tomato. Some of these compounds show promise, although the mechanism of action is not known (McNew and Sandholm, (1949). For a survey of the newer fungicides see Wellman (1948).

EVALUATING FUNGICIDES

The preliminary tests of fungicidal activity are made in the laboratory in order to eliminate inactive compounds or to compare the activities of different compounds under identical conditions. Evaluation in the greenhouse and field is the final test of a new fungicide. This discussion will be limited to a general consideration of laboratory testing of fungicides.

Fungus spores rather than mycelium are used in most laboratory tests because it is the function of a protectant fungicide to kill or inhibit spore germination. Three basic types of procedures may be used in laboratory tests (McCallan, 1947): (1) Spores are suspended in solutions or suspensions of the fungicide under test, and the inhibition of germination is noted as a function of time of exposure and concentration of the fungicide. This is a modification of the Rideal-Walker method of evaluating antiseptics. (2) The compound to be tested is incorporated in a suitable solid or liquid medium, which is then inoculated with spores of the test fungi. The amount of inhibition of germination or growth is determined. (3) Glass slides are covered uniformly with the fungicide, and after drying, the spores are sown on the treated slides. The inoculated plates are then placed in constant-humidity chambers and the percentage of germination determined after 20 to 24 hr.; or the effectiveness of a fungicide may be studied as a function of time of exposure.

The second and third methods appear to be the most useful. Fleury (1948) studied the fungistatic action of thiourea on *Aspergillus niger* by adding this substance to a liquid basal medium. Thiourea was a much more potent inhibitor when nitrate nitrogen was used than when ammonium or organic nitrogen was present in the medium. Agar medium has been used by Leben and Keitt (1949) to assay the amount of toxicant on leaf surfaces. A suspension of spores of *Glomerella cingulata* was prepared in warm (38 to 40°C.) agar medium. Five milliliters of this seeded medium was added to Petri dishes which contained 15 ml. of solidified agar medium. After the seeded agar had solidified, leaf disks of uniform size were cut from sprayed leaves and placed on the agar. The amount of toxicant present on the leaf surface was determined by measur-

ing the diameter of the zone of inhibition. Disks of blotting paper to which fungicides have been added may be used to determine their potency. Thornberry (1950) has suggested the use of filter-paper disks for the evaluation of fungicides and bactericides. Filter-paper disks appear to be more suitable than blotting paper. In this method seven filter-paper disks are uniformly spaced on a Petri dish, and 0.09 ml. of the toxicant in aqueous solution is added per disk. The zone of inhibition is a measure of the effectiveness of the fungicide.

The glass-slide method appears to simulate more closely the conditions under which the spores of plant pathogenic fungi germinate in nature. The Committee on the Standardization of Fungicidal Tests of the American Phytopathological Society has considered this method important enough to publish a detailed and documented summary (1943), to which the student is referred for further information and references. This committee recommended the use of spores of the following species for this test: *Alternaria solani, Glomerella cingulata, Macrosporium sarcinaeforme, Sclerotinia fructicola, Penicillium expansum,* and *Rhizopus nigricans.* For accurate work, at least two of these test fungi should be used. The effectiveness of a fungicide is determined by calculating the percentage of inhibition of spore germination. The methods of evaluating data obtained in fungicide tests are discussed by Horsfall (1945).

SUMMARY

A fungicide is an agent capable of killing some fungi. Fungicides may be either water-soluble or nearly insoluble. The action of fungicides of the first class is immediate; that of the second class is delayed. Eradicant fungicides are of the first class, while protective fungicides are of the second. Fungistasis is the complete or partial inhibition of one or more life processes of a fungus. This inhibition is reversible. The same chemical compound may cause fungistasis or may be a fungicide, depending upon the concentration and time of exposure. The same substance may be a fungicide for one species, cause fungistasis of a second, and be without effect upon a third. Fungistasis precedes fungicidal action.

Before a fungicide can act upon a fungus, the toxicant must get into the fungus cells, or at least reach the protoplasmic membrane. While other factors undoubtedly enter into the mechanism of fungicidal action, the principal point of attack appears to be enzyme systems. The heavy-metal fungicides appear to act by inhibiting various sulfhydryl enzymes. Fungus spores transform sulfur into hydrogen sulfide, which inhibits the metalloenzymes. Organic fungicides, so far as is known, are also enzyme inhibitors.

In the past, fungicides containing copper, mercury, and sulfur have been the most useful. Recently, organic fungicides have become impor-

tant and promise to be used even more extensively in the future. Organic fungicides are generally more specific than inorganic fungicides. Satisfactory fungicides for the control of certain diseases are still undiscovered.

REFERENCES

Barker, B. T. B., and C. T. Gimingham: The fungicidal action of bordeaux mixtures, *Jour. Agr. Sci.* **4**: 76–94, 1911.

Barratt, R. W., and J. G. Horsfall: Fungicidal action of metallic alkyl bisdithiocarbamates, *Conn. Agr. Expt. Sta. Bull.* 508, 1947.

Barron, E. S. G., and T. P. Singer: Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism, *Jour. Biol. Chem.* **157**: 221–240, 1945.

Colwell, C. A., and M. McCall: The mechanism of bacterial and fungus growth inhibition by 2-methyl-1,4-naphthoquinone, *Jour. Bact.* **51**: 659–670, 1946.

★Committee on the Standardization of Fungicidal Tests: The slide-germination method of evaluating protectant fungicides, *Phytopathology* **33**: 627–632, 1943.

Cook, E. S., G. Perisutti, and T. M. Walsh: The action of phenylmercuric nitrate. II. Sulfhydryl antagonism of respiratory depression caused by phenylmercuric nitrate, *Jour. Biol. Chem.* **162**: 51–54, 1946.

Dillon-Weston, W. A. R., and J. R. Booer: Seed disinfection. 1. An outline of an investigation on disinfectant dusts containing mercury, *Jour. Agr. Sci.* **25**: 628–649, 1935.

Fildes, P.: The mechanism of the anti-bacterial action of mercury, *Brit. Jour. Exptl. Path.* **21**: 67–73, 1940.

Fleury, C.: Action de la thio-urée sur l'*Aspergillus niger*. Rôle particulier joué par la source d'azote nitrique, *Bull. soc. botan. suisse* **58**: 462–476, 1948.

Foote, M. W., J. E. Little, and T. J. Sproston: On naphthoquinones as inhibitors of spore germination, *Jour. Biol. Chem.* **181**: 481–487, 1949.

Foster, A. A.: Acceleration and retardation of germination of some vegetable seeds resulting from treatment with copper fungicides, *Phytopathology* **37**: 390–398, 1947.

Frear, D. E. H.: Chemistry of Insecticides, Fungicides and Herbicides; 2d ed., D. Van Nostrand Company, Inc., New York, 1948.

Geiger, W. B.: The mechanism of the antibacterial action of quinones and hydroquinones, *Arch. Biochem.* **11**: 23–32, 1946.

Geiger, W. B.: Antibacterial unsaturated ketones and their mode of action, *Arch. Biochem.* **16**: 423–435, 1948.

Goldsworthy, M. C., and E. L. Green: Availability of the copper of Bordeaux mixture residues and its adsorption by the conidia of *Sclerotinia fructicola*, *Jour. Agr. Research* **52**: 517–533, 1936.

Gries, G. A.: Juglone (5-hydroxy-1,4-naphthoquinone)—a promising fungicide, *Phytopathology* **33**: 1112, 1943.

Gries, G. A.: Juglone—the active agent in walnut toxicity, *Northern Nut Growers Assoc. Ann. Rept.* **34**: 52–55, 1943*a*.

★Horsfall, J. G.: Fungicides and Their Action, Chronica Botanica Co., Waltham, 1945.

Large, E. C.: The Advance of the Fungi, Henry Holt and Company, Inc., New York, 1940.

Leben, C., and G. W. Keitt: Laboratory and greenhouse studies of antimycin preparations as protectant fungicides, *Phytopathology* **39**: 529–540, 1949.

Leonian, L. H.: Differential growth of Phytophthoras under the action of malachite green, *Am. Jour. Botany* **17**: 671–677, 1930.

Leonian, L. H.: Effects of position of inoculum on growth of some Trichophytons in the presence of dyes, *Arch. Dermatol. and Syphilol.* **25**: 1016–1020, 1932.

Liming, O. N.: The relation of pentathionic acid and its component constituents to the toxicity of sulphur fungicides, *Phytopathology* **22**: 143–165, 1932.

★Little, J. E., T. J. Sproston, and M. W. Foote: Isolation and antifungal action of naturally occurring 2-methoxy-1,4-naphthoquinone, *Jour. Biol. Chem.* **174**: 335–342, 1948.

McCalla, T. M.: Cation adsorption by bacteria, *Jour. Bact.* **40**: 23–32, 1940.

★McCallan, S. E. A.: Studies on fungicides. III. The solvent action of spore excretions and other agencies on protective copper fungicides, *Cornell Univ. Agr. Expt. Sta. Mem.* 128, 1929.

McCallan, S. E. A.: Outstanding diseases of agricultural crops and uses of fungicides in the United States, *Contribs. Boyce Thompson Inst.* **14**: 105–115, 1946.

McCallan, S. E. A.: Bioassay of agricultural fungicides, *Agr. Chemicals* **2**(9): 31–34, 67; **2**(10): 45, 1947.

McCallan, S. E. A., and F. R. Weedon: Toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide gases. II. Fungi and bacteria, *Contribs. Boyce Thompson Inst.* **11**: 331–342, 1940.

McCallan, S. E. A., and R. H. Wellman: Fungicidal versus fungistatic, *Contribs. Boyce Thompson Inst.* **12**: 451–463, 1942.

★McCallan, S. E. A., and F. Wilcoxon: The fungicidal action of sulphur. II. The production of hydrogen sulphide by sulphured leaves and spores and its toxicity to spores, *Contribs. Boyce Thompson Inst.* **3**: 13–38, 1931.

McCallan, S. E. A., and F. Wilcoxon: The action of fungous spores on Bordeaux mixture, *Contribs. Boyce Thompson Inst.* **6**: 151–165, 1936.

McGowan, J. C., P. W. Brian, and H. G. Hemming: The fungistatic activity of ethylenic and acetylenic compounds. I. The effect of the affinity of the substituents for electrons upon the biological activity of ethylenic compounds, *Ann. Applied Biol.* **35**: 25–36, 1948.

McNew, G. L., and N. K. Sanduolm: The fungicidal activity of substituted pyrazoles and related compounds, *Phytopathology* **39**: 721–751, 1949.

Marten, E. A., and J. G. Leach: Some factors influencing the solubility of cuprous oxide in relation to its toxicity as a fungicide, *Phytopathology* **34**: 459–470, 1944.

Parker-Rhodes, A. F.: Studies on the mechanism of fungicidal action. IV. Mercury, *Ann. Applied Biol.* **29**: 404–411, 1942.

Prévost, B.: Mémoire sur la cause immédiate de la carie ou charbon des blés, et de plusieurs autres maladies des plantes, et sur préservatifs de la carie, 1807. Trans. by G. W. Keitt, Phytopathological Classics No. 6, American Phytopathological Society, Menasha, 1939.

Rich, S., and J. G. Horsfall: Gaseous toxicants from organic sulfur compounds, *Am. Jour. Botany* **37**: 643–650, 1950.

Roach, W. A., and M. D. Glynne: The toxicity of certain sulphur compounds to *Synchytrium endobioticum*, the fungus causing wart disease of potatoes, *Ann. Applied Biol.* **15**: 168–190, 1928.

Singer, T. P., and E. S. G. Barron: Studies on biological oxidations. XX. Sulfhydryl enzymes in fat and protein metabolism, *Jour. Biol. Chem.* **157**: 241–253, 1945.

Starkey, R. L., and S. A. Waksman: Fungi tolerant to extreme acidity and high concentrations of copper sulfate, *Jour. Bact.* **45**: 509–519, 1943.

Ter Horst, W. P., and E. L. Felix: 2,3-Dichloro-1,4-naphthoquinone, a potent fungicide, *Ind. Eng. Chem.* **35**: 1255–1259, 1943.

Theis, E. H.: The collagen-quinone reaction. 1. Fixation and thermolability as a function of pH values, *Jour. Biol. Chem.* **157**: 23–33, 1945.

Thornberry, H. H.: A paper-disk plate method for the quantitative evaluation of fungicides and bactericides, *Phytopathology* **40**: 419–429, 1950.

Uppal, B. N.: Toxicity of organic compounds to the spores of *Phytophthora colocasiae* Rac., *Jour. Agr. Research* **32**: 1069–1097, 1926.

Wellman, R. H.: Synthetic chemicals for agriculture. II. Fungicides, nematocides, rodenticides and weed killers, *Chem. Inds.* **63**: 223–229, 1948.

Wilcoxon, F., and S. A. E. McCallan: The fungicidal action of sulphur. I. The alleged role of pentathionic acid, *Phytopathology* **20**: 391–417, 1930.

Yoe, J. H., and L. A. Sarver: Organic Analytical Reagents, John Wiley & Sons Inc., New York, 1941.

Young, H. C.: The toxic property of sulphur, *Ann. Missouri Botan. Garden* **9**: 403–435, 1922.

★Zentmeyer, G. A.: Inhibition of metal catalysis as a fungistatic mechanism, *Science* **100**: 294–295, 1944.

CHAPTER 13

METABOLIC PRODUCTS

The most important product of fungus metabolism is carbon dioxide, and the most important function of the fungi in the economy of nature is the destruction of plant and animal remains. The use of fungi for food antedates written history. The use of fungi for the preparation of bread and wine developed as a household art. From the time of Pasteur, the study of fermentation has led to an ever-increasing knowledge and understanding of the activities of microorganisms. The production of antibiotics and vitamins, alcohol and organic acids, and the potential utilization of waste agricultural products are current fields of research and industrial activity. For extensive treatment of these subjects the reader is referred to Prescott and Dunn (1949) and Foster (1949).

DECOMPOSITION OF ORGANIC MATERIALS

Brefeld (1908) called fungi "Organismen der Verwesung" and considered them to be indispensable agents in maintaining the essential-element balance of nature. Saprophytic fungi and bacteria prevent the accumulation of plant and animal debris and return the elements that compose these materials to the storehouse of nature, where they are reused by new generations of plants and animals. In this role, saprophytic fungi are designated as "vegetable vultures" by Rolfe and Rolfe (1926), for they act as scavengers in the plant world.

Green plants assimilate carbon in the form of carbon dioxide. Waksman (1938) has assembled the data with regard to the amount of carbon in the biosphere. It is estimated that the atmosphere contains 600 billion tons of carbon in the form of carbon dioxide, and plants are estimated to remove 16 billion tons yearly. Thus, the carbon content of the atmosphere is sufficient for about 40 years, if no carbon dioxide were returned to the air.

The complete destruction of plant and animal remains by fungi and bacteria requires a long time, although some plant constituents, such as soluble sugars and other carbohydrates, are quickly utilized. Presumably the fungi are the most important organisms in this process. Other plant constituents, such as the waxes and lignin, are attacked more slowly. The more resistant constituents are slowly modified to form

humus. Some of the carbon and other essential elements is converted into bacterial and fungus protoplasm, which after death is subject to decay. In the end, humus is converted into carbon dioxide, water, and other simple compounds, which are used again. The importance of humus as a soil constituent is ably discussed by Waksman (1938). In addition to the carbon cycle, the fungi also play an important part in the cycles involving the release and utilization of the other essential elements.

FUNGI AS FOOD

Many curious details about the early use of fungi as food have been collected from classical and other sources by Buller (1914) and by Rolfe and Rolfe (1926). The mushrooms were no doubt among the first fungi used as food by man. Yeast became part of his diet when the arts of brewing and baking were discovered. The widespread use of fermented beverages, under certain dietetic circumstances, has an important bearing on nutrition and health. J. S. Wallerstein (1939) has discussed primitive brewing practices and the geographical distribution of the art. The beer of the Middle Ages was turbid, owing to its content of suspended yeast cells (Thaysen, 1943).

The nutritive value of any food depends upon its composition and digestibility and the assimilability of its hydrolytic products. The early writers, in the absence of precise information, were of the opinion that fungi had little value as food. The nutritive value of fungi, of yeast in particular, will be discussed from the standpoint of protein content and value, vitamins, fats, and minerals.

Assuming good digestibility, the value of fungus protein is determined by its amino-acid composition. Rose (1938), in a long series of careful experiments, has determined which amino acids are essential for man and animals. Some nine or ten amino acids were found to be essential (Table 20). If the protein part of a diet is deficient in a *single* essential amino acid, nitrogen is lost from the body, or inefficient utilization of protein results. More of a poor protein must be consumed in order to increase the intake of essential amino acids to satisfactory levels. The amino-acid composition of yeast and some other proteins is given in Table 46. Yeast protein compares favorably with casein or meat with respect to essential amino acids.

Less complete data are available for the amino-acid composition of fleshy fungi. According to Lintzel (1941), the proteins of *Psalliota campestris*, *Cantherella cibarius*, *Boletus edulis*, and *Morchella esculenta* are about equal to animal protein. From 100 to 200 gr. (dry weight) of these mushrooms was required to maintain the nitrogen balance in a man weighing 70 kg. Fitzpatrick *et al.* (1946) found the tryptophane content of *P. campestris* to be 5 mg. per 100 g.

TABLE 46. APPROXIMATE AMINO-ACID COMPOSITION (IN PER CENT) OF SOME PLANT AND ANIMAL PROTEINS CALCULATED TO 16 PER CENT NITROGEN

(Block and Bolling, *Arch. Biochem.* **7**, 1945. Published by permission of Academic Press, Inc.)

Amino acid	Yeasts*		Meat	Casein	Corn gluten	Polished rice
	Max.	Min.				
Arginine	5.3	3.1	7.7	4.1	3.1	7.2
Histidine	3.1	2.3	2.9	2.5	1.6	1.5
Lysine	8.1	6.7	7.2	7.5	0.8	3.2
Tyrosine	3.7	3.4	3.4	6.4	6.7	5.6
Tryptophane	1.5	1.2	1.3	1.2	0.7	1.3
Phenylalanine	4.6	2.9	4.9	5.2	6.4	6.7
Cystine	1.1	0.9	1.3	0.4	1.1	1.4
Methionine	2.8	2.6	3.3	3.5	4.0	3.4
Threonine	6.0	5.1	5.4	3.9	4.1	4.1
Leucine	8.5	6.1	7.7	12.1	24.0	9.0
Isoleucine	6.2	5.5	5.2	6.5	5.0	5.3
Valine	5.9	4.6	5.7	7.0	5.0	6.3

* Eight strains analyzed.

The value of fungus protein in nutrition can be assessed only in relation to the amino-acid composition of the remainder of the diet. If the dietary proteins are low in certain essential amino acids, the supplementary value of yeast (or other) protein may be great. The cereal grains, which furnish the bulk of protein for the population of the world, are generally low in one or more essential amino acids. Usually cereal protein is low in lysine or tryptophane or both. Sure (1946, 1947) studied the effect on the growth of rats of adding 1, 3, and 5 per cent of dried yeast to diets which contained cereals as the sole source of protein. The most marked effect of yeast occurred on a maize diet. At the end of a 10-week experimental period the rats receiving only cereal weighed 27.3 g., while the rats which received an additional 1 per cent yeast weighed 50.5 g. Rats which received the cereal plus 3 and 5 per cent yeast weighed 91.8 and 109.9 g., respectively. The effect of yeast was not so great when wheat or rice supplied the protein in the diet. In general, the most promising use of yeast protein in human nutrition is as a supplement rather than as a sole source of protein.

Yeasts are efficient in absorbing and concentrating the vitamins present in the media in which they grow (Gorcica and Levine, 1942). The relative value of yeast as a source of vitamins depends upon the vitamin content of the other constituents of the diet. The prevalence of vitamin deficiency diseases (beriberi, pellagra, and others) is evidence that the vitamin content of many diets is inadequate.

A dramatic demonstration of the value of yeast as a source of vitamins is reported by Bray (1928), onetime medical officer, Nauru, Central Pacific. The mandating government prohibited the brewing of toddy (palm wine) and allowed the sale of refined sugar. The results of these dietary changes were appalling. Soon, 40 per cent of the infants born in 1 year perished of infantile beriberi (thiamine deficiency) before reaching the age of 6 months. The restoration of toddy and enforced consumption of the *dregs*, *i.e.*, the yeast, reduced the incidence of beriberi to one death in 16 months. Truly, Bray was right in calling toddy the elixir of life of the Nauruans. Platt and Webb (1945) have noted that a simple maize diet which was inadequate with respect to riboflavin and nicotinic acid was made adequate in these respects by converting a portion of the dietary maize into maize beer.

The vitamin content of yeasts depends upon the species or strain and the conditions of cultivation. Some representative data are presented in Table 47.

TABLE 47. VITAMIN CONTENT OF SEVEN FOOD YEASTS

Results in milligrams per 100 g. of dry yeast. (Von Loesecke, *Jour. Am. Dietet. Assoc.* **22,** 1946. Published by permission of the American Dietetic Association.)

Species	Thiamine	Riboflavin	Nicotinic acid	Pantothenic acid
Torula utilis	1.7	4.7	19.0	86.0
*Saccharomyces cerevisiae**	17.0	8.0	25.0	112.0
S. cerevisiae	20.5	7.6	29.0	122.0
S. cerevisiae†	17.5	4.2	48.0	86.0
S. cerevisiae†	17.5	4.5	37.0	72.0
S. cerevisiae†	16.0	3.6	32.0	74.0
S. cerevisiae‡	3.0	7.5	38.0	13.5

* Six per cent salt added.
† Debittered brewer's yeast.
‡ Primary yeast.

The production of fats by fungi is discussed elsewhere in this chapter. The usual fatty acids, including palmitic and oleic acids, are found in fat synthesized by fungi. Apparently few studies have been made on the value of fungi as sources of fat and essential minerals in human nutrition.

CULTIVATION OF FUNGI FOR FOOD

The ants were perhaps the first to cultivate fungi as a source of food (see Leach, 1940, for discussion and references). Fungi have been used for centuries in the Orient as food for man. The Chinese grow *Hirneola polytricha* and the Japanese grow *Armillaria shii-take* on oak saplings. The mushroom cultivated almost exclusively in the Occident is *Agaricus*

(*Psalliota*) *campestris*. The method of cultivating this species on composted horse manure was developed near Paris before 1700. For information on mushroom growing the reader is referred to Duggar (1915).

While attempts to replace composted horse manure by other substrates have been made, none appears to be entirely satisfactory. Humfield (1948) has suggested that *Psalliota campestris* be grown in large fermentors and the mycelium rather than the fruit bodies be used for food. Asparagus butt juice, a waste agricultural product, is a suitable medium. The chemical composition of mycelium and that of the fruit bodies is similar and the flavor comparable. This approach perhaps offers a way to cultivate other desirable species, including the morels and the truffles. Nord (1948) has suggested that the mycelium of *Fusarium lini* be used for food.

The use of yeasts to convert low-grade carbohydrates, such as wood sugar and molasses, into food has interesting possibilities. It is necessary to fortify these carbohydrates with other nutrients for the cultivation of yeast. Phosphates, a source of potassium, and nitrogen, in the form of urea, ammonia, or ammonium salts, are added. The function of yeast is to convert inorganic nitrogen into protein. Animals are unable to assimilate ammonia or urea directly but require nitrogen in the form of protein or amino acids. Inorganic nitrogen may be converted into proteins by green plants or by certain microorganisms. The use of urea, a derivative of ammonia, as cattle fodder is an example of the synthesis of protein by the microflora of the rumen.

The possibility of using wood waste for yeast propagation was investigated in Germany during the First World War. In 1944 it is reported that 9,000 tons of food yeast were produced in Germany. Fermentable carbohydrates are obtained from wood as a by-product of sulfite paper manufacture, or by direct hydrolysis. Before sulfite liquor or wood hydrolysate is used for yeast culture, it is treated with calcium carbonate to adjust the pH and precipitate impurities. After the addition of nutrients the solution is heavily inoculated with the desired strain of yeast. Aeration is necessary for high yields of yeast. The weight of yeast produced amounts to about half the weight of sugar utilized. Such yeast is approximately 50 per cent protein (Harris *et al.*, 1948). The economics of fodder-yeast production from sulfite liquor have been studied by Schleef (1948). The use of by-product molasses for the production of food and fodder yeasts should offer fewer technical difficulties than the use of wood sugar.

FAT PRODUCTION

Serious efforts to utilize fungi for the synthesis of fats were made in Germany during the First World War and continued thereafter. The technical problems encountered proved difficult, but some success was achieved by 1942 (Hesse, 1949). The controlling factor in fat production

appears to be the carbon-nitrogen ratio. As long as an adequate supply of nitrogen is present, little fat is synthesized. If the carbohydrate supply is high when the nitrogen is exhausted, assimilable fat is synthesized. Linder (1922) termed these two phases *protein generation* and *fat generation.* Fat-laden cells of many fungi appear to be incapable of cell division. Fat formation takes place only in the presence of an abundant supply of oxygen. The relation between sugar concentration and amount of fat synthesized by *Penicillium javanicum* is illustrated in Fig. 52.

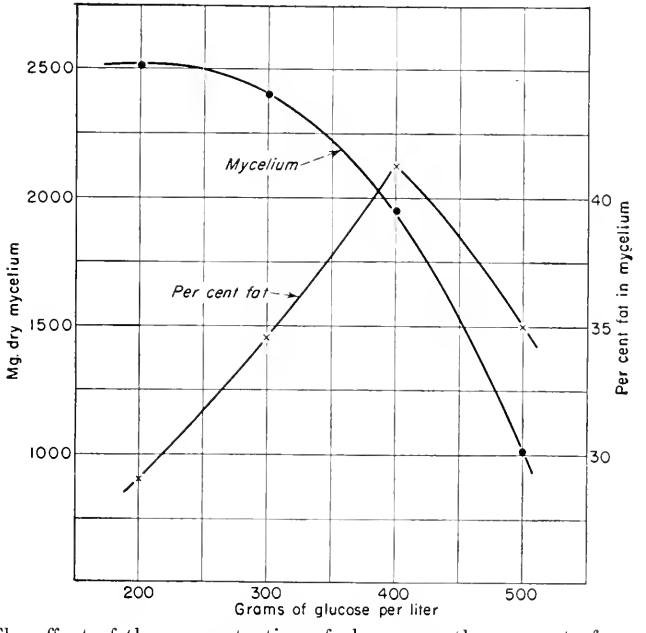


FIG. 52. The effect of the concentration of glucose on the amount of mycelium and amount of fat synthesized by *Penicillium javanicum* cultured in 75 ml. of medium for 12 days. (Drawn from the data of Lockwood, *Catholic Univ. of America Biol. Ser.* 13, p. 8, 1933. Published by permission of the Catholic University of America.)

Among the fungi investigated for fat synthesis are *Endomyces vernalis, Oidium lactis, Torula utilis, Rhodotorula glutinis,* and species of *Aspergillus, Penicillium, Mucor,* and *Fusarium.* From a practical standpoint, only fungi which are capable of synthesizing fat in submerged culture are of potential value. *E. vernalis* and *O. lactis* do not produce fat efficiently in submerged culture. The fat content of various filamentous fungi was determined by Preuss *et al.* (1934) and Ward *et al.* (1935). The use of *E. vernalis* for fat and protein synthesis has been reviewed by Raaf (1941). Starkey (1946) studied fat production by an unidentified soil yeast, which under favorable conditions contained from 50 to 63 per cent

crude lipide. A list of species of *Penicillium* and *Aspergillus* which synthesize considerable fat is given in Table 48.

TABLE 48. THE CRUDE FAT CONTENT OF DRIED MYCELIUM OF VARIOUS SPECIES OF *Penicillium* AND *Aspergillus* AS DETERMINED BY EXTRACTION WITH ETHER
(Ward *et al.*, *Ind. Eng. Chem.* **27**, 1935. Published by permission of the American Chemical Society.)

Species	Crude Fat, %
Penicillium flavo-cinereum	28.5
P. piscarum	26–28
P. oxalicum	24.4
P. roqueforti	22.9
P. javanicum	22.2
Aspergillus flavus	16.0

Various theories of the mechanism of fat synthesis have been published and are reviewed by Foster (1949) and Hesse (1949). Most of these consider acetaldehyde or acetate to be the product of intermediary metabolism used in fat synthesis. This emphasizes the importance of pyruvic acid in fungus metabolism. Various investigators have shown that acetaldehyde may be converted into fat by yeasts. The glycerol required for fat synthesis is thought to arise from the reduction and hydrolysis of dihydroxyacetone phosphate or 3-phosphoglyceric aldehyde (scheme VI, Chap. 7).

PRODUCTION OF VITAMINS

Only a few species of fungi and bacteria produce vitamins in large enough amounts to be of interest in industry. Biological synthesis must compete with chemical synthesis on a cost basis. The recovery of vitamins as a by-product of commercial processes or the use of waste materials as the basis of a cheap medium may make biological synthesis attractive.

Riboflavin is produced so abundantly by *Candida guilliermondi* under certain cultural conditions that it crystallizes in the medium (Burkholder, 1943). Among the factors found to influence the amount of riboflavin synthesized, the sources of carbon and nitrogen and aeration are important. Various investigators have found the concentration of iron in the medium to have a profound influence on the amount of riboflavin synthesized by various organisms. Iron concentrations in excess of 10 μg per liter decreased the amount of riboflavin synthesized by *C. guilliermondi* and *C. flareri* (Tanner *et al.*, 1945; Tanner and Van Lanen, 1947). The optimum iron concentration for riboflavin synthesis by *Clostridium acetobutylicum* is said to be 1 mg. per liter. Hickey (1945) has suggested the use of 2,2′-bipyridine to inactivate excessive concentrations of iron in industrial fermentations. By maintaining the iron concentration between 40 and 60 μg per liter, Levine *et al.* (1949) found the maximum yields of riboflavin produced by *C. guilliermondi* and *C. flareri* to be 175 and 567 μg per ml., respectively. Pilot-plant yields were somewhat less

Eremothecium ashbyi was shown to produce as much as 157 mg. per liter of riboflavin when cultivated on glucose-peptone medium (Renaud and Lachaux, 1945). Aeration was necessary. Foster (1947) has recommended a molasses medium for the commercial production of riboflavin by *E. ashbyi*. The closely related species, *Ashbya gossypii*, also synthesizes riboflavin in large amounts (Tanner *et al.*, 1949).

Peltier and Borchers (1947) determined the amount of riboflavin produced by 240 isolates of soil fungi when grown on wheat bran. Forty-five isolates produced 2 mg. or more of riboflavin per 100 g. of dry mold bran. An unidentified species of *Aspergillus* produced 5.8 mg. of riboflavin per 100 g. of substrate. Species of *Fusarium* and *Aspergillus* were outstanding producers of riboflavin.

The commercial microbiological synthesis of riboflavin depends upon the use of either *E. ashbyi* or *C. acetobutylicum* (Tanner *et al.*, 1949).

Vitamin B_{12} was isolated in crystalline form from liver and shown to contain cobalt (Rickes *et al.*, 1948; Smith, 1948). It is the only vitamin so far discovered which contains a metal as an integral part of the molecule. *Streptomyces griseus* and other microorganisms synthesize this vitamin. Sheep and cattle pastured on cobalt-deficient soils (Florida, Australia, New Zealand) develop a deficiency disease. Ingested cobalt is more effective than injected cobalt in overcoming this condition. It may be assumed that cobalt is used in the synthesis of vitamin B_{12} by the action of the microorganisms of the rumen and intestine. Vitamin B_{12} appears to be the anti-pernicious-anemia factor (West, 1948). Whether it is the animal protein factor is undecided. The cow-manure factor may be vitamin B_{12} (Lillie *et al.*, 1948).

Until the structure of vitamin B_{12} is determined and methods of synthesis developed, certain natural products will remain the only source of this vitamin. The only organic moiety of vitamin B_{12} so far disclosed is 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (Brink *et al.*, 1950). Vitamin B_{12} is obtained as a by-product from various industrial processes, especially streptomycin production. It is evident that the medium must contain cobalt; within limits, the amount of vitamin B_{12} synthesized by *Streptomyces griseus* is a function of the cobalt content of the medium. Maximum synthesis was observed when the medium contained 1 to 2 mg. of cobalt per liter (Hendlin and Ruger, 1950).

None of the other vitamins appears to be synthesized by fungi in amounts which would make the latter attractive sources for the isolation of pure vitamins. The value of these vitamins in fungi used for food was discussed previously. Yeast can be fortified with thiamine so that it may serve as a therapeutic agent. By adding synthetic thiamine to an aerated yeast culture, yeast was produced which contained 6 mg. of thiamine per g. (Van Lanen *et al.*, 1942).

ENZYME PRODUCTION

The industrial production and use of enzymes from microorganisms in the Occident is fairly recent, although the use of fungi as amylolytic agents by the peoples of the Orient for the preparation of *koji* and other foods is an old art. For this purpose, mixed cultures of species of *Aspergillus* and *Rhizopus* are grown upon the rice or soybean substrates, the enzymes being used without separation. The pioneering work of Takamine (1914) on the amylases of *A. oryzae* was especially important.

The ability of fungi to produce *amylase* is widely distributed, but only a few species are used commercially for this purpose. The amount of amylase produced varies with the species or isolate and the cultural conditions. Le Mense *et al.* (1947) screened 359 isolates of *Penicillium* and *Aspergillus* and found 42 isolates to produce amylase in submerged culture. The activity of the species of *Penicillium* ranged from 0.1 to 0.6 enzyme unit per milliliter of culture medium. One isolate of *A. niger* (NRRL 337) was found to be especially adapted for the production of amylase in submerged culture. The production of amylase was highly dependent upon the composition of the medium. Corn meal was especially valuable in increasing amylase production when added to basal media composed of corn steep liquor, dried tankage, soybean meal, or thin stillage. Amylase production was stimulated by the addition of 10 to 20 mg. of sodium chloride per liter of culture medium. Addition of a mixture of chlorinated phenols (Dowcide G) inhibited sporulation and increased amylase production (Erb *et al.*, 1948).

Others have found different isolates of the same species to produce varying amounts of amylase. Hao *et al.* (1943) studied the production of amylase by 27 isolates of various species of fungi when grown upon wheat bran. *A. oryzae*, *Rhizopus delemar*, and *R. oryzae* produced the largest amounts of amylase. *A. oryzae* was the fungus of choice because of ease of handling.

In practice, fungus amylases are produced and utilized in three general ways. (1) In the amylo process, starch is solubilized by autoclaving with a trace of a mineral acid, and the mash is inoculated with a species of *Rhizopus*, which produces amylase abundantly, and a species of yeast. The function of the *Rhizopus* species is to convert the starch into fermentable sugars, from which the yeast produces alcohol. For a description of this process see Owen (1933). (2) The fungus may be grown upon a solid substrate such as bran and the resulting moldy mass (mold bran) dried (Underkofler *et al.*, 1946). The fresh material may be used without drying (Roberts *et al.*, 1944). (3) Fungus amylases may be produced in submerged aerated cultures much as antibiotics are produced. The culture medium may be used directly to replace malt as a saccharifying agent.

Fungus amylases are used to replace malt amylase for the saccharification of starch. Myrbäck (1948) is of the opinion that amylase from *A. niger* is an α-amylase, but it differs from α-amylase of malt in that it has a higher capacity for saccharification. For a comparison of fungus and malt amylase and the economic considerations involved, see Underkofler *et al.* (1946). The yield of alcohol is said to be slightly higher when fungus amylase is used in place of malt for saccharification.

Fungi are the source of other enzymes of commercial interest, including *pectinase* and *sucrase*. Pectinase is used in the clarification of fruit juices. For a survey of the commercial production of fungus enzymes see L. Wallerstein (1939).

ALCOHOLIC FERMENTATION

Yeasts are used almost exclusively for the commercial production of fermentation alcohol, but alcoholic fermentation is not restricted to these fungi. Pasteur (1872) observed that *Penicillium glaucum*, *Aspergillus glaucus*, and *Mucor racemosus* produced alcohol under anaerobic conditions. Further information on alcohol production by filamentous fungi may be found in the monograph of Raistrick *et al.* (1931), who determined complete carbon balances for 96 species of *Aspergillus*, 75 species of *Penicillium*, 8 species of *Citromyces* (*Penicillium*), 23 species of *Fusarium* and 36 miscellaneous species. The original report should be consulted for details and the *quantitative* methods used. All the 23 species of *Fusarium* studied produced alcohol. From this and other reports in the literature, it must be concluded that this property is common among species of this genus. Many species of *Aspergillus* and *Penicillium* produced alcohol, as did species of other genera. Only a few of the species studied failed to produce detectable amounts of alcohol. The apparatus used in these studies is shown in Fig. 53.

The concentration of alcohol which inhibits the growth of fungi varies with the species or strain. In general, yeasts are more tolerant of alcohol than the filamentous fungi. The upper limit for most yeasts is about 12 per cent alcohol, although some strains are more tolerant. The susceptibility to alcohol limits the alcohol concentration of naturally fermented beverages. The rate of fermentation decreases as the concentration of alcohol increases.

Not all isolates of a species are equally efficient in producing alcohol. For example, eight isolates of *Fusarium lini* produced varying amounts of alcohol on the same medium. The more virulent pathogens on flax produced the most alcohol (Letcher and Willaman, 1926). A correlation between sporulation and alcohol production by *Aspergillus flavus* was noted by Yuill (1928). In general, sporulating cultures produced less alcohol than nonsporulating cultures.

The most important condition which governs alcoholic fermentation

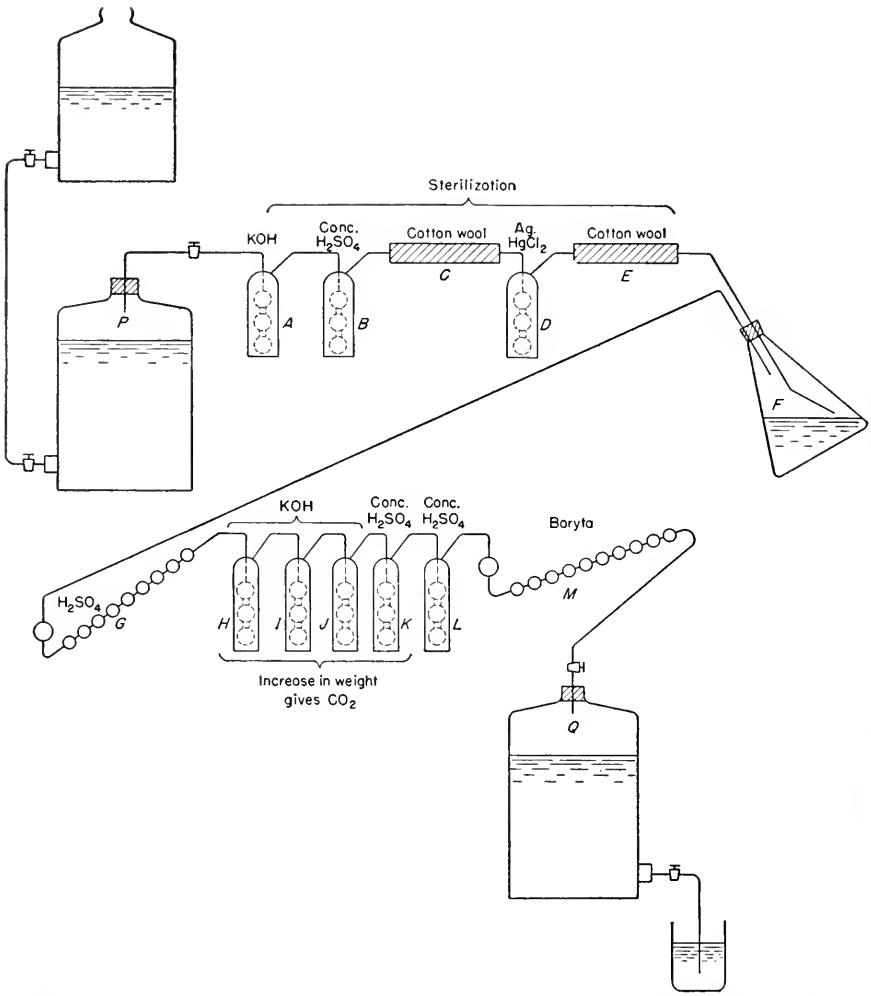


FIG. 53. Apparatus for studying the metabolic products of fungi and other microorganisms. The apparatus consists of five units: A gasholder, *P*; a train for the purification and sterilization of air or other gases, *A–E*; the culture flask, *F*; a train for the quantitative absorption of carbon dioxide, *H–M*; an aspirator, *Q*, for the collection of gaseous products of metabolism other than carbon dioxide. (Redrawn from Birkinshaw and Raistrick, *Trans. Roy. Soc.* (*London*), Ser. B, **220**: 14, 1931. Published by permission of the Royal Society.)

is the supply of oxygen. The relation between fermentation and anaerobic conditions was recognized by Pasteur, who summarized his extensive investigations on fermentation as "la vie sans air." The essential feature of fermentation is anaerobic dissimilation of carbohydrates. Growth and fermentation are competitive processes, for fungi require oxygen for growth. In practice it is advantageous to carry out fermentations in the

presence of some air, especially at the start. This allows some increase in the number of cells and reduces the amount of inoculum required. The amount of oxygen available to submerged mycelium or cells, unless vigorous aeration is used, is insufficient to inhibit alcoholic fermentation by certain species.

Alcoholic fermentation has been studied since the time of Lavoisier. Few fields of study have been so valuable in increasing our understanding of the life processes of microorganisms. Harden (1932) has concisely reviewed the early work and theories on fermentation. The idea that yeasts as living fungi were the proximate cause of fermentation did not gain acceptance for many decades. The eminent Wöhler (1839) ridiculed this idea in a lively skit, in which he declared that he had followed the entire process microscopically. Briefly, he states that the responsible organism developed from an egg and had the shape of a Beindorf distilling flask; " . . . diese Infusorien fressen Zucker, entleeren aus dem Darmkanal Weingeist, und aus den Harnorganen, Kohlensaure."

The enzymatic nature of alcohol fermentation was established by Buchner (1897). The enzymatic transformations involved in fermentation were discussed in Chap. 7. Further information and references may be found in Summer and Somers (1947), Tauber (1949), Prescott and Dunn (1949), Meyerhof (1944, 1949), Nord and Mull (1945), and Foster (1949).

The larger part of the world-wide fermentation industry is devoted to the production of ethyl alcohol. During the war year of 1945 some 600 million gallons of 95 per cent ethyl alcohol was produced in the United States alone. Less than one-third this amount was produced in 1948. Of this amount 64 per cent was produced by fermentation (Lee, 1949). While any source of fermentable sugars may be used for the production of alcohol, the more common raw materials include molasses, starch from various sources, hydrolyzed cellulose or wood sugar, and fruit juices. It is beyond the scope of this text to discuss the commercial production of industrial and beverage alcohol. For information on these subjects see Prescott and Dunn (1949).

ORGANIC ACIDS

Many fungi synthesize organic acids, which accumulate in the medium. These acids include oxalic, citric, succinic, fumaric, malic, lactic, itaconic, kojic, gluconic, and others. Commonly, a species may synthesize a variety of related acids. The isolates of a given species may differ widely in synthetic capacity. To obtain maximum yields, it is necessary to control nutritional and environmental factors closely. The optimum conditions for one isolate may differ from those of another isolate of the

same species. Acid production by fungi is discussed in detail by Foster (1949), Prescott and Dunn (1949), and Walker (1949).

The meaning of the term fermentation has been expanded by most authors to include aerobic as well as anaerobic processes. The production of most organic acids and antibiotics by fungi takes place in the presence of oxygen, and these processes are not fermentations in the restricted (anaerobic) sense of the term. Indeed, adequate aeration is one of the salient features of such processes. Aeration may be achieved by cultivating the fungi on the surface of shallow layers of medium in pans or trays; or the fungi may be cultivated in closed tanks, which may contain as much as 15,000 gal. of medium. Aeration is provided by mechanical stirring and blowing in sterile air under pressure.

The organic acids discussed in this chapter are derived from carbohydrates present in the medium. In general, media highly unbalanced with respect to carbohydrates are used. The balanced medium developed by Steinberg for the cultivation of *Aspergillus niger* (Chap. 2) has a carbon-to-nitrogen ratio of 29 to 1, while the medium recommended by Currie (1917) for the production of citric acid by *A. niger* has a carbon-to-nitrogen ratio of 72 to 1. A fungus first utilizes the nutrients in the unbalanced medium for the production of mycelium (growth phase). The excess carbohydrate which remains when the nitrogen is exhausted is dissimilated ("fermentation" phase). Advantage is taken of such preformed mycelium, for if the original medium is replaced by fresh medium, the mycelium continues to dissimilate carbohydrate. The replacement medium is frequently more unbalanced than the growth medium. For example, Karow and Waksman (1947) used for *A. wentii* a growth medium with a carbon-to-nitrogen ratio of 135 to 1, while the replacement medium had a carbon-to-nitrogen ratio of 270 to 1.

Economic amounts of organic acids may accumulate in the medium because the normal use of these compounds for the synthesis of mycelium is prevented by the imposed experimental conditions. If the nitrogen supply is exhausted, no more protoplasm can be formed. The mycelium then dissimilates sugars enzymatically. Enough nutrients are supplied in replacement media to repair and maintain the enzyme systems of the fungus in a vigorously functioning state. The enzymes, other than those concerned with certain phases of carbohydrate dissimilation, are largely idle because of the lack of suitable substrates.

A fungus commonly produces several organic acids at the same time. Citric and oxalic acids are produced by many isolates of *A. niger*, and the relative amounts of these acids may be varied by controlling the pH of the medium. In general, a highly acid medium (pH 2.0 to 3.0) favors the synthesis of citric acid, while less acid media favor the production of oxalic acid.

Citric acid. Wehmer was the first to recognize the commercial possibilities of citric acid synthesis by two species of *Citromyces* (*Penicillium*). Selected isolates of *Aspergillus niger* appear to be used in industry, although the property of producing citric acid is common to many fungi. The following fungi have been suggested for commercial citric acid production (Von Loesecke, 1945): *Citromyces pfefferianus, C. glaber, C. citricus, Aspergillus carbonarius, A. glaucus, A. clavatus, A. cinnamomeus, A. fumaricus, A. awamori, A. aureus, Penicillium arenarium, P. olivaceum, P. divaricatum, P. sanguifluus, P. glaucum, Mucor pyriformis.*

The production of citric acid in the United States increased from about 5 million to 26 million pounds between 1935 and 1945 (Von Loesecke, 1945). Presumably most of this was "fermentation" citric acid. At present it is believed that most citric acid is produced by surface cultures.

Citric acid is formed from many sources of carbon. Sucrose is said to be the best carbon source for the production of citric acid. There is less agreement upon the value of other sugars. Different investigators have found glucose, fructose, and maltose to vary from good to poor. In part, this is to be attributed to the use of different isolates and different experimental conditions. Beet molasses is used in industry. The suitability of this substrate is said to vary with the source and year of production (Bernhauer and Knobloch, 1941). The evaluation of carbon sources is complicated by the metallic elements they contain, especially iron and manganese. Methods of treating beet and cane molasses to remove inhibiting impurities are described by Perlman *et al.* (1946), Gerhardt *et al.* (1946), and Karow and Waksman (1947). The inhibiting effect of metallic ions on the production of citric acid from sugars is illustrated by the data in Table 49.

TABLE 49. THE EFFECT OF REMOVING METALLIC CONTAMINANTS FROM THREE SUGARS, BY THE PROCESS OF CATIONIC EXCHANGE, ON THE PRODUCTION OF CITRIC ACID BY *Aspergillus niger*, WISCONSIN STRAIN 62

(Perlman *et al.*, *Arch. Biochem.* **11**, 1946. Published by permission of Academic Press, Inc.)

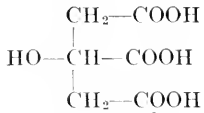
Sugar used	Treatment	Yield* of citric acid, %
Sucrose from cane	Not treated	21.4
	Treated	64.0
Sucrose from beet	Not treated	11.3
	Treated	66.8
Glucose	Not treated	20.5
	Treated	60.0

* Theoretical yield 123 per cent.

The production of citric acid in submerged culture was tried at an early date and abandoned in favor of surface culture. However, recent litera-

ture indicates that submerged culture may be the preferred process in the future. Average yields of 72 g. of anhydrous citric acid per 100 g. of sucrose in the medium have been obtained in the laboratory (Shu and Johnson, 1948).

The formula for citric acid is given below:



Citric acid

Any theory of citric acid formation must take into account the following facts: Citric acid, a branched-chain compound, is synthesized from carbon sources containing from two to seven carbon atoms. Yields of citric acid may approach 90 per cent of the sugar used (Wells *et al.*, 1936). The amount of carbon dioxide evolved is low, which suggests either reutilization or a mechanism of producing the necessary intermediates without the production of carbon dioxide. Reutilization of carbon dioxide seems the more probable, for Foster *et al.* (1941) showed *Aspergillus niger* to utilize radioactive carbon dioxide in the synthesis of citric acid. The more probable pathway of synthesis is via the Krebs cycle (Chap. 7) and the supplementary formation of oxalacetic from pyruvic acid and carbon dioxide (Wood-Werkman reaction).

Gluconic acid. A considerable number of fungi produce gluconic acid. These include *Aspergillus niger* (various isolates), *A. fuscus*, *A. cinnamomeus*, *A. oryzae*, *Penicillium glabrum*, *P. glaucum*, *P. purpurogenum* var. *rubrisclerotium*, *P. chrysogenum*, *P. crustaceum*, and *Fumago vagans*. Most investigators have used selected isolates of *A. niger* for the production of gluconic acid. Details of laboratory and semi-pilot-plant investigations may be found in the papers of Wells *et al.* (1937), Gastrock *et al.* (1938), and Porges *et al.* (1941).

Many factors influence the formation of gluconic acid. Isolates of *A. niger* differ in ability to synthesize this acid. Not all isolates produce the maximum amount of acid under identical conditions. Adequate aeration is necessary for the enzymatic conversion of glucose to gluconic acid. Gluconic acid is produced most abundantly when the pH of the medium is kept near 5. Calcium carbonate is used for neutralizing the gluconic acid formed. This is advantageous, for calcium gluconate is used in medicine as a source of readily assimilable calcium. Precipitation of calcium gluconate during formation may be prevented by the addition of boric acid or borax to the culture medium in amounts varying up to 2,000 p.p.m. (Moyer *et al.*, 1940). Boron compounds are added after the growth of mycelium is essentially complete. The mycelium may be used as many as thirteen times by removing the spent medium and adding

fresh medium with a high glucose content but low in other nutrients (Porges *et al.*, 1940, 1941).

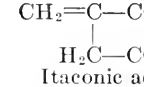
The production of gluconic acid appears to be a direct oxidation of glucose. The enzyme responsible for this transformation is called glucose aerodehydrogenase. This enzyme, when free from catalase, catalyzes a reaction between glucose and oxygen. Gluconic acid and hydrogen peroxide are the products formed. Glucose aerodehydrogenase was first isolated from *Penicillium chrysogenum* and was called notatin, or penicillin B, at first. Its antibiotic activity is due to liberation of hydrogen peroxide. For recent papers on this enzyme see Keilin and Hartree (1948, 1948*a*).

Lactic acid. Various lactic acid bacteria are used in the commercial production of lactic acid. These bacteria require a complex natural medium, which makes the purification of lactic acid laborious. Many species of Phycomycetes produce lactic acid, and species of *Rhizopus* are noteworthy in this respect. The following fungi produce lactic acid: *Rhizopus arrhizus*, *R. chinensis*, *R. elegans*, *R. japonicus*, *R. nodosus*, *R. oryzae*, *R. pseudochinensis*, *R. salebrosus*, *R. shanghaiensis*, *R. stolonifer*, *R. tritici*, *Mucor rouxii*, *Monilia tamari*, and *Blastocladia pringsheimii*. Most of these fungi appear to synthesize *d*-lactic acid, although *R. chinensis* synthesizes *l*-lactic acid (Saito, 1911).

The use of *R. oryzae* for production of lactic acid has been intensively investigated (Lockwood *et al.*, 1936; Ward *et al.*, 1936, 1938). Glucose appears to be the best sugar. Nitrate nitrogen is not used by this fungus. Calcium carbonate is used in the medium to neutralize lactic acid as it is formed. Yields increase when the cultures are aerated. As much as 75 per cent of the glucose utilized is converted into lactic acid. The presence of added zinc increases mycelial growth but depresses the yield of lactic acid.

The mechanism of lactic acid production by fungi is ably discussed by Foster (1949). Under anaerobic conditions, ethyl alcohol, carbon dioxide, and lactic acid are produced in equimolecular amounts. The amount of lactic acid produced under aerobic conditions increases, while the amount of alcohol decreases (Waksman and Foster, 1939). The most probable intermediate for the production of lactic acid is pyruvic acid.

Itaconic acid. *Aspergillus itaconicus* was the first fungus reported to synthesize itaconic acid. The structural formula below shows that this unsaturated acid is related to succinic acid.



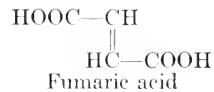
Itaconic acid

The fungi which have been tested for itaconic acid production are mainly selected isolates of *A. terreus*. Relatively few isolates produce sufficient itaconic acid to have commercial possibilities (Calam *et al.*, 1939; Moyer and Coghill, 1945).

Various attempts have been made to produce mutants of *A. terreus* by irradiating conidia with ultraviolet light (Raper *et al.*, 1945). Less success attended these efforts than comparable treatment of conidia of *Penicillium chrysogenum* for obtaining mutants with enhanced penicillin production.

Among the factors which affect the production of itaconic acid by isolates of *A. terreus* are the composition of the medium, hydrogen-ion concentration, temperature, and aeration. Glucose and ammonium nitrate appear to be the best sources of carbon and nitrogen. The pH range in which itaconic acid accumulates is narrow and low, 1.9 to 2.3. The aluminum ion is toxic to *A. terreus*, but aluminum trays may be used if the concentration of magnesium ion in the medium is high. As much as 4.75 g. of magnesium sulfate heptahydrate per liter of medium may be used. It is probable that this high concentration of magnesium ion also enables the fungus to withstand low pH values (Lockwood and Ward, 1945).

Fumaric acid. This unsaturated, four-carbon, dicarboxylic acid is produced by many fungi, although only a relatively few species synthesize large amounts. With few exceptions, the fungi which synthesize fumaric acid in significant amounts are Phycomycetes. The formula for fumaric acid is given below:



Fumaric acid

The factors which affect the production of fumaric acid by *Rhizopus nigricans* were studied by Foster and Waksman (1939). The concentration of zinc was found to be especially important. Optimum production of fumaric acid occurred in cultures receiving less zinc than that required for optimum growth. Not all isolates of *R. nigricans* synthesized fumaric acid in equal amounts or under the same conditions. One isolate studied by Foster and Waksman (1939*a*) produced fumaric acid anaerobically and aerobically, whereas another produced fumaric acid aerobically only.

Various proposals have been made to explain the mechanism of fumaric acid formation. Anaerobic synthesis is thought to involve the formation of oxalacetic acid from pyruvic acid and carbon dioxide (Foster and Davis, 1948). The following steps would convert oxalacetic acid to fumaric acid: oxalacetate → malate → fumarate. It is probable that fumaric acid is produced aerobically from acetic acid as follows: 2 (ace-

tate) → succinate → fumarate (Thunberg-Wieland condensation). *R. nigricans* produces high yields of fumaric acid from both ethyl alcohol and acetic acid, which is evidence in favor of this scheme of formation (Foster and Waksman, 1939).

Other organic acids. Apparently, the first organic acid to be discovered as a product of fungus metabolism was *oxalic acid.* Many fungi in nature contain calcium oxalate crystals. This was noted as early as 1887 by De Bary. Many species of *Aspergillus* and *Penicillium* produce large amounts of oxalic acid, especially if enough alkali is present in the medium to convert the acid into an oxalate. Many species of *Aspergillus* which produce oxalate in the presence of a neutralizing agent also produce citric acid in acid media (Currie, 1917). For a recent discussion of oxalic acid production by fungi see Foster (1949).

Various species of *Aspergillus*, including *A. oryzae*, *A. flavus*, *A. nidulans*, *A. giganteus*, and some other fungi produce *kojic acid.* Kojic acid is a cyclic compound, a pyrone, and has been shown to have antibiotic properties (Morton *et al.*, 1945).

ESTERS

Among the esters reported to be formed by fungi are ethyl acetate, methyl cinnamate, methyl *p*-methoxycinnamate, and isobutyl acetate. Various reports are in the literature concerning a "banana-oil" odor being produced by fungi, but apparently amyl acetate has not been isolated and identified as a product of fungus metabolism. Ethyl acetate is produced by *Penicillium digitatum* (Birkinshaw *et al.*, 1931) and by *Endoconidiophora moniliformis* (Gordon, 1950).

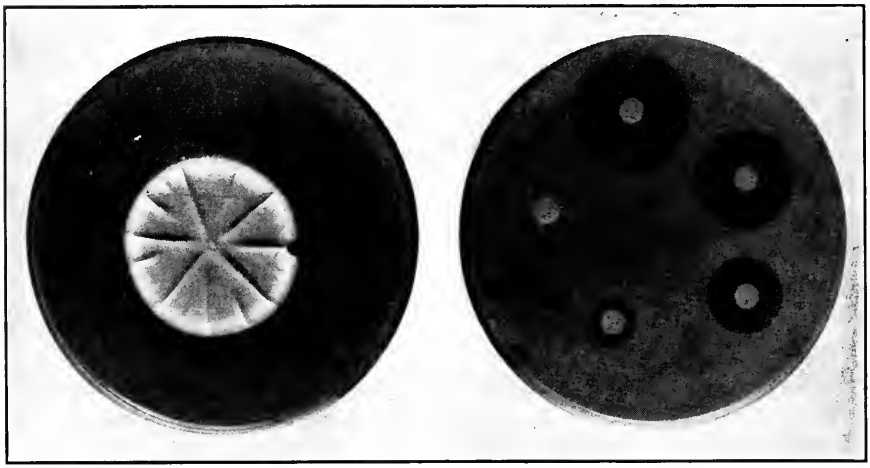
ANTIBIOTICS AND DRUGS

The inhibition of one organism by another is called *antagonism.* The phenomenon has been known since the time of Pasteur, and the subject has been reviewed by Waksman (1947) in a book containing over 1,000 references. Antagonism occurs in nature as well as in the laboratory and is of such common occurence that it is frequently overlooked. Examples are easily found by examining contaminated plates for clear areas around the contaminants. Antagonism may be due to competition for nutrients or to toxic substances. This discussion will deal with the toxic substances produced by fungi which inhibit fungi and bacteria.

General discussion. Fungi and other organisms produce a variety of toxic substances, which include enzymes, alkaloids, toxins, simple and complex organic compounds, and inorganic compounds. Organic compounds produced by fungi and other organisms, especially bacteria and actinomycetes, which inhibit the life processes of microorganisms are called *antibiotics.* Waksman (1947) would restrict the term antibiotic

to organic compounds produced by microorganisms which inhibit the functioning of other microorganisms. General usage of the term antibiotic is, however, wider than this and applies the term to those organic compounds of fairly simple structure produced by organisms which inhibit microorganisms. These substances are referred to more specifically as *antibacterial*, *antifungal*, or *antiviral* substances.

There are no universal antibacterial or antifungal substances. Antibiotics are specific in action. Penicillin, for example, is active against



A *B*

FIG. 54. Method of assay for antibiotics. *A*, control culture of *Penicillium notatum* on agar medium; radial series of plugs cut at 6 days. *B*, agar-plug assay plate showing zones of inhibition of *Staphylococcus* developed after agar blocks removed from *A* have been incubated for 16 hr. at 37°C. (Courtesy of Raper, Alexander, and Coghill, *Jour. Bact.* **48**: 644, 1944. Published by permission of The Williams & Wilkins Company.)

many Gram-positive bacteria and only a relatively few Gram-negative organisms.

The occurrence of antibiotics is probably far more widespread than suspected at present. The reason for this lies in the way in which antibiotics are discovered. Antibiotics are detected by their inhibiting action on living organisms. A susceptible test organism is essential for the detection of an antibiotic. For obvious reasons, human pathogenic bacteria are most used for screening tests. If one desires to obtain antifungal substances active against pathogenic fungi, these fungi should be used as test organisms.

The same principle underlies all methods for detecting antibiotic action. The test organisms are brought into contact with the products elaborated by the organism suspected of producing an antibiotic. This may be done by growing two organisms on the same Petri dish. A clear zone between

the colonies indicates inhibition (Fig. 45). A second method consists in growing an organism on agar and cutting radially a series of agar plugs and placing these agar disks, which contain the antibiotic, on agar plates sown uniformly with the test organism (Raper *et al.*, 1944). This method is illustrated in Fig. 54. Other methods of detecting antibiotics have been summarized by Waksman (1947).

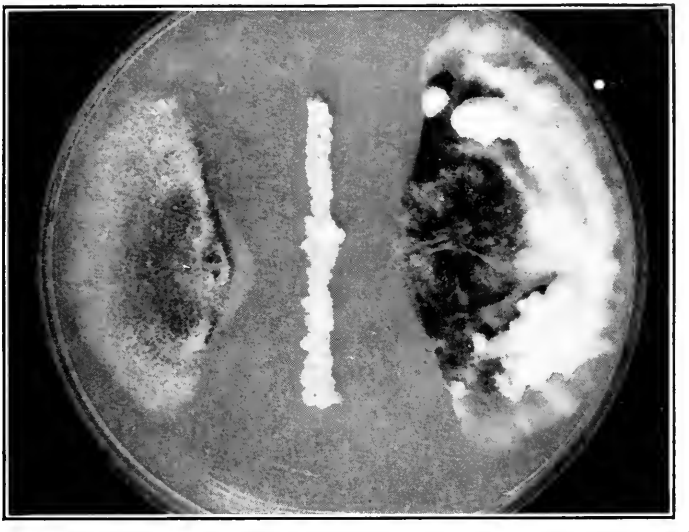


FIG. 55. The antibiotic effect of *Streptomyces* sp. on two plant pathogenic fungi, *Monilinia fructicola*, on the left, and *Helminthosporium sativum*, on the right.

The production of antibiotic substances by fungi is common. In a screening test of over 400 species, which included over 300 wood-inhabiting fungi and 22 dermatophytes, somewhat over 200 species produced substances active against *Staphylococcus aureus* and *Escherichia coli* (Robbins *et al.*, 1945). A large number of Basidiomycetes and other fungi have been tested for the presence of antibiotics by Wilkins and Harris (1944). The actinomycetes are the source of many useful antibiotics including streptomycin, chloromycetin, aureomycin, terramycin, and other unidentified compounds (Waksman, 1947). With the exception of *Phytophthora erythroseptica* none of the Phycomycetes appear to have been reported as producing antibacterial substances. For a survey of Fungi Imperfecti in the role of producing antibacterial substances (against *Staphylococcus aureus*) and antifungal substances (against *Botrytis allii*), see Brian and Hemming (1947). The inhibiting effect of *Streptomyces* sp. on two plant pathogenic fungi is shown in Fig. 55.

Many soil organisms produce antibiotics. Whether these organisms produce antibiotics in sufficient amounts to inhibit plant pathogens under natural conditions in the soil is not certain. It is known, however, that

the incidence of certain diseases may be decreased by adding certain bacteria, actinomycetes, and fungi to soil. For references, see Grossbard (1948), Henry (1931), Waksman (1937), and Anwar (1949).

The influence of various soil-inhabiting organisms in decreasing infection of barley by *Helminthosporium sativum* has been reported by Anwar (1949). Figure 56 illustrates some of these results. It is by no means certain that these effects were due to the antifungal substances produced by the antagonistic organisms.

FIG. 56. The effects of certain soil organisms on the pathonogenicity of *Helminthosporium sativum* on barley. Seedlings grown at 80°F. in steamed soil infested with: *A*, no organisms; *B*, *H. sativum* and *Bacillus subtilis: C*, *H. sativum* and *Penicillium* sp.; *D*, *H. sativum* and *Trichoderma lignorum: E*, *H. sativum*. (Courtesy of Anwar, *Phytopathology* **39**: 1011, 1949.)

The situation in soil is very complicated. Basic antibiotics such as streptomycin are adsorbed on clay; acidic antibiotics like clavacin are apparently held less firmly. Gottlieb and Siminoff (1950) are of the opinion that competition is more of a factor than antibiotic action as the cause of one organism inhibiting another in the soil. Thus, either *Aspergillus clavatus* or *Streptomyces griseus* inhibits the growth of *Bacillus subtilis* in soil. No difference was noted between a strain of *S. griseus* which produced streptomycin and one which did not.

Schatz and Hazen (1948) reported that 124 of the 243 soil *Actinomyces*

tested were antagonistic to four test human pathogens, *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton gypseum*, and *T. rubrum*.

TABLE 50. ANTIBIOTICS PRODUCED BY SOIL-INHABITING ACTINOMYCETES AND FUNGI (Brian, *Chem. Industry* **1949**. Published by permission of the Society of Chemical Industry.)

Organism	Antibiotic	Properties
Streptomyces griseus	Grisein	Antibacterial (Gram positive and negative); antirickettsial; *not* antifungal
	Actidione	Not antibacterial; antifungal
	Streptomycin	Antibacterial (Gram positive and negative and acid fast)
Nocardia gardneri	Proactinomycin	Antibacterial (Gram positive)
Actinomyces lavendulae	Streptothricin	Antibacterial (Gram positive); antifungal
Proactinomyces cyaneus	Litmocidin	Antibacterial (Gram positive and negative)
Streptomyces venezuelae	Chloromycetin	Antibacterial (Gram positive and negative); *not* antifungal; antirickettsial
Aspergillus flavus	Aspergillic acid	Antibacterial (Gram positive and negative); antifungal
A. terreus	Citrinin	Antibacterial (Gram positive and negative); antifungal
Fusarium orthoceras	Enniatin B	Antibacterial (Gram positive and acid fast); *not* antifungal
Penicillium brevi-compactum	Mycophenolic acid	Antibacterial (Gram positive and negative); antifungal
P. chrysogenum	Penicillin	Antibacterial (Gram positive); *not* antifungal
P. griseofulvum	Griseofulvin	Not antibacterial; antifungal
P. janczewiskii	Griseofulvin	Not antibacterial; antifungal
P. patulum	Patulin	Antibacterial (Gram positive and negative); antifungal
Trichoderma viride	Gliotoxin	Antibacterial (Gram positive and negative and acid fast); antifungal
	Viridin	Not antibacterial; antifungal

A list of antibiotics produced by some soil-inhabiting actinomycetes and fungi is given in Table 50. Note that some organisms produce more than one antibiotic and that the same antibiotic substance may be produced by more than one species. Organisms differ in susceptibility to antibiotics. This range of effectiveness is frequently called the *antibiotic spectrum*. Thus, *Penicillum luteum-purpurogenum* is some 12 thousand times as sensitive to gliotoxin as to streptomycin. Not all fungi are equally inhibited by the same concentration of an antibiotic; some 11

times as much clavacin is required to inhibit the growth of *Aspergillus clavatus* as *Trichophyton mentagrophytes* (Reilly *et al.*, 1945).

Fungi produce substances which are capable of inactivating certain plant viruses. The Basidiomycetes are especially noteworthy in this respect (Utech and Johnson, 1950). Extracts of *Trichothecium roseum* reduce infectivity of southern bean mosaic, tobacco mosaic, and tobacco necrosis viruses (Gupta and Price, 1950). These authors believe that this reduced infectivity is due to increased resistance of the host. There is no evidence which indicates that any of the known antibiotics are involved in the destruction of plant viruses. However, antibiotics are known which are effective against virus diseases in man.

Preliminary studies indicate that certain antibiotics may be used to control fungi which cause plant diseases. Actidione has been reported by Vaughn *et al.* (1949) to control powdery mildew on beans and roses. Actidione was toxic to young rose leaves at a concentration of 2.5 p.p.m. but less toxic to bean plants. Laboratory tests indicated that actidione is a fungistatic substance for a considerable number of plant pathogenic fungi, including *Sclerotinia fructicola, Cladosporium cucumerinum,* and *Colletotrichum lagenarium.* Further data on the effect of actidione on plant pathogenic fungi are reported by Whiffin (1950).

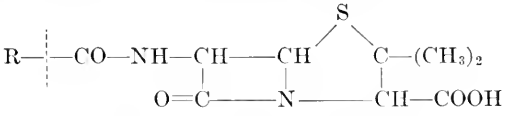
The protective action of an antibiotic obtained from an unidentified species of *Streptomyces* against *Venturia inaequalis* on apple has been reported by Leben and Keitt (1949). This antibiotic has been named *antimycin.*

Penicillin has been used successfully, to a limited extent, in controlling necrosis of giant cactus, caused by *Erwinia carnegieana* (Boyle, 1949). Injections of penicillin into the necrotic tissue apparently diffused through the plant tissues for some distance, killing the bacteria. This is one of the few cases in which an antibiotic has been used successfully in therapeutic treatment of plant disease.

The principal use of antibiotics is to control disease in man and animals. Only a relatively few antibiotics are useful for this purpose. In addition to killing or inhibiting pathogenic organisms, an antibiotic, to be useful in medicine, must be relatively nontoxic to the host. Some of the older and more useful antibiotics used in medicine will be discussed in greater detail on the following pages.

Penicillin. This antibiotic drug is produced in industry by selected isolates or mutants of *Penicillium chrysogenum* and *P. notatum.* The original isolate of Fleming produced from 2 to 4 units of penicillin per milliliter of culture filtrate. *P. chrysogenum Q*-176 has produced in excess of 1,000 units per milliliter. The synthesis of penicillin is not limited to species of the *P. chrysogenum-notatum* group but includes certain species of *Aspergillus* belonging to the *A. flavus* group. A few fungi

belonging to other genera also produce penicillin. Sterile culture conditions must be maintained at all times, as penicillin is rapidly destroyed by the enzyme, penicillinase, which is excreted by many bacteria. Penicillin is extracted from the "fermentation" liquid, or penicillin beer, either by extraction at pH 2.0 to 2.5 with water-immiscible solvents such



Type formula of the penicillins

as amyl acetate, or by adsorption on activated carbon. The extraction must be carried out quickly from acid solutions, owing to the instability of penicillin under these conditions. Penicillin forms crystalline salts with the alkali metals. The sodium salt is usually produced.

Table 51. Names and American and British-type Designations of Four Naturally Occurring Penicillins

R	Name	Type	
		American	British
$C_6H_5CH_2$—	Benzylpenicillin	G	II
CH_3—CH_2—CH=CH—CH_2—	Δ^2-Pentenylpenicillin	F	I
$HOC_6H_4CH_2$—	*p*-Hydroxybenzylpenicillin	X	III
$CH_3(CH_2)_5$—CH_2—	*n*-Heptylpenicillin	K	IV

Penicillin, as produced by *P. chrysogenum*, is a mixture of related compounds. The ratios among the various penicillins depends upon the isolate and conditions used. By the use of suitable precursors the yield of the desired compound, penicillin G, is greatly increased. This is desirable because penicillin G salts crystallize well and are most useful in medicine. The type formula for the penicillins is shown below. The precursors used for the production of penicillin G are related to phenylacetic acid. Many other penicillins are known, some of which are produced only in the presence of precursors which do not occur in nature (Behrens, 1949).

The growth of the penicillin industry in the United States is shown by the production figures in Table 52.

Penicillin is chiefly active against Gram-positive bacteria. A few important Gram-negative pathogens, including *Neisseria gonorrhoeae* and *Treponema pallidum*, are controlled by penicillin. Penicillin is not active against acid-fast bacteria or fungi. Many bacteria may become resistant, or fast, to penicillin. Whether natural selection or mutation or both are involved in this phenomenon is uncertain. The morphology and

physiology of penicillin-fast bacteria may be abnormal (Bellamy and Klimek 1948). Penicillin is most active against young cells, in that it inhibits the process of cell division. For papers on the mechanism of penicillin action see Cavallito *et al.* (1945), Chain and Duthie (1945); Bailey and Cavallito (1948); and a series of papers by Pratt and Dufrenoy (1949).

TABLE 52. THE PRODUCTION OF PENICILLIN IN THE UNITED STATES FOR THE YEARS 1943 TO 1948

A unit of penicillin is 0.6 μg. (Coghill and Koch, *Chem. Eng. News* **23,** 1945; Lee, *Ind. Eng. Chem.* **41,** 1949. Published by permission of the American Chemical Society.)

Year	Billions of Units
1943	21
1944	1,633
1945	6,852
1946	25,809
1947	41,426
1948	95,855

Further details may be found in the following selected references. For a concise authoritative account of all phases of penicillin, see Foster (1949). The medical aspects of penicillin therapy are discussed by Fleming (1949). The chemistry of penicillin is covered in the monograph edited by Clarke *et al.* (1949). The early history of penicillin is presented by Chain and Florey (1944) and Waksman (1947).

Streptomycin. This antibiotic was discovered in Waksman's laboratory in 1943, and three years later, commercial production of this drug began. Streptomycin is synthesized by some isolates of *Streptomyces griseus*. The techniques used in industry resemble those used for the production of penicillin in submerged aerated culture. Streptomycin is adsorbed on activated carbon as the first step in isolation and purification. In contrast to penicillin, streptomycin is a basic compound. The production of streptomycin in the United States increased from 1,175 billion units in 1946 to 37,710 billion units in 1948 (Lee, 1949). The chemistry of streptomycin is reviewed by Lemieux and Wolfrom (1948).

Streptomycin is mainly active against Gram-negative bacteria and certain acid-fast organisms, including *Mycobacterium tuberculosis*. This drug controls many pathogens which are unaffected by penicillin. Organisms exposed to streptomycin frequently become fast. Indeed, some bacteria have been reported to become dependent upon the drug.

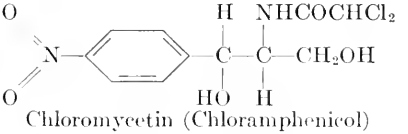
The composition of the medium influences streptomycin production. Soybean meal appears to be a suitable source of nitrogen for commercial production (Rake and Donovick, 1946). The influence of carbon and nitrogen sources in synthetic media has been studied by Dulaney (1948, 1949). These results may be summarized as follows: Glucose and man-

nose are the best hexoses; maltose is the best disaccharide; starch and its degradation product dextrin are good carbon sources. *Streptomyces griseus* does not utilize nitrate nitrogen. L-Proline is the most favorable single amino acid, but addition of this amino acid to other sources of nitrogen does not increase yields (see also Thornberry and Anderson, 1948). A popular account of the development of streptomycin is to be found in Epstein and Williams (1946).

Aureomycin. This antibiotic is produced by *Streptomyces aureofaciens*. Aureomycin has been prepared in pure crystalline form (Duggar, 1948). In addition to being effective against many Gram-positive and Gram-negative bacteria, aureomycin is also active against certain rickettsial and viral agents. Preliminary reports on the use of aureomycin for treating Rocky Mountain spotted fever have been favorable (Schoenbach *et al.*, 1948). Aureomycin appears to be of great clinical value in treating lymphogranuloma venereum and granuloma inguinale, two virus diseases of man (Wright *et al.*, 1948).

Chloromycetin. This antibiotic was discovered independently in two laboratories (Ehrlich *et al.*, 1947, and Carter *et al.*, 1948). It is produced by *Streptomyces venezuelae* and is the first useful antibiotic to be produced synthetically on a commercial scale. Chloromycetin is active against certain Gram-positive and Gram-negative bacteria, acid-fast bacteria, and *Rickettsia* (Smith *et al.*, 1948). It has been reported that this antibiotic has been used successfully to treat epidemic typhus (Payne and Knaudt, 1948).

Chloromycetin contains non-ionic chlorine and a nitro group, two unusual features in compounds of biological origin. The formula is given below:



Chloromycetin (Chloramphenicol)

Ergot. Among the alkaloids produced by fungi, only those obtained from the sclerotia of *Claviceps purpurea* appear to be used in medicine. Seven related alkaloids have been isolated from ergot. These alkaloids have different pharmacological properties. The most useful alkaloid, ergonovine, is isolated from the others before use. Certain undesirable effects of the other alkaloids are avoided by this procedure. Ergonovine, and formerly a mixture of ergot alkaloids, is used to stimulate uterine contraction. At present there is no satisfactory synthetic substitute for ergonovine (Mass, 1950).

In 1941, 571,000 lb. and in 1944, 85,000 lb. of ergot sclerotia were imported into the United States. If the sclerotia are consumed in large

enough quantities by man or animals, they cause ergotism, a disease also known as St. Anthony's fire.

C. purpurea has been cultured under laboratory conditions but forms neither sclerotia nor alkaloids under these conditions (Michener and Snell, 1950). Apparently alkaloids are formed only in the sclerotia. Ergotamine, when added to mycelial cultures of *C. purpurea*, was largely destroyed.

TOXINS

Numerous toxic substances are produced by fungi in nature, and their effects on man and animals are varied. The most severe toxins are produced by some of the Agaricaceae, particularly by species of *Amanita*. It is not known whether the toxins are present in the mycelium of these species as well as in the fruit bodies. A few of the outstanding examples of fungus toxins will be discussed briefly.

Amanita toxin (phalloidin) is stable to heat and drying and to the action of digestive juices. The great majority of the deaths due to mushroom poisoning are caused by *Amanita phalloides*, *A. virosa*, and *A. verna*, which contain amanita toxin. The action of this toxin is slow, the symptoms being delayed for 6 to 15 hr. after the mushrooms are eaten. By this time the toxin has been absorbed, and the patient seldom responds to treatment. No antidote for this toxin is known. The mortality rate is high, varying from 60 to 100 per cent (Fischer, 1918). In addition to the three species of *Amanita* mentioned above, the same or a similar toxin is present in *A. spreta*, *A. porphyria*, *A. strobiliformis*, *A. radicata*, and *A. chlorinosma*. *Hygrophorus conicus* and *Pholiota autumnalis* produce similar symptoms and may contain this toxin (Krieger, 1936).

About 1 g. of pure crystalline toxin can be extracted from 40 kg. of *A. phalloides* fruit bodies. The toxic dose for white mice is 50 μg; death results in from 1 to 2 days. Chemically, phalloidin is a polypeptide containing six amino-acid residues. Wieland and Witkop (1940) report that phalloidin, on hydrolysis with sulfuric acid, yields 1 mole each of *l*-α-oxytryptophane and cysteine, and 2 moles each of *l*-hydroxyproline b (not found in protein digests) and *l*-alanine. Kuhn *et al.* (1939) found methionine in addition to cysteine (ratio 1 to 5) in phalloidin. Among the antibiotics, gramicidin and tyrocidine are polypeptides which contain "unnatural" amino acids and are toxic when injected into experimental animals.

Muscarin is the principal toxic agent present in *A. muscaria*. It is a quick-acting toxin, producing symptoms within 1 to 6 hr. after being consumed. The patient usually responds well to treatment and recovery is rapid, although death may occur. Atropin is an antidote for muscarin which is closely related to choline. Muscarin has also been demonstrated in *A. pantherina*, *Russula emetica*, *Boletus luridus*, and *B. satanas*. A

similar or the same toxin is present in *Clitocybe illudens*, *Inocybe infelix*, *I. infida*, *Lactarius torminosus*, and *B. miniato-olivaceus* var. *sensibilis* (Krieger, 1936; Wolf and Wolf, 1947).

Helvellic acid is known to be present only in *Gyromitra esculenta*. Apparently there is considerable variability in the reaction of individuals who eat this fungus. Many people have eaten it with no ill effects, although a number of cases of poisoning and even a few deaths have been reported. Helvellic acid is soluble in hot water. Its toxic action is due to its blood-dissolving power.

While a number of other toxic substances have been detected in the fleshy fungi, the exact identity of most of them is not known. For example, species of *Paneolus* may cause temporary paralysis or intoxication similar to alcoholic intoxication. Some species of *Amanita* have been reported to contain other toxins in addition to those discussed above (Fischer, 1918).

The alkaloids produced by *Claviceps purpurea* and *C. paspali* are toxic to man and animals if consumed in large enough quantities. The specific alkaloids produced were mentioned previously. The production of toxins is also common in the genus *Fusarium*. *Gibberella zeae*, the cause of scab of small grains, produces toxic substances which are poisonous to livestock fed on scabby grain (Christensen and Kernkamp, 1936). Numerous other fungi which cause plant diseases are known to produce toxic substances which kill the host or modify its activity (see Chap. 17).

PIGMENTS

Colored compounds produced by fungi and other organisms are called *pigments*. In the fungi, some pigments accumulate in the mycelium and spores, while others diffuse into the culture medium. The pigments produced by a fungus are in part determined by genetic factors and in part by the environment. Mycelium, fruit bodies, and spores may be pigmented, or in some species the pigment is confined to the spores.

Among the fleshy fungi, brown is one of the most common colors of fruit bodies, with yellow, orange, and red being somewhat less common. Often a number of pigments are obviously present. Few fungi are green. Yet, *Chlorosplenium aeruginosum* produces a green pigment, sylindein (Wolf and Wolf, 1947), which stains the wood in which it grows. Blue-stain fungi (*Ceratostomella* spp.) excrete blue pigments into wood. Some species of *Boletus* produce a blue or bluish-green pigment when bruised or wounded. *Tricholoma personatum* and *Laccaria amythestina* are among the mushrooms producing purple or violet pigments. It is said that the red-orange pigment of the fruit bodies of *Echinodontium tinctorium*, the Indian paint fungus, was used by the Indians as make-up. Few of the

larger fruit bodies of the fungi are entirely black, although this is a common color for perithecia, pycnidia, and spores.

Among the nutritional factors which modify the production of pigments by fungi in culture, the micro essential elements, the carbon and nitrogen sources, the initial pH of the medium, and the temperature are important. Perhaps the first of these factors to be studied was the effect of iron, copper, zinc, and other micro elements upon the spore color of *Aspergillus niger*. Copper seems to play an outstanding role in the production of dark spores by this fungus (Mulder, 1939), but low concentrations of other micro essential elements also affect spore color of this fungus. The influence of iron, copper, and zinc on the pigmentation of mycelium and spores, and the production of soluble pigments by certain species was studied by Metz (1930).

The investigation of the chemical structure of fungus pigments has formed an essential part of a comprehensive study of the products of fungus metabolism at the University of London. The citations in this paragraph will give the reader entry into this excellent work. Many fungi produce anthraquinone pigments. *Helminthosporium gramineum* stores in its mycelium two pigments (helminthosporin and hydroxyisohelminthosporin), which may account for 30 per cent of the dry weight of the mycelium. Helminthosporin is 2-methyl-4,5,8-trihydroxyanthraquinone (Charles *et al.*, 1933). *H. cynodontis* and *H. cuchlaenae* form cynodontin, 1,4,5,8-tetrahydroxy-2-methylanthraquinone, which is closely related to helminthosporin (Raistrick *et al.*, 1933). Some 12 anthraquinone pigments are produced by fungi (Howard and Raistrick, 1949). Xanthone pigments are produced by *H. ravenellii* and *H. turcicum* (Raistrick *et al.*, 1936). The production of anthraquinone pigments is not restricted to species of *Helminthosporium*, for *Penicillium islandicum* synthesizes chrysophanic acid, 4,5-dihydroxy-2-methylanthraquinone (Howard and Raistrick, 1950). In general, the production of these and other pigments is modified by the cultural conditions used. The production of helminthosporin by *H. gramineum* was increased when nitrate or organic sources of nitrogen were used. Ammonium nitrogen was not favorable for pigment production. More pigment was produced when the initial pH was 8 than in more acid media.

Many of the water-soluble pigments produced by fungi are indicators. *P. phoeniceum* and *P. rubrum* produce such an indicator pigment, phoenicine (2,2′-dihydroxy-4,4′-ditoluquinone). The color changes of this indicator are from yellow to red in the pH range of 1.8 to 3.4 and from red to violet in the range 5.4 to 6.4. As much as 2 g. of this pigment is produced by *P. rubrum* per liter of medium (Curtin *et al.*, 1940).

The functions of fungus pigments are not well understood. It is known that certain of these pigments are enzyme inhibitors. Others, like

citrinin, are antibiotics. The physiological activity of solanione, a purple pigment produced by *Fusarium solani*, decreases growth and the efficiency of fat formation by *F. lini* (Weiss and Nord, 1949). Solanione is a 1,4-naphthoquinone, and this activity is in accord with the effects of other compounds of this series. *F. graminearum* synthesizes an orange-red pigment, rubofusarin, which is a xanthone (Ashley *et al.*, 1937). Rubofusarin was found to stimulate growth and to inhibit the enzymatic dehydrogenation of isopropyl alcohol by *F. lini* (Sciarini *et al.*, 1943). It is suggestive that pigment production frequently occurs near the time of maximum development of mycelium. Perhaps pigments influence sporulation in some way.

Carotene is produced by *Mucor hiemalis* and *Phycomyces blakesleeanus*, and probably by *Mucor mucedo*, *Pilairia anomala*, and *Dicranophora fulva* (Schopfer, 1935). The amount of carotene produced by *Phycomyces blakesleeanus* was increased by increasing the concentration of asparagine in the medium. The plus strain of this fungus synthesized more carotene than the minus strain. Carotene occurs in nature as three isomeric compounds, all of which may be converted into vitamin A. The carotene found in *M. hiemalis* and *P. blakesleeanus* is β-carotene. Emerson and Fox (1940) found γ-carotene to be associated with the male gametangia of a certain species of *Allomyces*. Apparently carotene is also common among the yellow or orange Ascomycetes and Basidiomycetes.

SUMMARY

The saprophytic fungi play an important, if not indispensable, part in the degradation and decay of plant and animal residues. The most important product of fungus metabolism in nature is carbon dioxide. Humus is in part a result of the activities of soil-inhabiting fungi.

Various species of fungi have been used for the preparation of food and beverages. Fungi may be used to increase the world's supply of food. Yeasts and other fungi are able to convert waste carbohydrate and inorganic nitrogen compounds into protein, fats, and vitamins. Yeast protein, because of its content of essential amino acids, has value as a protein supplement.

Alcoholic fermentation is not restricted to the yeasts, although these fungi are used almost exclusively in industry for this purpose. The production of alcohol requires anaerobic or partially anaerobic conditions.

Fungi may be used to produce organic acids, of which citric acid is the most important commercially. In general, a medium high in carbohydrate and low in nitrogen favors the production of organic acids, which are synthesized in quantity only after growth is essentially complete.

In nature one organism may be antagonistic to another because of the competition for nutrients or because of the production of antibiotics.

Relatively few of the antibiotics known to be produced meet the prerequisite of being nontoxic to man, but some of those which do are enormously important.

Ergonovine, one of the ergot alkaloids obtained from the sclerotia of *Claviceps purpurea*, is a useful drug for which no synthetic substitute is available.

A number of fleshy fungi produce toxins, some of which are deadly to man if consumed in sufficient quantity. The toxins vary in chemical nature, in severity, and in the symptoms they produce.

Pigments are assumed to serve a definite function in fungi, at least in some instances. Some pigments are known to be antibiotics; others such as carotene are provitamins; but in general the functions of the fungus pigments remain unknown.

REFERENCES

Anwar, A. A.: Factors affecting the survival of *Helminthosporium sativum* and *Fusarium lini* in soil, *Phytopathology* **39**: 1005–1019, 1949.

Ashley, J. N., B. C. Hobbs, and H. Raistrick: Studies in the biochemistry of micro-organisms. LIII. The crystalline colouring matters of *Fusarium culmorum* (W. G. Smith) Sacc. and related forms, *Biochem. Jour.* **31**: 385–397, 1937.

Bailey, J. H., and C. J. Cavallito: The reversal of antibiotic action, *Jour. Bact.* **55**: 175–182, 1948.

Behrens, O. K.: Biosynthesis of penicillins *in* The Chemistry of Penicillin, Princeton University Press, Princeton, N.J., 1949.

Bellamy, W. D., and J. W. Klimek: Some properties of penicillin-resistant Staphylococci, *Jour. Bact.* **55**: 153–160, 1948.

Bernhauer, K., and H. Knobloch: Ueber die Saürebildung aus Zucker durch *Aspergillus niger*. XI. Mitteilung. Factoren der Citronensäureanhäufung 2, *Biochem. Zeit.* **309**: 151–178, 1941.

Birkinshaw, H., J. H. V. Charles, and H. Raistrick: Studies in the biochemistry of micro-organisms. XVIII. Biochemical characteristics of species of *Penicillium* responsible for the rot of citrus fruits, *Trans. Roy. Soc.* (*London*), Ser. B, **220**: 355–362, 1931.

Birkinshaw, J. H., and H. Raistrick: Studies in the biochemistry of micro-organisms. II. Quantitative methods and technique of investigation of the products of metabolism of micro-organisms, *Trans. Roy. Soc.* (*London*), Ser. B, **220**: 11–26, 1931.

Block, R. J., and D. Bolling: The amino acids yielded by various yeasts after hydrolysis of the fat-free material. A comparative investigation, *Arch. Biochem.* **7**: 313–321, 1945.

★Boyle, A. M.: Further studies of the bacterial necrosis of the giant cactus, *Phytopathology* **39**: 1029–1052, 1949.

Bray, C. W.: Vitamin B deficiency in infants: its possibility, prevalence and prophylaxis, *Trans. Roy. Soc. Trop. Med. Hyg.* **22**: 9–42, 1928.

Brefeld: O.: Untersuchungen aus dem Gesamtgebeit der Mykologie, Vol. XIV, Kommissions-Verlag von Heinrich Schöningh, Muenster, 1908.

Brian, P. W.: The production of antiobiotics by soil microorganisms, *Chem. & Industry*, **1949**, 391–393.

Brian, P. W., and H. G. Hemming: Production of antifungal and antibacterial

substances by fungi: Preliminary examination of 166 strains of Fungi Imperfecti, *Jour. Gen. Microbiol.* **1**: 158–167, 1947.

Brink, N. G., F. W. Holly, C. H. Shunk, E. W. Peel, J. J. Cahill, and K. Folkers: Vitamin B_{12}. IX. 1-*alpha*-D-ribofuranosido-5,6-dimethylimidazole, a degradation product of vitamin B_{12}, *Jour. Am. Chem. Soc.* **72**, 1866, 1950.

Buchner, E.: Alcoholische Gärung ohne Hefezellen, *Ber. d. deut. chem. Ges.* **30**: 117–124, 1897.

Buller, A. H. R.: The fungus lore of the Greeks and Romans, *Trans. Brit. Mycol. Soc.* **5**: 21–66, 1914.

Burkholder, P. R.: Influence of some environmental factors upon the production of riboflavin by a yeast, *Arch. Biochem.* **3**:121–129, 1943.

Calam, C. T., A. E. Oxford, and H. Raistrick: Studies in the biochemistry of micro-organisms. LXIII. Itaconic acid, a metabolic product of a strain of *Aspergillus terreus* Thom., *Biochem. Jour.* **33**: 1488–1495, 1939.

Carter, H. E, D. Gottlieb, and H. W. Anderson: Chloromycetin and streptothricin, *Science* **107**: 113, 1948.

*Cavallito, C. J., J. H. Bailey, T. H. Haskell, T. H. McCormick, and W. F. Warner: The inactivation of antibacterial agents and their mechanism of action, *Jour. Bact.* **50**: 61–69, 1945.

Chain, E., and E. S. Duthie: Bacteriocidal and bacteriolytic action of penicillin on the Staphylococcus, *Lancet* **248**: 652–657, 1945.

Chain, E., and H. W. Florey: Penicillin, *Endeavour* **3**: 3–14, 1944.

Charles J. H. V., H. Raistrick, R. Robinson, and A. R. Todd: Studies in the biochemistry of micro-organisms. XXVIII. Helminthosporin and hydroxy*iso*-helminthosporin, metabolic products of the plant pathogen *Helminthosporium gramineum* Rabenhorst., *Biochem. Jour.* **27**: 499–511, 1933.

Christensen, J. J., and H. C. H. Kernkamp: Studies on the toxicity of blighted barley to swine, *Minn. Agr. Expt. Sta. Tech. Bull.* 113, 1936.

Clarke, H. T., J. R. Johnson, and R. Robinson (Editors): The Chemistry of Penicillin, Princeton University Press, Princeton, N.J., 1949.

Coghill, R. D., and R. S. Koch: Penicillin, a wartime accomplishment, *Chem. Eng. News* **23**: 2310–2316, 1945.

Currie, J. N.: The citric acid fermentation of *Aspergillus niger*, *Jour. Biol. Chem.* **31**: 15–37, 1917.

Curtin, T., G. Fitzgerald, and J. Reilly: Production of phoenicine on synthetic media. 1. *Penicillium phoeniceum* van Beyma. 2. *Penicillium rubrum* Grassberger-Stoll., *Biochem. Jour.* **34**: 1605–1610, 1940.

Duggar, B. M.: Mushroom Growing, Orange Judd Publishing Co., Inc., New York, 1915.

Duggar, B. M.: Aureomycin: a product of the continuing search for new antibiotics, *Ann. N.Y. Acad. Sci.* **51**: 177–181, 1948.

Dulaney, E. L.: Observations on *Streptomyces griseus*. III. Nitrogen sources for growth and streptomycin production, *Jour. Bact.* **56**: 305–313, 1948.

Dulaney, E. L.: Observations on *Streptomyces griseus*. II. Carbon sources for growth and streptomycin production, *Mycologia* **41**: 1–10, 1949.

Ehrlich, J., Q. R. Bartz, R. M. Smith, D. A. Joslyn, and P. Bunkholder: Chloromycetin, a new antibiotic from a soil Actinomycete, *Science* **106**: 417, 1947.

Emerson, R., and D. L. Fox: *Gamma*-carotene in the sexual phase of the aquatic fungus *Allomyces*, *Proc. Roy. Soc. (London)*, Ser. B, **128**: 275–293, 1940.

*Efstein, S., and B. Williams: Miracles from Microbes, the Road to Streptomycin, Rutgers University Press, New Brunswick, 1946.

Erb, N. M., R. T. Wisthoff, and W. L. Jacobs: Factors affecting the production of

amylase by *Aspergillus niger*, strain NRRL 337, when grown in submerged culture, *Jour. Bact.* **55**: 813–821, 1948.

Fischer, O. E.: Mushroom poisoning *in* C. H. Kauffman, The Agaricaceae of Michigan, Vol. I, Publication 26, Biological Series of the Michigan Geological and Biological Survey, Lansing, 1918.

Fitzpatrick, W. H., W. B. Esselen, and E. Weir: Composition and nutritive value of mushroom protein, *Jour. Am. Dietet. Assoc.* **22**: 318–322, 1946.

Fleming, A.: Penicillin, Its Practical Application, The Blakiston Company, Philadelphia, 1949.

Foster, J. W.: Riboflavin, British patent 593,027, Oct. 7, 1947. *Chem. Abs.* **42**: 1710, 1948.

Foster, J. W.: Chemical Activities of Fungi, Academic Press, Inc., New York, 1949.

Foster, J. W., S. F. Carson, S. Ruben, and M. D. Kamen: Radioactive carbon as an indicator of carbon dioxide utilization. VII. The assimilation of carbon dioxide by molds, *Proc. Natl. Acad. Sci. U.S.* **27**: 590–596, 1941.

Foster, J. W., and J. B. Davis: Anaerobic formation of fumaric acid by the mold *Rhizopus nigricans*, *Jour. Bact.* **56**: 329–338, 1948.

Foster, J. W., and S. A. Waksman: The specific effect of zinc and other heavy metals on growth and fumaric acid production by *Rhizopus*, *Jour. Bact.* **37**: 599–617, 1939.

Foster, J. W., and S. A. Waksman: The production of fumaric acid by molds belonging to the genus *Rhizopus*, *Jour. Am. Chem. Soc.* **61**: 127–135, 1939*a*.

Gastrock, E. A., N. Porges, P. A. Wells, and A. J. Moyer: Gluconic acid production on pilot-plant scale. Effect of variables on production by submerged mold growths, *Ind. Eng. Chem.* **30**: 782–789, 1938.

Gerhardt, P., W. W. Dorrell, and I. L. Baldwin: Citric acid fermentation of beet molasses, *Jour. Bact.* **52**: 555–564, 1946.

Gorcica, H. J., and H. Levine: Vitamin B_1 assimilation by yeast, U.S. patent 2,295,036, Sept. 8, 1942.

Gordon, M. A.: The physiology of a blue stain mold with special reference to production of ethyl acetate, *Mycologia* **42**: 167–185, 1950.

Gottlieb, D., and P. Siminoff: The role of antibiotics in soil, *Phytopathology* **40**: 11, 1950.

Grossbard, E.: Production of an antibiotic substance on wheat straw and other organic materials in the soil, *Nature* **161**: 614–615, 1948.

Gupta, B. M., and W. C. Price: Production of plant virus inhibitors by fungi, *Phytopathology* **40**: 642–652, 1950.

Hao, L. C., E. I. Fulmer, and L. A. Underkofler: Fungal amylases as saccharifying agents in the alcoholic fermentation of corn, *Ind. Eng. Chem.* **35**: 814–818, 1943.

Harden, A.: Alcoholic Fermentation, 4th ed., Longmans, Roberts and Green, London, 1932.

Harris, E. E., M. L. Hannan, and R. R. Marquardt: Production of food yeast from wood hydrolysates, *Ind. Eng. Chem.* **40**: 2068–2072, 1948.

Hendlin, D., and M. L. Ruger: The effect of cobalt in the microbial synthesis of LLD-active substances, *Science* **111**: 541–542, 1950.

Henry, A. W.: The natural microflora of the soil in relation to the foot-rot problem of wheat, *Can. Jour. Research* **4**: 69–77, 1931.

Hesse, A.: Industrial biosyntheses. I. Fats, *Advances in Enzymol.* **9**: 653–704, 1949.

Hickey, R. J.: The inactivation of iron by 2,2′-bipyridine and its effect on riboflavin synthesis by *Clostridium acetobutylicum*, *Arch. Biochem.* **8**: 439–447, 1945.

★Howard, B. H., and H. Raistrick: Studies in the biochemistry of micro-organisms. 80. The colouring matters of *Penicillium islandicum* Sopp. Part 1. 1,4,5-trihydroxy-2-methylanthraquinone, *Biochem. Jour.* **44**. 227–233, 1949.

Howard, B. H., and H. Raistrick: Studies in the biochemistry of micro-organisms. 81. The colouring matters of *Penicillium islandicum* Sopp. Part 2. Chrysophanic acid, 4,5-dihydroxy-2-methylanthraquinone, *Biochem. Jour.* **46**: 49–53, 1950.

Humfield, H.: The production of mushroom mycelium (*Agaricus campestris*) in submerged culture, *Science* **107**: 373, 1948.

Karow, E. O., and S. A. Waksman: The production of citric acid in submerged culture, *Ind. Eng. Chem.* **39**: 821–825, 1947.

Keilin, D., and E. F. Hartree: Properties of glucose oxidase (Notatin), *Biochem. Jour.* **42**: 221–229, 1948.

Keilin, D., and E. F. Hartree: The use of glucose oxidase (Notatin) for the determination of glucose in biological material, and for the study of glucose-producing systems by manometric methods, *Biochem. Jour.* **42**: 230–238, 1948*a*.

Krieger, L. C. C.: The Mushroom Handbook, The Macmillan Company, New York 1936.

Kuhn, R., L. Birkhofer, and F. W. Quackenbush: Jodometrische Titration von SH-gruppen: Mikromethode zur Bestimmung von Cystein und Methionin in Proteinen, *Ber. d. deut. chem. Ges.* **72**: 407–416, 1939.

Leach, J. G.: Insect Transmission of Plant Diseases, McGraw-Hill Book Company, Inc., New York, 1940.

★Leben, C., and G. W. Keitt: Laboratory and greenhouse studies of antimycin preparations as protectant fungicides, *Phytopathology* **39**: 529–540, 1949.

Lee, S. B.: Fermentation, *Ind. Eng. Chem.* **41**: 1838–1879, 1949.

Le Mense, E. H., J. Corman, J. M. van Lanen, and A. F. Langlykke: Production of mold amylase in submerged culture, *Jour. Bact.* **54**: 149–159, 1947.

Lemieux, R. U., and M. L. Wolfrom: The chemistry of streptomycin, *Advances in Carbohydrate Chem.* **3**: 337–384, 1948.

Letcher, H., and J. J. Willaman: Biochemistry of plant diseases. VIII. Alcoholic fermentation of *Fusarium lini*, *Phytopathology* **16**: 941–949, 1926.

Levine, H., J. E. Oyaas, L. Wanerman, J. C. Hoogerheide, and R. M. Stern: Riboflavin production by *Candida* yeasts, *Ind. Eng. Chem.* **41**: 1665–1668, 1949.

Lillie, R. J., C. A. Denton, and H. R. Bird: Relation of vitamin B_{12} to the growth factor present in cow manure, *Jour. Biol. Chem.* **176**: 1477–1478, 1948.

Linder, P.: Das Problem der biologischen Fettbildung und Fettgewinnung, *Zeit angew. Chem.* **35**: 110–114, 1922.

Lintzel, W.: Ueber den Nährwert des Eiweisses der Speisepilze, *Biochem. Zeit.* **308**: 413–419, 1941.

Lockwood, L. B.: A study of the physiology of *Penicillium javanicum* van Beijma with special reference to the production of fat, *Catholic Univ. of America Biol. Ser.* 13, 1933.

Lockwood, L. B., G. E. Ward, and O. E. May: The physiology of *Rhizopus oryzae*, *Jour. Agr. Research* **53**: 849–857, 1936.

Lockwood, L. B., and G. E. Ward: Fermentation process for itaconic acid, *Ind. Eng. Chem.* **37**: 405–406, 1945.

Mass, J. M.: Personal communication, 1950.

Metz, O.: Ueber Wachstum und Farbstoffbildung einiger Pilze unter dem Einfluss von Eisen, Zink und Kupfer, *Arch. Mikrobiol.* **1**: 197–251, 1930.

Meyerhof, O.: Energy relationships in glycolysis and phosphorylation, *Ann. N.Y. Acad. Sci.* **45**: 377–393, 1944.

Meyerhof, O.: Glycolysis of animal tissue extracts compared with the cell-free fermentation of yeast, *Wallerstein Labs. Communs.* **12**: 255–264, 1949.

Michener, H. D., and N. Snell: Studies on cultural requirements of *Claviceps purpurea* and inactivation of ergotamine, *Am. Jour. Botany* **37**: 52–59, 1950.

Morton, H. E., W. Kocholaty, R. Junowicz-Kocholaty, and A. Kelner: Toxicity and antibiotic activity of kojic acid produced by *Aspergillus lutco-virescens*, *Jour. Bact.* **50**: 579–584, 1945.

Moyer, A. J., and R. D. Coghill: The laboratory-scale production of itaconic acid by *Aspergillus terreus*, *Arch. Biochem.* **7**: 167–183, 1945.

★Moyer, A. J., E. J. Umberger, and J. J. Stubbs: Fermentation of concentrated solutions of glucose to gluconic acid. Improved process, *Ind. Eng. Chem.* **32**: 1379–1383, 1940.

Mulder, E. G.: Ueber die Bedeutung des Kupfers für das Wachstum von Mikroorganismen und über eine mikrobiologische Methode zur Bestimmung des pflanzenverfügbaren Bodenkupfers, *Arch. Mikrobiol.* **10**: 72–86, 1939.

Myrbäck, K.: Products of the enzymatic degradation of starch and glycogen, *Advances in Carbohydrate Chem.* **3**, 1948.

Nord, F. F.: Food composition containing Fusaria, U.S. patent 2,450,055, Sept. 28, 1948.

Nord, F. F., and R. P. Mull: Recent progress in the biochemistry of Fusaria, *Advances in Enzymol.* **5**: 165–205, 1945.

Owen, W. L.: Production of industrial alcohol from grain by the amylo process, *Ind. Eng. Chem.* **25**: 87–89, 1933.

Pasteur, L.: Etudes sur la bière, 1872. Studies on Fermentation. The Diseases of Beer (trans. F. Faulkner and D. C. Robb), Macmillan & Co., Ltd., London, 1879.

Payne, E. H., and J. H. Knaudt: Treatment of epidemic typhus with chloromycetin, *Proc. Intern. Cong. Trop. Med. and Malaria*, 4th Congr., **4**: 426–428, 1948.

Peltier, G. L., and R. Borchers: Riboflavin production by molds, *Jour. Bact.* **54**: 519–520, 1947.

Perlman, D., W. W. Dorrell, and M. J. Johnson: Effect of metallic ions on the production of citric acid by *Aspergillus niger*, *Arch. Biochem.* **11**: 131–143, 1946.

Platt, B. S., and R. A. Webb: Fermentation and human nutrition, *Biochem. Jour.* **39**: 31, 1945.

Porges, N., T. F. Clark, and E. A. Gastrock: Gluconic acid production. Repeated use of submerged *Aspergillus niger* for semi-continuous production, *Ind. Eng. Chem.* **32**: 107–111, 1940.

Porges, N., T. F. Clark, and S. I. Aronovsky: Gluconic acid production. Repeated recovery and re-use of submerged *Aspergillus niger* by filtration, *Ind. Eng. Chem.* **33**: 1065–1067. 1941.

★Pratt, R., and J. Dufrenoy: Cytochemical mechanisms of penicillin action. VIII. Involvement of ribonucleic acid derivatives, *Jour. Bact.* **57**: 9–13, 1949.

Prescott, S. C., and G. G. Dunn: Industrial Microbiology, 2d ed., McGraw-Hill Book Company, Inc., New York, 1949.

Preuss, L. M., E. C. Eichinger, and W. H. Peterson: The chemistry of mold tissue. III. Composition of certain molds with special reference to the lipid content, *Cent. Bakt.*, Abt. II, **89**: 370–377, 1934.

Raaf, H.: Beiträge zur Kenntnis der Fett- und Eiweissyntheses bei *Endomyces vernalis* und einigen anderen Mikroorganismen, *Arch. Mikrobiol.* **12**: 131–182, 1941.

Raistrick, H., J. H. Birkinshaw, J. H. V. Charles, P. W. Clutterbuck, F. P. Coyne, A. C. Hetherington, C. H. Lilly, M. L. Rintoul, W. Rintoul,

R. Robinson, J. A. R. Stoyle, C. Thom, and W. Young: Studies in the biochemistry of micro-organisms, *Trans. Roy. Soc. (London)*, Ser. B,**220**: 1–367, 1931.

Raistrick, H., R. Robinson, and A. R. Todd: Studies in the biochemistry of micro-organisms. XXXII. Cynodontin (1,4,5,8-tetrahydroxy-2-methylanthraquinone), a metabolic product of *Helminthosporium cynodontis* Marigroni and *Helminthosporium euchlaenae* Zimmermann, *Biochem. Jour.* **27**: 1170–1175, 1933.

Raistrick, H., R. Robinson, and D. E. White: Studies in the biochemistry of micro-organisms. L. Ravenelin (3-methyl-1,4,8-trihydroxyxanthone), a new metabolic product of *Helminthosporium ravenellii* Curtis and *H. turcicum* Passerini, *Biochem. Jour.* **30**: 1303–1314, 1936.

Rake, G., and R. Donovick: Studies on the nutritional requirements of *Streptomyces griseus* for the formation of streptomycin, *Jour. Bact.* **52**: 223–228, 1946.

★Raper, K. B., D. F. Alexander, and R. D. Coghill: Penicillin. II. Natural variation and penicillin production in *Penicillium notatum* and allied species, *Jour. Bact.* **48**: 639–659, 1944.

Raper, K. B., R. D. Coghill, and A. Hollaender: The production and characterization of ultraviolet-induced mutants in *Aspergillus terreus*. II. Cultural and morphological characteristics of the mutations, *Am. Jour. Botany* **32**: 165–176, 1945.

Reilly, H. C., A. Schatz, and S. A. Waksman: Antifungal properties of antibiotic substances, *Jour. Bact.* **49**: 585–594, 1945.

Renaud, J., and M. Lachaux: Recherches sur la formation de lactoflavine à partir de l' *Eremothecium ashbyii*. Influence des constituants du milieu sur la production du pigment, *Compt. rend. acad. sci.* **221**: 187–188, 1945.

Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers: Comparative data on vitamin B_{12} from liver and a new source, *Streptomyces griseus*, *Science* **108**: 634–635, 1948.

Robbins, W. J., A. Hervey, R. W. Davidson, R. Ma, and W. C. Robbins: A survey of some wood-destroying and other fungi for antibacterial activity, *Bull. Torrey Botan. Club* **72**: 165–190, 1945.

Roberts, M., S. Laufer, E. D. Stewart, and L. T. Saletan: Saccharification of wheat by fungal amylases for alcohol production, *Ind. Eng. Chem.* **36**: 811–812, 1944.

Rolfe, R. T., and F. W. Rolfe: The Romance of the Fungus World, J. B. Lippincott Company, Philadelphia, 1926.

Rose, W. C. The nutritive significance of the amino acids, *Physiol. Revs.* **18**: 109–136, 1938.

Saito, K.: Ein Beispiel von Milchsäurebildung durch Schimmelpilze, *Cent. Bakt.*, Abt. II, **29**: 289–290, 1911.

Schatz, A., and E. L. Hazen: The distribution of soil microorganisms antagonistic to fungi pathogenic for man, *Mycologia* **40**: 461–477, 1948.

Schleef, M. L.: The economics of fodder yeast from sulfite waste liquor, *Wash. State Coll. Economic and Business Studies Bull.* 7, 1948.

Schoenbach, E. B., M. S. Bryer, and P. H. Long: The pharmacology and clinical trial of aureomycin: a preliminary report, *Ann. N.Y. Acad. Sci.* **51**: 267–279, 1948.

Schopfer, W. H.: Etude et identification d'un carotinoide de champignon, *Compt. rend. soc. biol.* **118**: 3–5, 1935.

Sciarini, L. J., R. P. Mall, J. C. Wirth, and F. F. Nord: Concerning the relation between structure and action of xanthones on dehydrogenations by Fusaria, *Proc. Natl. Acad. Sci. U.S.* **29**: 121–126, 1943.

★Shu, P., and M. J. Johnson: Citric acid production by submerged fermentation with *Aspergillus niger*, *Ind. Eng. Chem.* **40**: 1202–1205, 1948.

Smith, E. L.: Purification of antiperinicious anemia factors from liver, *Nature* **161**: 638–639, 1948.

Smith, R. M., D. A. Joslyn, O. M. Gruhzit, I. W. McLean, M. A. Penner, and J. Ehrlich: Chloromycetin: biological studies, *Jour. Bact.* **55**: 425–448, 1948.

Starkey, R. L.: Lipid production by a soil yeast, *Jour. Bact.* **51**: 33–50, 1946.

Sumner, J. B., and G. F. Somers: Chemistry and Methods of Enzymes, 2d ed., Academic Press, Inc., New York, 1947.

★Sure, B.: Nutritional improvement of cereal flours and cereal grains, *Jour. Am. Dietet. Assoc.* **22**: 494–502, 1946.

Sure, B.: Further studies on nutritional improvement of cereal flours and cereal grains with yeast, *Jour. Am. Dietet. Assoc.* **23**: 113–119, 1947.

Takamine, J.: Enzymes of *Aspergillus oryzae* and the application of its amyloclastic enzyme to the fermentation industry, *Jour. Ind. Eng. Chem.* **6**: 824–828, 1914.

Tanner, F. W., Jr., and M. J. van Lanen: Production of riboflavin by *Candida flareri*, U.S. patent 2,424,003, July 15, 1947. *Chem. Abs.* **41**: 6368, 1947.

Tanner, F. W., Jr., C. Vojnovich, and J. M. van Lanen: Riboflavin production by *Candida* species, *Science* **101**: 180–181, 1945.

★Tanner, F. W., Jr., C. Vojnovich, and J. M. van Lanen: Factors affecting riboflavin production by *Ashbya gossypii*, *Jour. Bact.* **58**: 737–745, 1949.

Tauber, H.: Chemistry and Technology of Enzymes, John Wiley & Sons, Inc., New York, 1949.

Thaysen, A. C.: Value of microorganisms in nutrition (food yeast), *Nature* **151**: 406–408, 1943.

★Thornberry, H. H., and H. W. Anderson: Synthetic medium for *Streptomyces griseus* and the production of streptomycin, *Arch. Biochem.* **16**: 389–397, 1948.

Underkofler, L. A., G. M. Severson, and K. J. Goering: Saccharification of grain mashes for alcoholic fermentation, *Ind. Eng. Chem.* **38**: 980–985, 1946.

Utech, N. M., and J. Johnson: The inactivation of plant viruses by substances obtained from bacteria and fungi, *Phytopathology* **40**: 247–265, 1950.

Van Lanen, J. M., H. P. Broquist, M. J. Johnson, I. L. Baldwin, and W. H. Peterson: Synthesis of vitamin B_1 by yeast, *Ind. Eng. Chem.* **34**: 1244–1247, 1942.

Vaughn, J. R., J. L. Lockwood, G. S. Randwa, and G. Hamner: Action of actidione on plant tissue and upon certain fungi, *Mich. Agr. Expt. Sta. Quart. Bull.* **31**: 456–464, 1949.

Von Loesecke, H. W.: A review of information on mycological citric acid production, *Chem. Eng. News* **23**: 1952–1959, 1945.

Von Loesecke, H. W.: Controversial aspects: yeast in human nutrition, *Jour. Am. Dietet. Assoc.* **22**: 485–493, 1946.

Waksman, S. A.: Associative and antagonistic effects of microorganisms. I. Historical review of antagonistic relationship, *Soil Sci.* **43**: 51–68, 1937.

Waksman, S. A.: Humus, Origin, Chemical Composition and Importance in Nature, 2d ed., The Williams & Wilkins Company, Baltimore, 1938.

Waksman, S. A.: Microbial Antagonism and Antibacterial Substances, Commonwealth Fund, Division of Publication, New York, 1947.

Waksman, S. A., and J. W. Foster: Respiration and lactic acid production by a fungus of the genus *Rhizopus*, *Jour. Agr. Research* **57**: 873–899, 1939.

Walker, T. K.: Pathways of acid formation in *Aspergillus niger* and in related molds, *Advances in Enzymol.* **9**: 537–584, 1949.

Wallerstein, J. S.: On the genealogy of beer, *Wallerstein Labs. Communs.* **2**: 35–45, 1939.

★Wallerstein, L.: Enzyme preparations from microorganisms. Commercial production and industrial application, *Ind. Eng. Chem.* **31**: 1218–1224, 1939.

Ward, G. E., L. B. Lockwood, O. E. May, and H. T. Herrick: Production of fat from glucose by molds. Cultivation of *Penicillium javanicum* van Beyma in large scale laboratory apparatus, *Ind. Eng. Chem.* **27**: 318–322, 1935.

Ward, G. E., L. B. Lockwood, O. E. May, and H. T. Herrick: Studies in the genus *Rhizopus*. I. The production of dextro-lactic acid, *Jour. Am. Chem. Soc.* **58**: 1286–1288, 1936.

Ward, G. E., L. B. Lockwood, B. Tabenkin, and P. A. Wells: Rapid fermentation process for dextrolactic acid, *Ind. Eng. Chem.* **30**: 1233–1235, 1938.

Weiss, S., and F. F. Nord: On the mechanism of enzyme action. XXXVII. Solanione, a pigment from *Fusarium solani* D_2 purple, *Arch. Biochem.* **22**: 288–313, 1949.

Wells, P. A., A. J. Moyer, and O. E. May: The chemistry of citric acid fermentation. I. The carbon balance, *Jour. Am. Chem. Soc.* **58**: 555–558, 1936.

Wells, P. A., A. J. Moyer, J. J. Stubbs, H. T. Herrick, and O. E. May: Gluconic acid production. Effect of pressure, air flow, and agitation on gluconic acid production by submerged mold growths, *Ind. Eng. Chem.* **29**: 653–656, 1937.

West, R.: Activity of vitamin B_{12} in Addisonian pernicious anemia, *Science* **107**: 398, 1948.

Whiffin, A. J.: The activity *in vitro* of cycloheximide (acti-dione) against fungi pathogenic to plants, *Mycologia* **42**: 253–258, 1950.

Wieland, H., and B. Witkop: Giftstoffe des Knollenblätterpilzes. V. Zur Konstitution des Phalloidins, *J. Liebigs Ann. d. Chem.* **543**: 171–183, 1940.

Wilkins, W. H., and G. C. M. Harris: Investigations into the production of bacteriostatic substances by fungi. VI. Examination of the larger Basidiomycetes, *Ann. Applied Biol.* **31**: 261–270, 1944.

Wöhler, F.: Das Entrathselte Geheimniss der geistigen Gärung (Vorläufige briefliche Mittheilung), *J. Leibigs Ann. d. Chem.* **29**: 100–104, 1839.

Wolf, F. A., and F. T. Wolf: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

Wright, L. T., M. Sanders, M. A. Logan, A. Prigot, and L. M. Hill: The treatment of lymphogranuloma venereum and granuloma inguinale in humans with aureomycin, *Ann. N.Y. Acad. Sci.* **151**: 318–330, 1948.

Yuill, J. L.: Alcoholic fermentation by *Aspergillus flavus* Brefeld, *Biochem. Jour.* **22**: 1504–1507, 1928.

CHAPTER 14

FACTORS INFLUENCING SPORULATION OF FUNGI

The life of the individual fungus is usually short and of uncertain duration. The continuance of the species (in most instances) depends upon the production and dissemination of sexual or asexual spores. The importance of spore production in the spread of epiphytotics is sufficient reason to study the factors which control, modify, or inhibit this stage of development in the life of the fungi. Some of the most difficult problems which arise in the study of the life processes of the fungi are to be found in the events and conditions which control the production of spores.

In nature, we find many examples of the influence of certain environmental and nutritional factors upon reproduction of the fungi. A number of parasitic fungi produce the perfect stage only in the spring, on or in dead host tissue. This is true of *Venturia inaequalis*, *Claviceps purpurea*, *Gnomonia ulmi*, *Monilinia fructicola*, *Coccomyces hiemalis*, *Guignardia bidwellii*, and others. Is the production of the sexual stage dependent upon the pretreatment of cold, or freezing and thawing? Is it dependent upon a favorable temperature and perhaps favorable intensity and duration of light? Or is it a matter of the proper nutrients which are made available only after decay of the host tissues? These are difficult questions to answer, for it is likely that the production of the sexual stage depends upon the proper balance of a number of factors. Similarly, we may speculate about the stimuli involved in the formation of the perithecia by the Erysiphales. Most of these obligate parasites form fruit bodies late in the growing season. Perhaps, at least in some cases, this is a reaction to cooler weather; or perhaps the formation of perithecia is a result of a decreasing or changing food supply as the host nears maturity. Other physical factors are probably involved, since we know that the abundance of perithecia varies from year to year. It is also of physiological interest that many parasitic fungi produce conidia only while the mycelium is actively attacking the living host.

A critical investigation of the factors influencing reproduction requires that the fungi be brought into the laboratory or greenhouse where external factors can be controlled. Only one variable should be studied at a time, and all other influencing factors must be controlled. It is, therefore, of great advantage in physiological studies to be able to grow a fungus in pure culture on synthetic or semisynthetic media. However, it must be

pointed out that the responses of a fungus in nature cannot always be duplicated in the laboratory.

Snyder and Hansen (1947) have given a brief and clear statement regarding the advantages of culturing fungi on natural media and under natural environmental conditions. These conditions are important, if one desires to obtain reproduction of a fungus which does not sporulate readily in culture. However, if one desires to study critically the individual nutritional and environmental requirements and their effects upon reproduction of a fungus which sporulates abundantly on the usual cultural media, it is often necessary to subject the fungus to unfavorable conditions. Thus, only by preventing sporulation, by varying but one factor at a time, may we discover the need for that factor.

Riker and Riker (1936) have listed 11 methods which have been successfully employed to induce sporulation of different fungi in culture. Since the writing of their manual much has been learned about this phase of fungus physiology. A revised list of the conditions known to influence sporulation of fungi is presented in the summary of this chapter.

Kauffman (1929) called attention to the views of Klebs, who held that living cells are influenced during their lifetime in three ways: (1) by the specific structure; (2) by the internal conditions; and (3) by the external conditions. Kauffman equated the first of these to *heredity* and the last two to *environment.* The *external environment* comprises the various physical and chemical factors, such as temperature, light, composition of the medium, and the like. Kauffman used the term *internal environment* to designate the complicated influences and reactions between cells within the organism. The physical and chemical effects of the external environment may be transmitted through the cells and become evident at some distance from the point of the stimulus.

The meaning of these statements may be clearer if we consider the effect of various external environmental factors upon fruiting. It is well known that various external stimuli may initiate the reactions which lead to reproduction. These stimuli must act through the internal environment. Most of the discussion that follows will be concerned with the external environment and the resulting development of the fungus. Some external factors may so modify the internal milieu as to favor sporulation, while others may inhibit or prevent sporulation.

Not all fungi respond in the same way to the external factors such as light, temperature, or nutrition. Each species produces spores when the internal environment is suitable, but the external factors do not operate upon the internal environment of all fungi alike. *Thus, there is no universal set of external conditions which lead to fructification in all fungi.* The external conditions favorable for sporulation must be studied for each species. This does not imply, however, that no two fungi react alike or

that certain helpful generalizations concerning sporulation cannot be drawn. It does, however, imply that the only sure way of understanding the conditions governing reproduction in a specific fungus lies in the experimental approach.

Again it must be emphasized that all the physical and chemical conditions may be at the optima, but no reproduction can occur without the presence of favorable genetic factors. Too often we may fail to realize the genetic requirements. The appropriate steps should be taken to determine whether the fungi under study are homothallic or heterothallic. It may be difficult, indeed, to determine whether failure of a fungus to reproduce in culture is due to unfavorable environmental conditions or to unfavorable genetic factors. There is much yet to be learned regarding the physiology of reproduction, but each new investigation is certain to add to our knowledge of this interesting and important phase of fungus physiology.

Vegetative growth must precede reproduction. The length of the vegetative phase varies from organism to organism, and the same organism may remain in the vegetative phase for a longer or shorter period of time depending upon the external environment. One of the functions of the vegetative phase is concerned with the building up of protoplasm and the storage of energy reserves. Reproduction is a process that draws heavily on the reserve food. The spore is usually well stocked with these materials. Asexual reproduction differs less from vegetative growth than does sexual reproduction. We shall find that the conditions limiting sexual reproduction are usually more narrow than conditions which allow asexual reproduction and growth.

Klebs (1900) summarized his views on reproduction in the fungi in the form of four laws or principles as follows: (1) Growth and reproduction are life processes, which, in all organisms, depend upon different conditions. In the lower organisms the external conditions mainly determine whether growth or reproduction takes place. (2) Reproduction in the lower organisms does not occur as long as characteristic external conditions are favorable for growth. The conditions which are favorable for reproduction are always more or less unfavorable for growth. (3) The processes of growth and reproduction differ, in that growth may take place under a wider range of environmental conditions than reproduction. Growth may take place, therefore, under conditions which inhibit reproduction. (4) Vegetative growth appears to be mostly a preliminary step for reproduction in that it creates a suitable internal environment for it. To a certain degree it is not growth in itself but the prolonged period of assimilation accompanying growth that is decisive for reproduction.

These generalizations were published in 1900 and were based upon Klebs's own work, as well as that of others. Many more fungi have been

studied during the past 50 years, and some new factors have been brought to light. It would not be surprising if some modifications in these conclusions would be necessary in the light of 50 years of research. We shall find, however, that, in the main, many of these "laws" are still valid.

ENVIRONMENTAL FACTORS

Temperature. Temperature was recognized by Bisby (1943) as an important natural factor governing the geographical distribution of the fungi. The temperature must be favorable not only for growth but also for the production and germination of the spores, if the fungus is to survive. Certain fungi are limited by high temperatures. Among these are *Plasmodiophora brassicae*, *Colletotrichum lindemuthianum*, *Urocystis cepulae*, and certain Phycomycetes. On the other hand, certain genera of the Gasteromycetes, such as *Podaxis*, *Battarrea*, *Chlamydopus*, and *Phellorina*, are confined to the hot arid regions of southwestern United States, northern Africa, central Australia, and western India. Between these extremes we may observe many examples where seasonal temperature limits or favors reproduction.

Klebs (1900) pointed out that the temperature range which allowed sporulation was more narrow than the range for growth. In general, the temperature limits for sexual reproduction are narrower than the limits for asexual reproduction. Some of Klebs's data are presented in Table 53.

TABLE 53. MINIMUM AND MAXIMUM TEMPERATURES (IN DEGREES CENTIGRADE) FOR GROWTH AND SPORULATION OF VARIOUS FUNGI
(Klebs, *Jahrb. wiss. Botan.* **35**, 1900.)

Fungus	Growth		Asexual spores		Sexual spores	
	Min.	Max.	Min.	Max.	Min.	Max.
Aspergillus repens	7–8	37–38	8–9	35–36	—	33–34
Sporidinia grandis	1–2	31–32	5–6?	29–30	5–6	27–28
Pilobolus microsporus	2–4	33–34	10–12	28–30	—	—
Saprolegnia mixta	0–1	36–37	1–2	32–33	1–2	26–27

It will be noted that the upper temperature which allowed the production of oospores by *Saprolegnia mixta* is a full 10°C. less than the upper temperature limit at which growth took place. Coons (1916) found the temperature limits for the growth of *Plenodomus fuscomaculans* to be 0 to 33°C., while pycnidia formed between 6 and 30°C. Perithecia failed to form in cultures of *Ceratostomella fimbriata* kept at 18°C. for 60 days (Barnett and Lilly, 1947*a*). Cultures of this fungus on the same medium

produced abundant peritheeia and ascospores at 25°C. within 11 days. Conidia were formed at 18°C.

The most noteworthy effect of culturing a fungus at temperatures below the optimum is the decrease in the rate of growth. It has been found by various investigators that there is an optimum temperature for sporulation as well as for growth. The two optima may be different. Figure 57 shows the effect of temperature on the time required to produce conidia by *Aspergillus repens.*

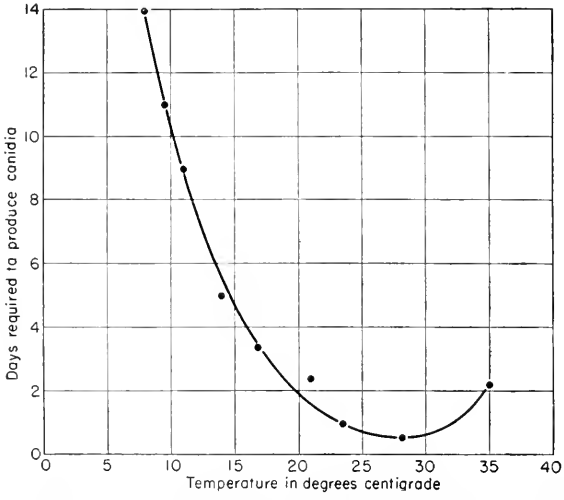


FIG. 57. The influence of temperature on the time required to produce conidia by *Aspergillus repens.* (Drawn from data of Klebs, *Jarhb. wiss. Botan.* **35**: 137, 1900.)

A temperature of 28°C. was optimum for sporulation of *Piricularia oryzae* (Henry and Andersen, 1948). Higher and lower temperatures of incubation decreased the numbers of spores produced. At 32°C. the number of spores was only 10 to 15 per cent of that produced at the optimum temperature. Reducing the temperature of incubation to 24°C. reduced the numbers of spores to about 80 per cent of the maximum. Thus, a small temperature increase above the optimum has a much greater effect upon the number of spores produced than a small decrease in temperature below the optimum (Fig. 58).

In nature, fungi are exposed to fluctuating temperatures. Whether a fluctuating temperature is more favorable in inducing sporulation than a constant temperature appears to have been studied but little. Jones (1946) concluded that temperature was the important controlling factor in the production of resistant sporangia of *Allomyces arbuscula* in culture, and he believed that "the total amount of temperature" to which the cultures were subjected was more important than the maximum, mini-

mum, or degrees of fluctuation. Mathur *et al.* (1950) reported that 15 to 20°C. favors conidium formation by *Colletotrichum lindemuthianum* in culture. Sporulation was less at 25°C. and ceased at 30°C. Mrak and Bonar (1938) found that temperature influenced the relative size of asci and spores of *Debaryomyces*. The ascus was much larger than the spore cluster at 4°C., but the spores nearly filled the ascus at 25°C.

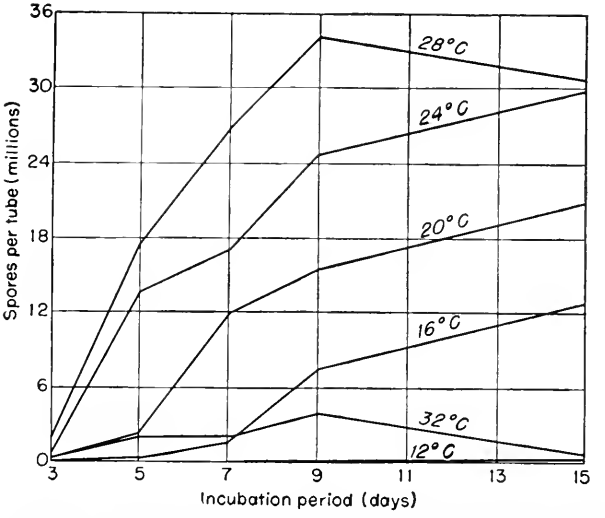


FIG. 58. The effects of temperature and time of incubation on sporulation of *Piricularia oryzae* on rice-polish agar. (Courtesy of Henry and Andersen, *Phytopathology* **38**: 272, 1948.)

An interesting selective effect of temperature upon type of asexual sporulation is found in *Choanephora cucurbitarum* (Barnett and Lilly, 1950). This fungus produces two types of asexual spores, those produced in typical sporangia and conidia borne in heads. Only the conidia are found commonly in nature, while both types are abundant in culture. When the fungus was grown in Petri dishes at 25°C., 87 per cent of the reproductive structures were conidial heads, while 13 per cent were sporangia (Table 54). When the temperature was increased to 30°C., this proportion was nearly reversed. At 31°C. many sporangia but no conidia were formed. No sporulation occurred at 34°C., but mycelial growth was abundant. Temperature also affected, either directly or indirectly, the size of the sporangia. Those produced at 25°C. averaged 60 to 90 μ in diameter, while those formed at 30 or 31°C. were much larger, averaging approximately 145 μ. It seems likely that this effect is indirect, being a reflection of the relative number of conidia, which are formed first under favorable conditions. We may assume that the production of abundant conidia uses much of the food materials which might also go

into the formation of sporangia. Under conditions unfavorable to conidium production, yet favorable to sporangium formation, both the size and abundance of sporangia are increased. The effect of temperature was also evident when pumpkin flowers artificially inoculated with *C. cucurbitarum* were brought into the laboratory and placed at 30°C. Under these conditions both conidia and sporangia were produced.

TABLE 54. THE EFFECT OF TEMPERATURE UPON ASEXUAL REPRODUCTION OF *Choanephora cucurbitarum*
(Barnett and Lilly, *Phytopathology* **40**: 83, 1950.)

Temperature during sporulation, °C.	Conidial heads per culture	Sporangia per culture	Average size of sporangia, μ
25	2,000	300	60–90
30	150	1,300	148
31	0	1,200	145
34	0	0	

Other critical temperature studies are needed, particularly those designed to show the interrelated effects of temperature with other environmental or nutritional factors and to determine the effects of temperature upon the "internal environment" of the fungi. The temperature of incubation affects zygospore formation by *Phycomyces blakesleeanus* indirectly through the amount of acid formed in the medium (Robbins and Schmitt, 1945).

Light. Light has been a neglected and often ignored factor in many studies of sporulation. Too often we place fungi in the laboratory or refrigerator according to our own convenience, not to their needs, and expect them to reproduce as they would in nature. Under natural conditions many fungi fruit only when exposed to light, often to the direct rays of the sun, for a part of the time. Numerous observations have been reported regarding the need for light, but too few of these reports give data as to the intensity, duration, or quality of the light required to initiate sporulation. We should not conclude that intensity and duration are without effect.

A review of the early work on the influence of light on the growth and fruiting of the fungi is presented by Coons (1916). Brefeld (1877) found that some species of *Coprinus* failed to fruit in the dark. A culture of *Coprinus* exposed to light for 2 or 3 hr. was then able to fruit in the normal manner when removed from the light. He also found that higher temperatures replaced, in part, the beneficial effect of light for some species.

Sphaerographium fraxini produced a few pycnidia in the dark at 30°C., whereas none were produced at room temperature in the dark (Leonian, 1924). Pycnidia were produced at room temperature in the light.

Ascochyta nymphaeae, Cytosporella mendax, Endothia parasitica, Kellermania yuccagena, Naemosphaera sp., *Plenodomus destruens,* and *Phoma urens* formed more pycnidia at 30°C. in the dark than in the light at room temperature. The following fungi failed to fruit in the dark at 8°C. but fruited at the same temperature in the presence of light: *Hendersonia* sp., *Melanconium betulinum, Naemosphaera* sp., *Pestalotia guepinia, Phoma urens, Phyllosticta opuntiae, Sphaerographium fraxini,* and *Sphaeronema pruinosum.* Light favored pycnidial formation by *Plenodomus fuscomaculans* (Coons, 1916). The above examples make it clear that light and temperature may serve as interchangeable stimuli to sporulation in some, but not all, instances. Since the response (sporulation) is the same whether light or temperature is the stimulus, this means that these stimuli in some way brought about the same or equivalent changes in the internal environment of the fungus.

Drayton (1937) was able to produce the perfect stage of *Botryotinia convoluta* by controlling light, temperature, and nutrition. The technique is somewhat involved, but it should be remembered that in nature the external environment varies a great deal during the course of a year. Fluctuations in temperature, moisture, light, and food supply are the normal result of the procession of the seasons. Drayton found autoclaved whole wheat to be an excellent substratum for this fungus. The most favorable results were obtained by allowing the culture to develop at 14°C. in the dark for 45 days. At the end of this time the sclerotia were placed in moist quartz sand at 0°C. for 3 to 4 months, then stored at 5°C. When the apothecial fundaments were 2 to 3 mm. long, the cultures were moved to a greenhouse and placed under cheesecloth and the temperature held at 7°C. at night and below 15°C. during the day. The apothecia matured within 4 weeks.

Yarwood (1936, 1941) observed parasitic fungi under natural conditions and found that the production and liberation of the conidia of *Erysiphe polygoni* and the ascospores of *Taphrina deformans* followed a definite diurnal pattern in nature.

The combined effects of temperature and light upon sporulation of *Helminthosporium gramineum* are clearly shown by Houston and Oswald (1946). Best sporulation was obtained under outdoor conditions, with 14 to 15 hr. of daylight and the average maximum and minimum temperatures 26.8 and 8.2°C., respectively. No conidia were produced on potato-glucose agar in the absence of light, either outdoors or inside. Artificial light apparently was less effective than daylight. However, continuous light at 13°C. allowed the formation of a few conidia. On pieces of infected barley leaves, conidia were formed without exposure to light, over a considerable range in temperature. As an explanation of these differences, the authors believe that the mycelium in the leaf in

nature stored up the "necessary potentialities," which then permitted conidium production in darkness. Mycelium growing from the pieces of leaf into the agar did not produce spores in darkness. This is an interesting theory regarding a possible delayed action of light upon sporulation. It also seems possible that the leaf tissue of the host may furnish some nutrient necessary for sporulation which is not contained in potato-glucose agar. Perhaps light is essential to the synthesis of this material by the fungus.

It was demonstrated recently (Barnett and Lilly, 1950) that an isolate of *Choanephora cucurbitarum* requires *both light and darkness* for the formation of conidia, but these factors have little or no apparent influence upon the formation of sporangia. This fungus was grown under a number of conditions, but none was found which overcame the need for either light or darkness. Cultures incubated in the laboratory under natural alternating light and darkness produced abundant conidial heads during the second and third nights after inoculation. Exposure to artificial light for 2 days after inoculation followed by darkness gave similar results, but an exposure in the reverse order resulted in no conidia. Cultures under continuous artificial light (65 foot-candles) and those in total continuous darkness failed to form conidial heads. Continuous light of low intensity (less than 1 foot-candle), however, did permit the formation of numerous conidial heads in the usual period. A summary of the important results is presented in Fig. 59, together with an outline of a proposed hypothesis to explain the results. We may assume that light, or its absence, affects two metabolic reactions, or groups of reactions, which are essential to conidium formation by *C. cucurbitarum*. Light, which is essential to reaction *A*, apparently inhibits reaction *B*, which must occur in darkness or weak light. The reaction in light must be followed by the reaction in darkness, if conidia are to be formed. Continuous bright light favors only reaction *A*, while continuous darkness permits only reaction *B*. Both reactions occur simultaneously in continuous light of low intensity.

A different isolate of *C. cucurbitarum* was studied by Christenberry (1938), who found that alternating periods of light and dark, 12 hr. each, gave the best sporulation. Red-yellow light was more favorable to conidium formation than the shorter rays. This isolate formed conidia in total darkness.

The beneficial effect of alternating light, or a period of light followed by darkness, was demonstrated (Timnick *et al.*, 1951) for the formation of ascospores by *Diaporthe phascolorum* var. *batatatis*. Cultures grown in continuous darkness formed only a few perithecia, which contained abundant ascospores. In continuous bright light numerous perithecia were formed, but relatively fewer ascospores were produced. A long period of light followed by darkness gave many perithecia with abundant ascospores.

Marked morphologic differences were found in strains of *Fusarium* subjected to different exposures of light and darkness (Snyder and Hansen, 1941). Some of the characters affected were color, zonation, type of colony, presence or absence of sporodochia, occurrence of the perithecial stage, and size, shape, and septation of macroconidia. Light was usually found necessary for the formation of macroconidia. Exposures were made to continuous total darkness but not to continuous light. Evidence in these experiments indicated that the effect of light is only upon the actively growing portion of the mycelium.

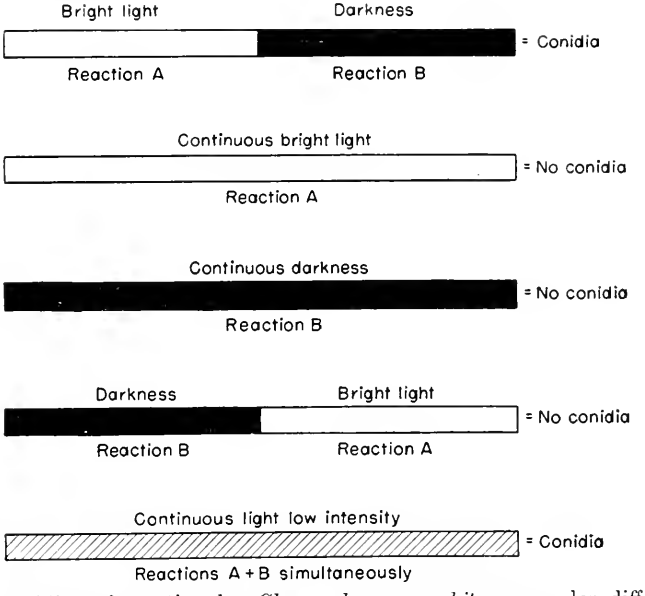


FIG. 59. Conidium formation by *Choanephora cucurbitarum* under different light conditions, showing the possible metabolic reactions controlled by light. Under variable conditions, the cultures were exposed to the first condition (on the left) for 2 days and to the second condition for 24 hr. (After Barnett and Lilly, *Phytopathology* **40**: 88, 1950.)

The length of exposure necessary to stimulate spore formation may be very short, as demonstrated by Bisby (1925) for *Fusarium discolor sulphureum*. He observed that brief exposure to light, while Petri dish cultures were being examined, resulted in the formation of rings of conidia. Using a photographic shutter, he further demonstrated that an exposure as brief as $\frac{1}{4}$ sec. to outdoor light on a bright day was sufficient to stimulate the formation of a ring of conidia.

Coons (1916), in his work with *Plenodomus fuscomaculans*, reasoned that the effect of light might be replaced by various oxidizing agents, since light is known to promote various oxidations. Cultures treated with hydrogen peroxide and other oxidizing agents produced a few pycnidia. The age of the culture when these chemicals were added was important.

For these chemicals to stimulate pycnidium formation, the culture had to be in such a physiological condition that 1-hr. exposure to light would induce sporulation.

The sporulation of a number of other species in our laboratory has been observed to be influenced by the presence or absence of light (Figs. 60, 61). Among these are *Dendrophoma obscurans*, *Trichoderma lignorum*,

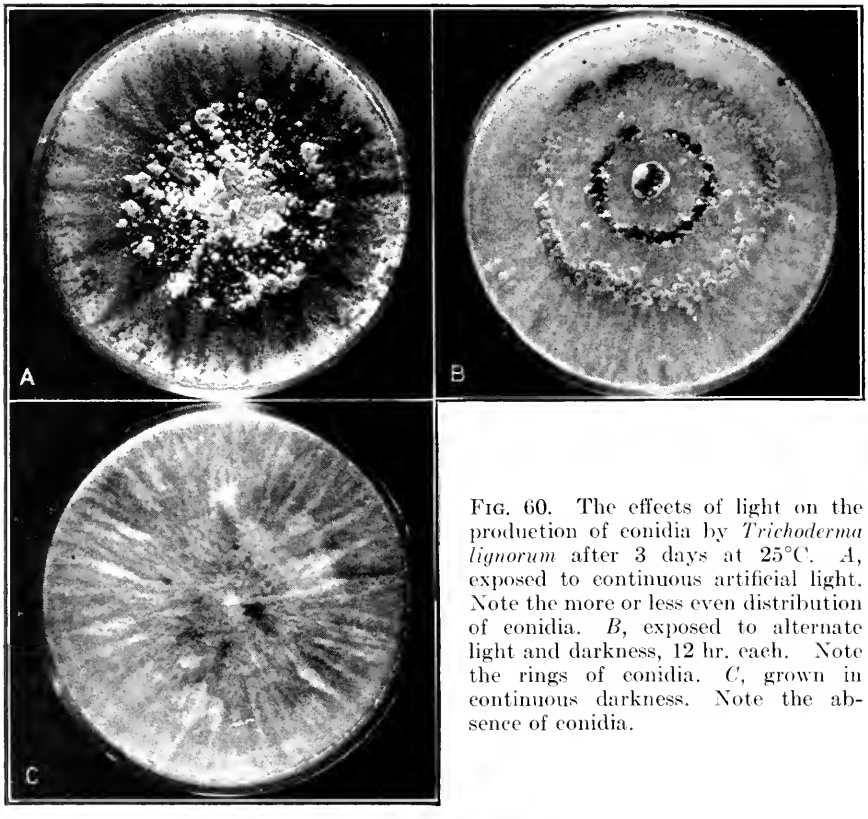


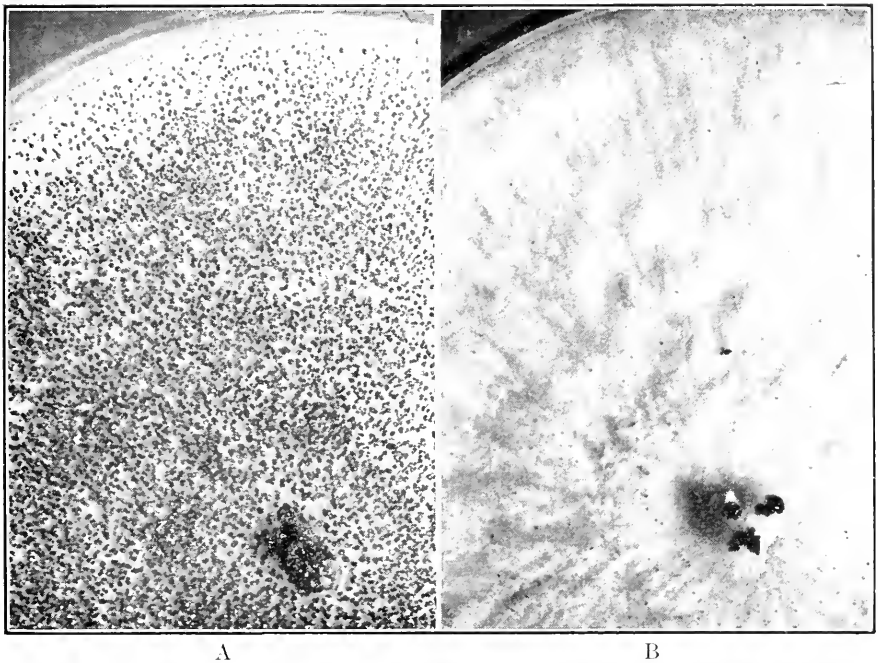
FIG. 60. The effects of light on the production of conidia by *Trichoderma lignorum* after 3 days at 25°C. *A*, exposed to continuous artificial light. Note the more or less even distribution of conidia. *B*, exposed to alternate light and darkness, 12 hr. each. Note the rings of conidia. *C*, grown in continuous darkness. Note the absence of conidia.

Sphaeropsis malorum, *Ceratostomella ulmi*, *Botrytis* sp., *Endothia parasitica*, *Septoria nodorum*. The reaction of some fungi to light is apparently dependent, to a certain extent, upon the composition of the medium.

Still another effect of light should be emphasized, *i.e.*, the inhibitory effect. The depressing effect of strong light upon growth and length of sporangiophores of *Phycomyces blakesleeanus* is easily demonstrated. Elfving (1890) noted that the amount of inhibition of growth by light varied with the composition of the medium.

Ultraviolet light. The destructive action of sunlight upon microorganisms, especially bacteria, was recognized about the time that pure-

culture methods came into wide use. The lethal action of ultraviolet light is conditioned by the wave length of the irradiation, by the time of exposure, and by the particular nature of the microorganism. A considerable number of investigators have studied the effect of ultraviolet radiation upon sporulation. Both favorable and unfavorable results have been obtained. It should be recognized that length of exposure



A B

FIG. 61. The effect of light on the production of pycnidia by an isolate of *Dendrophoma obscurans* when grown on malt extract–agar plates at 25°C. *A*, grown under continuous artificial light. Alternate light and darkness gave similar results. *B*, grown in continuous darkness.

is a very important factor in these experiments. In addition, the medium used, the age of the culture, and the temperature rise during irradiation also modify the results.

Stevens (1928) found that ultraviolet radiation induced the formation of perithecia by various isolates of *Glomerella cingulata* a few days after irradiation. While old cultures produced a few perithecia without irradiation, many more were produced by young cultures within a short time following irradiation. One effect of such irradiation is the killing of the aerial mycelium. Short exposures allowed the formation of superficial perithecia, while long exposures prevented their formation. The majority of the perithecia formed following intermediate dosages were embedded in the medium. It was noted that the age of the mycelium

at the time of irradiation had an effect on the number of perithecia formed. Colonies 4 days old when irradiated produced perithecia, which were most abundant on mycelium 1 day old at the time of irradiation. Irradiation of colonies 12 days old led to the formation of but few perithecia. No evidence was obtained that irradiation of the medium alone had any effect on perithecium formation. A species of *Coniothyrium* which formed pycnidia only after the cultures were very old was stimulated to produce pycnidia within 3 days after irradiation. This work of Stevens is apparently the first which demonstrated that ultraviolet radiation stimulated sporulation by fungi.

Spore production by *Macrosporium tomato* and *Fusarium cepae* was greatly increased by the proper exposure to ultraviolet radiation (Ramsey and Bailey, 1930). A 12- to 15-fold increase in the numbers of spores produced by these two species was obtained by the optimum exposure. These investigators also showed that irradiation of the medium before inoculation had no subsequent effect on sporulation by these two fungi. The range of wave lengths which stimulated the most abundant sporulation was found to be 2,300 to 2,800 Å. Smith (1935) points out that many workers have neglected the precaution of controlling the temperature of cultures during irradiation. She found it necessary to control the temperature of the cultures of *Fusarium eumartii* in order to separate the effects of increased temperature and ultraviolet radiation.

Ultraviolet radiation stimulated or depressed sporulation of *Diaporthe phaseolorum* var. *batatatis* depending on the medium used (Timnick *et al.*, 1951). Neither stromata nor perithecia were formed on casein hydrolysate–glucose medium, unless the cultures were irradiated. Cultures grown on potato-glucose agar produced stromata and long-beaked perithecia without irradiation. Irradiation of cultures on potato-glucose medium resulted in the formation of fewer and smaller short-beaked perithecia. Although the mode of action of ultraviolet radiation in stimulating sporulation is unknown, long exposures are known to be lethal. We may assume that even short exposures injure or kill some of the exposed cells. Perhaps some substance is thereby released which stimulates sporulation. The presence of such a substance in the potato-glucose medium might explain why irradiation was not necessary for the production of perithecia by *D. phaseolorum* var. *batatatis* on this medium

Aeration. Although the fungi are aerobic organisms, the amount of free oxygen that they need to carry out their life processes varies from fungus to fungus. The amount of oxygen required is less for growth than for reproduction. The aquatic fungi would be expected to grow and reproduce in a more limited supply of oxygen than terrestial forms. While many aquatic Phycomycetes produce their spores under water, a large number of fungi fail to fruit until some aerial mycelium has been

formed. Examples of the inhibiting effect of insufficient aeration on sporulation are numerous. Coons (1916) found that lowered oxygen tension inhibited pycnidium formation by *Plenodomus fuscomaculans*, though there was still sufficient oxygen supply to allow some growth. Leonian (1924) tested the effect of reduced oxygen on pycnidium formation by various Sphaeropsidales. This experiment was carried out by culturing these fungi in Petri dishes, some of which were placed in desiccators, while the controls were placed on a table. The following fungi produced fewer pycnidia in sealed desiccators than in the control cultures: *Ascochyta nymphaeae*, *Phoma urens*, *Plenodomus destruens*, *Phyllosticta opuntiae*, and *Septosporium acerinum*. It is possible that this effect may have been due to the increased concentration of carbon dioxide in the closed vessels.

Denny (1933) made an accurate study of the effect of oxygen supply on growth and formation of perithecia by *Neurospora sitophila*. Only a trace of oxygen was required for limited growth, for it was necessary to keep cultures in the presence of alkaline pyrogallol to inhibit growth entirely. Oxygen concentrations of less than 0.5 per cent inhibited perithecium formation for 30 days, while perithecia formed in air within 4 days. This paper should be consulted for the details of conducting experiments of this nature under closely controlled conditions. Some of Denny's data are given in Table 55.

TABLE 55. THE EFFECT OF OXYGEN CONCENTRATION ON THE FORMATION OF PERITHECIA BY *Neurospora sitophila*

(Prepared from the data of Denny, 1933. *Contribs. Boyce Thompson Inst.* **5**, 1933.)

Oxygen Concentration, %	Days Required to Form Perithecia
20.8	4
9.4	7
3.75	9
1.5	12
0.24	None at 30 days

Conidium production by *Choanephora cucurbitarum* was poor in tight-fitting Petri dishes (Barnett and Lilly, 1950). Sealing the dishes prevented conidium formation, while well-aerated dishes allowed abundant conidial heads to form. Failure to form conidia under these conditions may be due to (1) insufficient oxygen supply, (2) the accumulation of toxic, volatile, metabolic by-products, (3) increased carbon dioxide content, or (4) unfavorable humidity.

Adequate aeration was one of the most important environmental factors necessary for conidium formation by *Piricularia oryzae* (Henry and Andersen, 1948). The cultures emitted a strong odor of ammonia after a few days' incubation. It was believed that aeration removed the ammonia and other volatile metabolic by-products which prevented

abundant sporulation. Forced aeration of the culture flasks at the rate of 4 ml. of air per minute per milligram of oats-sorghum medium was found to be optimum for sporulation. Mader (1943) discussed the factors inhibiting fruiting of *Agaricus campestris* and concluded that volatile substances are important, and that they must be removed by aeration of mushroom cellars.

Hydrogen-ion concentration. The early workers recognized that the acidity of the medium influenced sporulation. Lockwood (1937) studied the formation of perithecia and asci by *Penicillium javanicum*, *Aspergillus herbariorum*, and *Chaetomium globosum* in buffered media of various

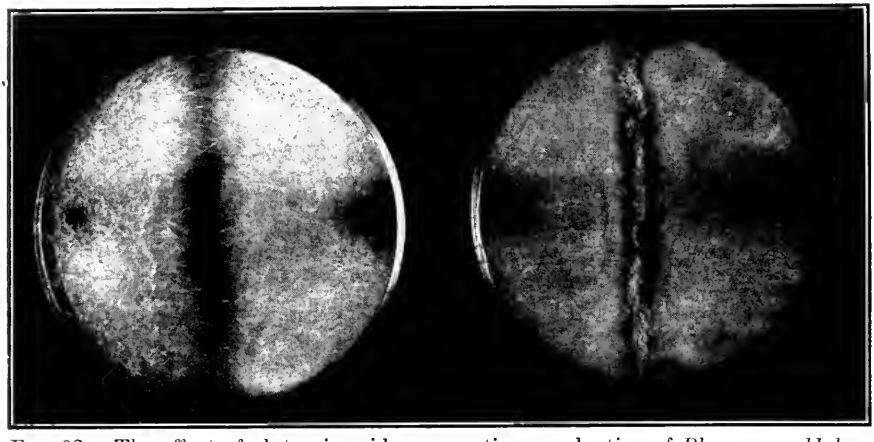


FIG. 62. The effect of glutamic acid on gametic reproduction of *Phycomyces blakesleeanus* at 26°C. Left, basal medium; right, basal medium plus 10 mg. *d*-glutamic acid, neutralized with $CaCO_3$. Note the line of progametes in the plate on the right. Age, 6 days. (Courtesy of Robbins and Schmitt, *Am. Jour. Botany* **32**: 321, 1945.)

hydrogen-ion concentrations and found that the perithecia produced in the more acid solutions contained few if any asci with ascospores. The percentage of fertile perithecia increased as the pH was increased to 7 or 8. Similarly, in our laboratory, we have noted that *A. rugulosus* produces many perithecia and few conidia at an initial pH value of 6 to 8, while conidia but no perithecia form at pH 3 to 4.

Robbins and Schmitt (1945) studied the sexual reproduction of *Phycomyces blakesleeanus* on glucose-asparagine medium and found that mature zygospores did not form at 26°C. Zygospores formed when various protein hydrolysates, amino acids (especially glutamic acid), or various organic acids were added to the medium. These buffers prevented the pH from falling low enough to inhibit zygospore formation (Fig. 62). These authors also noted that *P. blakesleeanus* on glucose-asparagine medium produced zygospores at 20°C. This is evidence that the composition of the medium has a profound effect on reproduction. In this

instance, it was possible to trace the connection between temperature and the composition of the medium to a specific factor, *i.e.*, acidity.

Perithecia were not formed by *Sordaria fimicola* until the pH of the culture medium was 6.5 or greater (Lilly and Barnett, 1947). While acidity of the medium was not the only controlling factor affecting the formation of perithecia by *S. fimicola*, perithecia never formed when the pH was less than 6.5, however favorable the other external conditions were.

OTHER PHYSICAL FACTORS

It has frequently been observed that many species of fungi fruit more readily when grown upon a solid or semisolid substratum than they do in liquid media. Leonian (1924) reported that only 6 out of 20 species studied formed pycnidia as readily in liquid medium as on solid medium. He concluded that the beneficial effect of solid media was due to better aeration and free transpiration.

The favorable effect of ozone upon the formation of pycnidia and spores of a limited number of fungi was recently reported by Richards (1949). The production of viable conidia of three species of *Alternaria* was greatly increased on exposure to ozone. Although conidium formation of *Mycosphaerella citrullina* was increased by exposure to ozone, the spores formed did not germinate.

The transformation and elongation of basidia of certain Polyporaceae in nature and under controlled conditions has been correlated with high humidity by Bose (1943). It seems likely that the humidity of the atmosphere may have a greater influence upon conidium formation in the aerial fungi than is generally supposed. In *Rhizopus*, for instance, much more liquid moves upward through the sporangiophore than can be contained within the sporangium. A high percentage of this water must be transpired in order to condense the protoplasm and food materials stored in the spores. A change in relative humidity must affect the rate of transpiration. On the other hand, Ternetz (1900) found that a humidity of 98 per cent or higher was necessary for fruit-body production by *Ascophanus carneus*. Actually, we know little about the influence of humidity, and much more information is needed on this subject.

Emerson and Cantino (1948) showed that the presence of high concentrations of carbon dioxide favored the production of resistant sporangia by *Blastocladia pringsheimii*.

Mutilation of the mycelium, which would cause the death and release of cellular constituents, has been used to stimulate sporulation (see Rands, 1917; Kunkel, 1918; and McCallan and Chan, 1944). Scraping of the mycelium of *Alternaria solani* followed by a brief exposure to ultraviolet rays was used successfully by McCallan and Chan (Fig. 63).

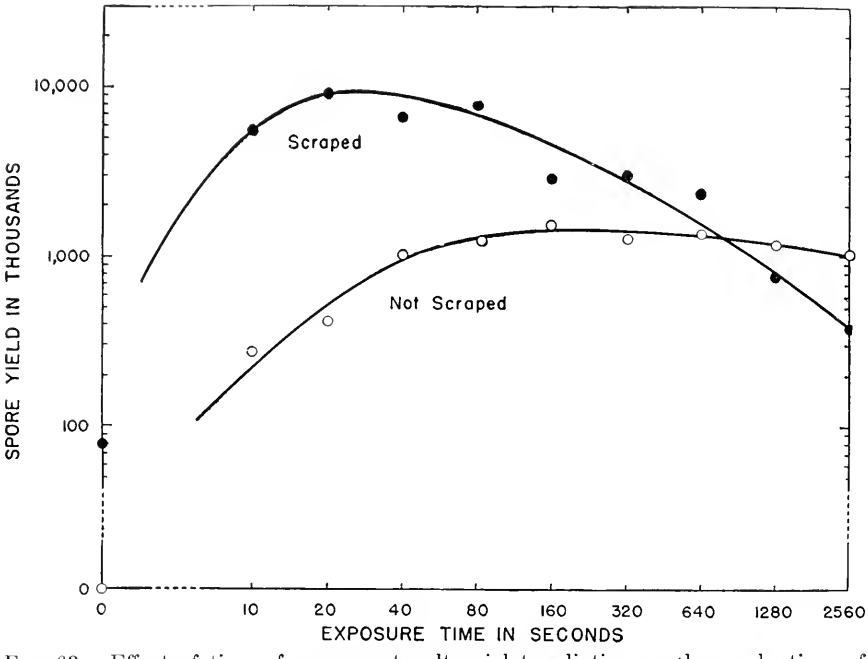


FIG. 63. Effect of time of exposure to ultraviolet radiation on the production of spores from scraped and unscraped cultures of *Alternaria solani*. (Courtesy of McCallan and Chan, *Contribs. Boyce Thompson Inst.* **13**: 327, 1944.)

NUTRITIONAL FACTORS

The nutritional conditions under which a fungus produces reproductive bodies and spores are often quite different from those which are optimum for vegetative growth. Not all media are equally suitable for sporulation. The frequent failure to obtain sporulation of many common fungi in culture, even though they grow profusely, testifies to the extent of our ignorance regarding the necessary nutritional factors. However, the following factors have been shown to be important: concentration of medium, carbon and nitrogen sources, carbon-nitrogen ratio, micro essential elements, specific reproductive factors, and vitamins.

Concentration of nutrients. Among the early workers, Klebs (1900) gave a great deal of attention to the effect of nutrient concentration upon reproduction. For most of the fungi with which he worked, exhaustion of the food supply favored sporulation. Klebs (1899) kept a culture of *Saprolegnia mixta* in the vegetative condition for 2½ years by constant renewal of the nutrient solution. Yet, this fungus produced spores within a few days when the food supply became exhausted. The same principle holds true for the Myxomycetes as well as the filamentous fungi. Camp (1937) grew *Physarum polycephalum* and studied the effect of the number

of feedings upon the time of fruiting. The protoplasm continued to grow as long as there was abundant food, but when the food was exhausted, the slime mold passed into the fruiting stage.

Leonian (1923, 1924) used a technique in studying sporulation which consisted in growing a fungus in a medium suitable for vigorous vegetative growth, and then transferring it to solutions of different concentrations to stimulate sporulation. When sterile mycelium of *Valsa leucostoma* was transferred from a medium containing 1.5 per cent nutrients to a medium containing 0.37 per cent nutrients the ratio of perithecia to pycnidia increased. Transferring sterile mycelium to more concentrated nutrient solutions favored the production of pycnidia and decreased the number of perithecia formed. *Endothia parasitica* showed a decrease in the number of pycnidia when the mycelium was transferred from a weak to a concentrated medium. When the sterile mycelium was grown in a concentrated medium and transferred to distilled water, the pycnidia did not mature but an enormous number of pycnidium initials were formed. If such a culture were then transferred back to a concentrated medium, maximum sporulation was obtained. A review of the literature on the effect of concentration on fruiting is given by Leonian (1924).

From the above examples we may conclude that the concentration of nutrients in a medium may have a profound influence upon fruiting, and that the different types of fruiting (sexual and asexual) may have different requirements. Not only the amounts of the different nutrients but the proper balance between the components of the medium may be essential for maximum sporulation.

Nitrogen source. The source of nitrogen influenced the formation of pycnidia and spores by *Phyllosticta solitaria* (Mix, 1933). The specificity of the nitrogen source was greater for the production of spores than for the formation of pycnidia. The different isolates of this fungus responded differently to the various nitrogen sources. Nitrate nitrogen was the most favorable. This may have been due to an indirect effect on the pH of the medium, for this fungus sporulates only between pH 4.2 and 5.8.

In our laboratory we have observed that sporulation of some fungi is favored by certain sources of nitrogen, which are not necessarily the same as those which are favorable for growth (Fig. 64). A few of these species with the more favorable nitrogen sources for sporulation are *Monilinia fructicola*, ammonium tartrate, glycine; *Phoma betae*, glycine; *Neocosmopara vasinfecta*, glutamic acid, glycine; *Septoria nodorum*, glycine; *Diaporthe phaseolorum* var. *batatatis*, asparagine; *Choanephora cucurbitarum*, organic nitrogen.

Carbon source. Not all carbon sources are equally suitable for fruiting of fungi. Some which are favorable for mycelial growth do not favor sporulation. Hawker (1939) found the number of perithecia produced

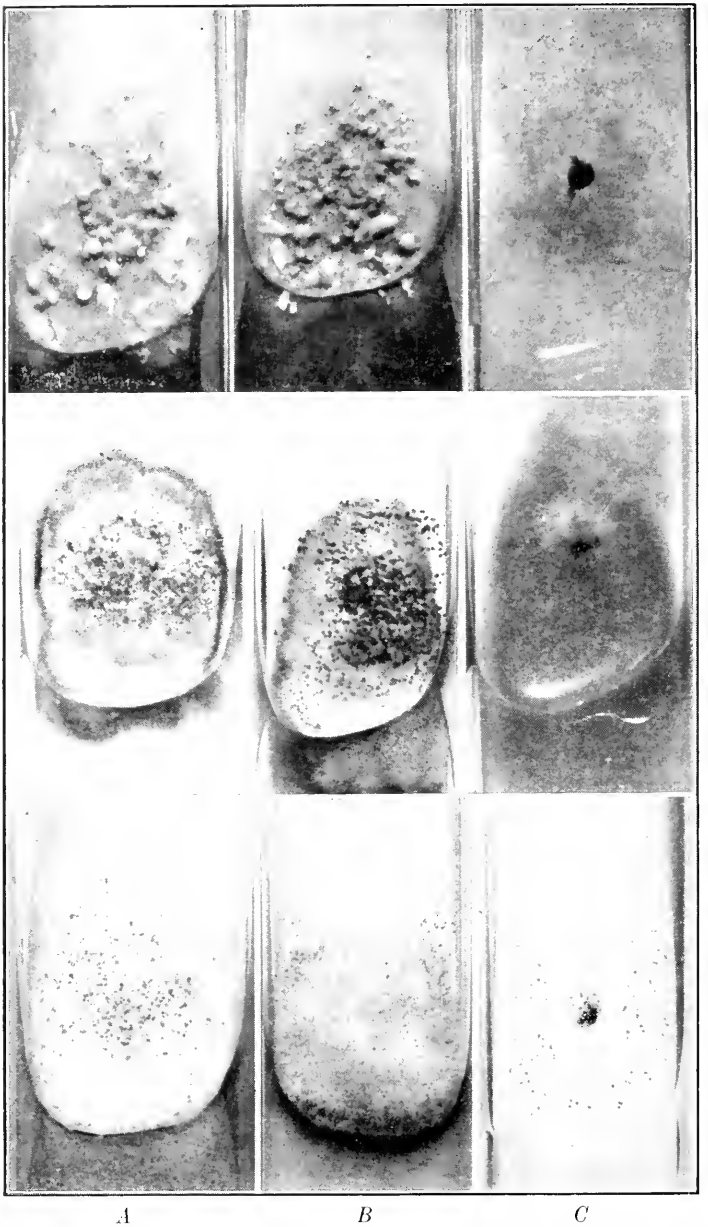
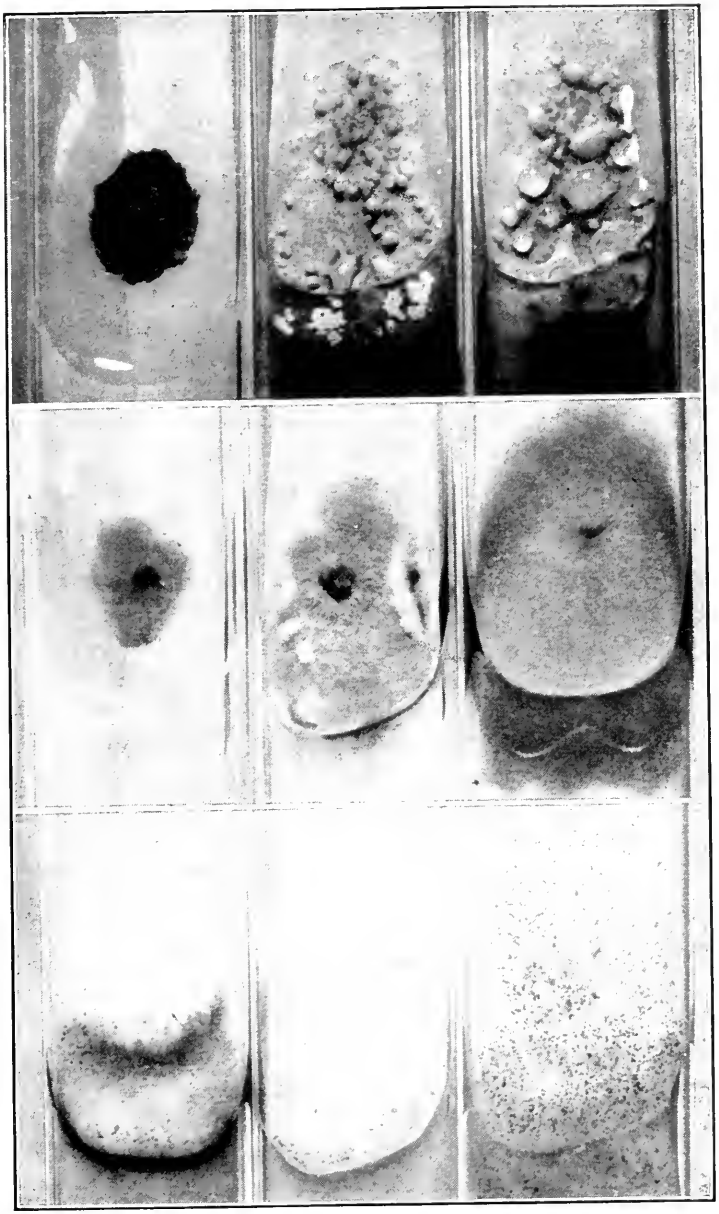


FIG. 64. The effects of different nitrogen sources on sporulation of three fungi after 19 days on a glucose-sucrose medium at 25°C. The nitrogen sources are: *A*, apsaragine; *B*, casein hydrolysate; *C*, potassium nitrate; *D*, ammonium sulfate; *E*, ammonium tartrate; *F*, glycine. The fungi are: top, *Glomerella cingulata;* middle, *Pleurage*



D *E* *F*

curvicolla; bottom, *Melanospora* sp. Note that asparagine and casein hydrolysate are good sources of nitrogen for spore production of all three fungi. Discharged spores of *P. curvicolla* are evident only on these two media.

by *Melanospora destruens* to be influenced by the concentration and kind of sugar used. Glucose, fructose, or an equimolar mixture of these sugars, when used at the rate of 5 g. per liter, allowed the production of perithecia, but no perithecia were formed when 50 g. was used. Many perithecia were produced when 50 g. per liter of sucrose was used.

The favorable effect of sucrose on perithecial formation was replaced by various hexose phosphate esters. Glucose-1-phosphate and fructose-1,6-diphosphate were equally active. In view of their ready enzymatic interconvertibility in organisms, this would be expected. These results

A *B* *C* *D* *E*

FIG. 65. The effects of different carbon sources on the production of conidia by *Glomerella cingulata* after 22 days on asparagine medium at 25°C. The carbon sources are: *A*, glucose; *B*, sucrose; *C*, maltose; *D*, sorbose; *E*, starch. Note that sporulation is greatest on sucrose and least on starch.

suggest that *M. destruens* phosphorylates sucrose with greater ease than either glucose or fructose. This is in line with the experiments of Doudoroff (1945) with growth of *Pseudomonas saccharophila*. Since *M. destruens* makes better growth upon glucose than upon sucrose, it may be suggested that the pathway of carbohydrate utilization is different in *growth* and *reproduction*. These findings emphasize again that the requirements for growth and reproduction may be different.

Glucose, mannose, fructose, lactose, and sucrose are reported (Mix, 1933) as favorable for pycnidium formation by *Phyllosticta solitaria*. Lactose was the most favorable sugar for the production of perithecia by *Diaporthe phaseolorum* var. *batatatis* (Timnick *et al.*, 1951). Brodie (1948) induced *Cyathus stercoreus* to produce normal, fertile fruit bodies on semisynthetic media containing filter paper.

In our laboratory we have observed that the carbon source affects reproduction of a number of other fungi, and that the best source for sporulation is not always the same which yields maximum vegetative growth (Figs. 65 and 66). Some of these fungi with some more favorable

carbon sources for reproduction are *Aspergillus niger*, glucose, sorbose, sucrose; *Glomerella cingulata*, sucrose; *Phoma betae*, sucrose; *Monilinia fructicola*, sorbose, sucrose; *Neocosmopara vasinfecta*, maltose, starch, glucose; *Pleurage curvicolla*, maltose, starch.

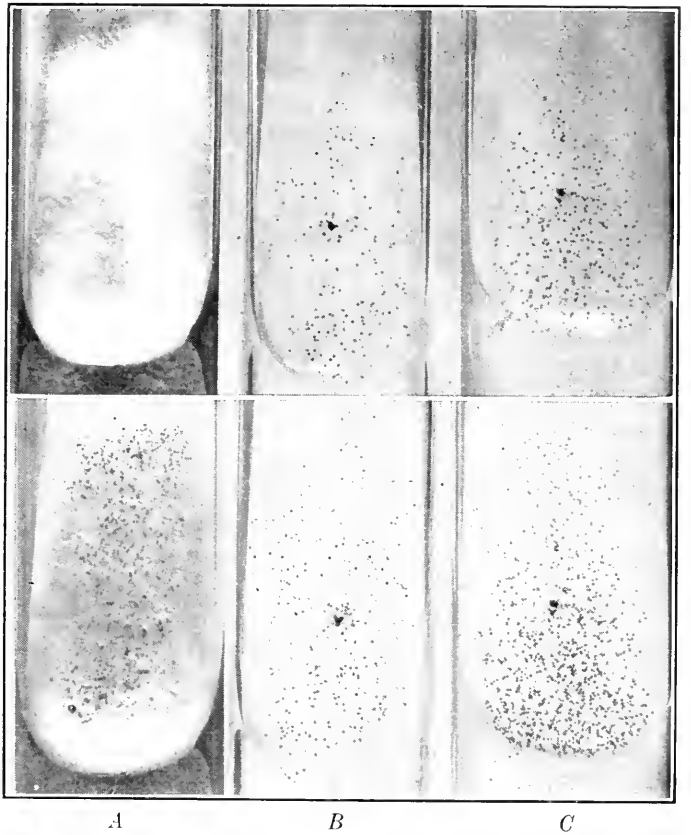


FIG. 66. The effect of three carbon sources and time on the production of perithecia by *Melanospora* sp. on asparagine medium at 25°C. The carbon sources were: *A*, glucose; *B*, sucrose; *C*, maltose. Above, cultures 11 days old; below, the same cultures 22 days old. Note the poor vegetative growth but presence of perithecia on sucrose and maltose, and the abundant early vegetative growth but delayed production of perithecia on glucose.

For further information on the effects of nutritional factors on sporulation, see Hawker (1950).

Carbon-nitrogen ratio. It seems to be generally held that a proper balance among the constituents of the medium is quite important in growth and sporulation. Westergaard and Mitchell (1947) investigated, among other factors, the influence of the carbon-nitrogen ratio of the medium on formation of perithecia by *Neurospora crassa*. Some of their data are given in Table 56. It is evident that high concentrations of

glucose and potassium nitrate are unfavorable for the production of perithecia by *N. crassa.*

TABLE 56. THE EFFECT OF THE CARBON-NITROGEN RATIO OF THE MEDIUM ON THE PRODUCTION OF PERITHECIA BY *Neurospora crassa*

Production rated on scale of 10. Age, 11 days. (Westergaard and Mitchell, *Am. Jour. Botany* **34**, 1947.)

Glucose concentration, %	KNO_3 concentration, %					
	0.001	0.01	0.05	0.1	0.5	1.0
0.2	1	3	3	3	2	1
0.6	2	4	6	5	3	1
1.0	2	5	7	7	3	1
1.4	1	4	7	9	3	1
1.8	3	5	9	10	3	2
2.2	1	4	7	9	2	0

Micro essential elements. Steinberg (see references in Chap. 5) found the sporulation of *Aspergillus niger* to be depressed by the omission of various of the essential elements. Lockwood and Ward (1936) found that *Rhizopus oryzae* sporulated on the thirteenth day of incubation when zinc was not added to the medium. When zinc was added, sporulation occurred on the third day of incubation. In general, when any essential element is low, sporulation tends to be depressed before growth is inhibited.

Specific reproductive factors. While many factors may influence reproduction, there is little evidence that the fungi need specific chemical substances to induce reproduction. Such factors, however, do exist among certain of the Phycomycetes.

Four specific regulatory substances, called *hormones* (Raper, 1942, and Raper and Haagen-Smit, 1942), were shown to initiate and control sexual reproduction of *Achlya bisexualis.* A specific substratum is required for the production of hormone A in large quantities. Hempseed allows a 2- to 10-fold production of hormone A over that produced by similar cultures grown upon corn, rice, lentils, or other substances. Hormone A has been concentrated 70,000-fold but has not yet been obtained in pure form. This concentrate of hormone A is active in dilutions of 1×10^{-12}.

Sexual reproduction in *Phytophthora cactorum* was greatly stimulated by an extract of garden peas (Leonian, 1936). This substance had no growth-promoting properties. It was concluded (Leonian and Lilly, 1937) that the sexuality factor was none of the known vitamins, and that it probably was not carotene or xanthophyll, although it was concentrated by methods which would concentrate these substances. When vigorous

sterile mycelium of *P. cactorum* was washed in distilled water and transferred to the optimum concentration of the sexuality factor in 0.1 per cent agar, oogonia began to appear within 15 hr., reaching the maximum development in 3 days. This factor was also effective in inducing sexual reproduction by *P. erythroseptica*, *P. bochmeriae*, and *P. megasperma*. In addition, the presence of this sexuality factor induced the formation of abundant oogonia within a week by 15 of the 20 species of *Phythium* tested. However, it failed to induce sporulation of various Zygomycetes, Ascomycetes, and Basidiomycetes.

γ-Carotene is associated with the male cells of the sexual phase and not with the female cells of *Allomyces* (Emerson and Fox, 1940). This specificity of association with the male cells indicates that γ-carotene may be associated with sexual reproduction in some species of this genus. No γ-carotene was found in the cells of these fungi in the asexual phase.

Association with other organisms. That one fungus may influence the sporulation of another has been known for a long time. Sporulation of *Alternaria* and *Helminthosporium* was increased when they were grown in association with certain other organisms (Porter, 1924). This paper has a valuable bibliography on associative effects.

The metabolic products of *Aspergillus niger* are known to promote conjugation in three species of yeasts belonging to the genus *Zygosaccharomyces* (Nickerson and Thimann, 1943). As a result of extensive investigations, these authors found that part of the activity of *Aspergillus* filtrate could be replaced by glutaric acid and riboflavin. While these substances had some activity when tested separately, the combination of glutaric acid and riboflavin greatly exceeded the activity of either alone. Riboflavin was shown definitely to be a component of the *Aspergillus* filtrate. An autolysate from *Zygosaccharomyces* cells had a favorable influence on sporulation by the same organism. From this experiment and from the work of Lindegren and Hamilton (1944), who found that ascus formation in yeast would take place only in portions of the yeast colony where autolysis had taken place, it may be concluded that autolytic products favor sporulation in some instances. Lindegren and Lindegren (1944) found that addition of 2 per cent dried brewer's yeast to a presporulation medium very favorably influenced the sporulation of *Saccharomyces cerevisiae*.

The presence of *Bacillus weidmaniensis* greatly stimulated growth and production of macroconidia by *Microsporum audouini* (Benedek, 1943, and Hazen, 1947). The addition of yeast extract to the medium had a similar effect. A part of this stimulating effect was attributed to pyridoxine. The addition of yeast extract to a basal medium of honey agar resulted in a marked increase in vegetative growth and macroconidium production (Hazen, 1947). This stimulation was attributed to the pres-

ence of growth factors in the yeast extract. The addition of pyridoxine to the basal medium caused little change in mycelial growth, but a great increase in abundance of macroconidia resulted. On the other hand, the addition of thiamine or of a mixture of thiamine and pyridoxine caused no increase in growth or production of macroconidia.

An interesting observation of the constant natural association between *Nectria coccinea* and *Gonatorrhodiella highlei* is reported by Ayres (1941). Because of this constant association it was believed that *G. highlei* was either parasitic upon *N. coccinea* or dependent upon it for some nutritional substance. On potato-glucose, malt extract, and other common

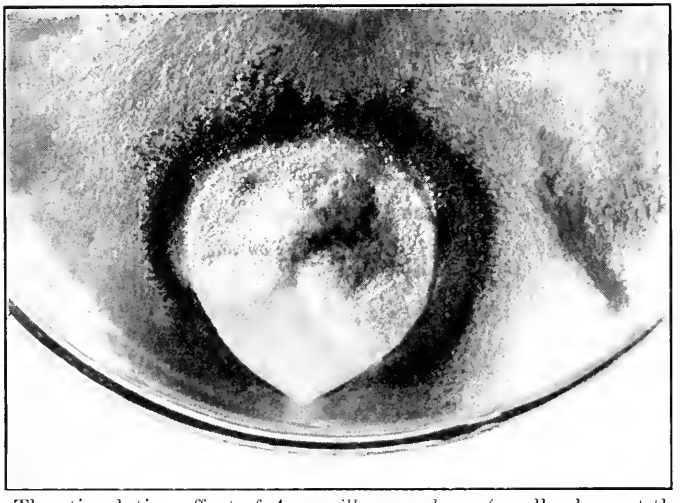


FIG. 67. The stimulating effect of *Aspergillus rugulosus* (small colony at the bottom) on the production of perithecia by *Sordaria fimicola* grown on glucose-asparagine medium low in biotin. The zone of black perithecia of *Sordaria* around the colony of *Aspergillus* is attributed to the biotin excreted by the latter fungus.

media *G. highlei* made only slight growth and formed no conidiophores or conidia. However, the fungus grew well and produced numerous conidiophores and conidia on the same media in the presence of *N. coccinea*, *N. galligena*, or *N. cucurbitula*. Neither *N. cinnabarina* nor *N. coryli* caused stimulation. *G. highlei* was cultivated successfully with production of abundant conidia on oatmeal mush, without the presence of other fungi. These results strongly suggest a nutritional relation between *G. highlei* and *N. coccinea*, other than that of parasitism.

The beneficial effect of one fungus upon reproduction of another can easily be demonstrated by placing *Aspergillus rugulosus* and *Sordaria fimicola* on a plate of agar containing little or no biotin (Fig. 67). *S. fimicola*, being a more rapid grower, produces a sparse mycelium, which surrounds the slow-growing colony of *Aspergillus*. The *Sordaria* myce-

lium next to the *Aspergillus* colony soon shows stimulated growth, which is followed by the formation of abundant perithecia in this area. Since we know that *Sordaria* is deficient for biotin and requires an exogenous supply of this vitamin for reproduction, we may assume that the stimulating effect is due to the extra biotin produced by *A. rugulosus* diffusing into the medium.

Vitamins. Many fungi cannot synthesize sufficient amounts of certain vitamins and must depend upon an outside source of these vitamins for optimum growth. Since many nutritional factors may influence both growth and reproduction, it would be logical to expect that the vitamin supply would affect the reproduction of vitamin-deficient fungi. Robbins and Ma (1942) ventured the opinion that, although the sex organs of certain deficient fungi were formed only in the presence of the growth factor for which these fungi were deficient, they were doubtful if there was any direct relation between vitamin supply and the formation of sex organs. They regarded the failure of *Ceratostomella pluriannulata* to produce perithecia in the absence of thiamine to be a disturbance of the physiology of the fungus and ventured the prediction that the formation of sex organs in other deficient fungi would be found associated with the vitamins for which the fungi was deficient.

Melanospora destruens is able to grow in the presence of biotin as the only growth factor, but it produces perithecia only when thiamine, too, is added to the medium (Hawker, 1939). The relationship between the amount of sugar and the amount of thiamine necessary for maximum fruiting is clearly brought out by the thiamine-deficient fungus, *C. fimbriata* (Barnett and Lilly, 1947*a*). Whether perithecia are formed on a given medium is determined by the amount of thiamine relative to the amount of food in the medium (Table 57). In a reduced supply of sugar the concentration of thiamine necessary to induce fruiting is also reduced. The abundance of perithecia is conditioned both by the amount of thiamine and by the amount of nutrients. Less thiamine is required for vegetative growth than for the production of perithecia. A similar relation between amounts of sugar and biotin and sporulation of *Memnoniella echinata* was described by Buston and Basu (1948). It should be noted from Table 57 that, in a medium high in sugar but low in thiamine, no perithecia were formed even though as much as 30 mg. of mycelium was present. On the other hand, perithecia were produced on as little as two mg. of mycelium when the medium contained the same amount of thiamine but was very low in sugar. These results are not in accord with the idea that vigorous or abundant mycelium is essential to the formation of perithecia; they indicate a more direct relation between vitamin supply and sexual reproduction.

This direct relationship may be further illustrated by a simple experi-

ment. Sterile thiamine-starved mycelium may be obtained by growing *C. fimbriata* on a synthetic medium containing 25 g. glucose and less than 2 μg of thiamine per liter. When a small portion of this sterile mycelium is transferred to the surface of distilled water, no perithecia are formed. However, under the same treatment, but with the addition of thiamine to the water, fertile perithecia are formed within a few days. We may assume that, when thiamine is added, the fungus uses this vitamin as a coenzyme in transforming the protoplasmic reserves into perithecia and

TABLE 57. THE EFFECT OF VARIOUS CONCENTRATIONS OF THIAMINE UPON GROWTH AND ESTIMATED ABUNDANCE OF PERITHECIA FORMED BY *Ceratostomella fimbriata* IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF NUTRIENTS

Growth in milligrams. Abundance of perithecia indicated by: 0 = none; + = less than 20; ++ = 20 to 200; +++ = 200 to 1,000; ++++ = more than 1,000. (Barnett and Lilly, *Mycologia* **39,** 1947.)

Thiamine, μg per culture (25 ml.)	Dilution of medium			
	Undiluted	1/4	1/16	1/64
2.5	110 ++++	33 ++++	10 ++++	2 ++
0.156	59 ++	36 ++++	9 ++++	2 ++
0.04	30 0	17 ++	10 ++++	2 +
0.02	18 0	14 0	9 +++	3 +

ascospores. This experiment has been successfully conducted using other fungi deficient for thiamine, and similar results also were obtained with biotin and biotin-deficient fungi. The amount of biotin added to the medium affected not only the number of perithecia formed by *Sordaria fimicola,* but also the time required for this fungus to form mature perithecia (Lilly and Barnett, 1947). This period ranged from 13 to 41 days, depending on the concentration of biotin used.

There is a pronounced effect of biotin deficiency upon the formation and development of the ascospores of *S. fimicola* (Barnett and Lilly, 1947). This fungus is well suited for such a study, for normally nearly all the ascospores mature at the same time. Figure 68 shows the effects of biotin starvation upon the formation of ascospores. Severe effects are evident by the failure of the protoplasm of the asci to be delimited into ascospores or by the failure of many of the ascospores to mature. Other conditions being equal, the amount of biotin required for the pro-

duction of mature ascospores is greater than the amount required for the formation of perithecia.

A *B*

FIG. 68. The effect of biotin starvation on the formation of ascospores by *Sordaria fimicola*. *A*, asci from a perithecium developed in a suboptimum concentration of biotin. Note the few mature ascospores and the majority of asci in which no spores, or only aborted ascospores, have formed. *B*, normal asci with mature ascospores developed on medium with optimum biotin concentration.

Since the vitamins are not considered as specific reproductive substances, it may be expected that an adequate vitamin supply may be necessary for the formation of asexual spores as well as the sexual fruiting structures. However, it should be kept in mind that asexual reproduction is more nearly like vegetative growth in its requirements than is sexual reproduction. A reduction in the supply of a necessary vitamin to the point where asexual reproduction is inhibited may also allow but little vegetative growth. *Piricularia oryzae* is deficient for both thiamine and biotin (Leaver *et al.*, 1947). Conidia did not form unless both vitamins were present in the medium. The concentration of biotin could be reduced to such a level that conidial production was inhibited but some mycelial growth was still allowed. In an adequate supply of biotin, growth and sporulation were apparently parallel. It is also

TABLE 58. THE EFFECTS OF CONCENTRATION OF GLUCOSE AND THIAMINE UPON THE NUMBER OF CONIDIAL HEADS FORMED BY *Choanephora cucurbitarum*
(Barnett and Lilly, *Phytopathology* **40**, 1950.)

Glucose, g. per liter	Thiamine, μg per liter	Conidial heads per plate
25	0	8
25	1.5	210
25	25	450
2	25	2,000

evident that a proper balance between the amounts of vitamins and supply of nutrients is necessary for maximum production of asexual spores. The effects of the concentrations of thiamine and glucose upon the production of conidial heads by *Choanephora cucurbitarum* are illustrated in Table 58. On the other hand, we have observed that an excess of certain vitamins may cause a decrease in sporulation of some fungi.

OTHER FACTORS

Method of inoculation. The method of inoculation and the type of inoculum used are often important factors affecting sporulation in culture. Some fungi sporulate more quickly, and often more abundantly, on agar when the medium is flooded with a spore suspension than when the inoculum is placed at only one point. This is particularly applicable to certain pycnidium- or acervulus-producing fungi, which usually produce abundant mycelium before fruiting.

It is possible that the spores of certain fungi carry over sporulation-inducing substances to the next generation. The production of conidia of *Colletotrichum lindemuthianum* was greater on agar media inoculated at one point with spores than when bits of mycelium were used as inoculum (Mathur *et al.*, 1950).

Method of sterilizing media. The most common method of sterilizing culture media is the use of steam pressure in an autoclave. The standard time is usually 15 to 20 min. at 15 lb. pressure. This temperature is known to cause a breakdown of certain sugars, with an accompanying change in acidity of the medium. When natural media are used, other chemical changes occur, which may or may not be beneficial to reproduction of the fungi. Shanor (1936) reported that fruiting structures of *Cordyceps militaris* were not formed when autoclaved insects were inoculated, whereas the fungus produced stromata and perithecia when living pupae were inoculated. A new approach to the problem of sterilization is suggested by Hansen and Snyder (1947), who propose the use of propylene oxide. This method has great possibilities but has not been used extensively enough to determine all its virtues and limitations.

Influence of the host. Numerous parasitic fungi which have been grown on artificial media have not been induced to form the sexual reproductive stages under any conditions in culture. Some of these will produce the reproductive stages when grown on their respective hosts. For instance, most smuts will produce mycelium in culture, but few have produced teliospores ("chlamydospores") under these conditions. *Claviceps* has never been induced to form sclerotia (which necessarily precede the perfect stage) in artificial culture. Other fungi seem to lose their ability to sporulate profusely in culture but may regain that ability when grown on the appropriate host. It may be pointed out that many of the fungi

pathogenic on man do not produce the same reproductive stages in culture that they do in their host (Conant *et al.*, 1944). Much more intensive study needs to be made of these pathogens.

SUMMARY

Reproduction in the fungi, particularly sexual reproduction, with all the necessary preliminary metabolic activities, is a complex phenomenon. Some fungi appear to be relatively indifferent to and independent of their environment with respect to sporulation, while others appear to require a special combination of environmental conditions. If we assume that all fungi require similar internal conditions for sexual reproduction—and we do not believe this to be an unreasonable assumption—we must recognize the existence of numerous fundamental differences in the metabolic activities of the many different fungi. Some are capable of creating the necessary internal conditions in spite of external conditions which may be limiting factors for other fungi. Other species may not have the ability to create the necessary internal conditions without specific action of certain external factors, such as light, proper nutrients, vitamins, and others. As has been previously emphasized, these processes are often dependent upon the enzyme systems of the fungus, and these systems and their activity vary widely among the different species.

Many fungi reproduce sexually only as the vegetative growth is near or past the maximum. At this time many of the cells of the mycelium are dead or dying. Autolysis follows, and the cells that remain alive absorb certain of these products of autolysis and are thus enabled to increase their concentration of certain essential substances to such a degree that reproduction is possible. If these speculations are valid, it might be expected that nearly any type of injury which causes death of some of the cells would have a favorable effect on reproduction of some fungi. Ultraviolet radiation, which inhibits growth and often favors sporulation, may act by killing some of the cells. Heat may also act by speeding up the life processes of a fungus so that maturity is reached more quickly, which, followed by death of cells and autolysis, would furnish the necessary stimulus for reproduction. Treatment with hydrogen peroxide and certain other chemicals may also result in death and autolysis of some cells. No claim is advanced that these speculations are the true explanation in all instances, but they do provide a hypothesis for interpreting certain puzzling problems connected with sexual reproduction in the fungi.

Factors which may initiate or stimulate sporulation of fungi which grow well but fruit only sparingly or not at all under the usual conditions of artificial culture (assuming that genetic factors are favorable), are as follows: (1) A change in the concentration of one or more of the nutrients.

A reduction in sugar alone may be effective. (2) A change in the source of carbon. Replacing glucose by the same amount of sucrose, lactose, starch, or other carbon source may favor reproduction. (3) A change in the source of nitrogen. (4) A change in the carbon-nitrogen ratio. (5) The addition of an adequate supply of vitamins for vitamin-deficient fungi. (6) The addition of certain micro elements to the medium, if it is made up of highly purified chemicals. (7) The addition to the medium of certain natural products, such as pieces of stems or leaves. (8) The addition of culture filtrate containing metabolic products from the same or other fungus. (9) The addition of special compounds, such as glutaric acid, which has been shown to be effective in a few cases. (10) The addition of certain specific sexual factors, or hormones, known to be effective for certain Phycomycetes. (11) The addition of certain chemicals, such as hydrogen peroxide, to the mycelium. (12) Exposure of cultures to ozone. (13) The use of spores instead of mycelium as inoculum. Flooding of agar plates with spore suspension has given excellent results with some fungi. (14) The sterilization of media by means other than heat. (15) Transfer of certain parasitic fungi to their living hosts. (16) Growing the fungus in the presence of certain bacteria or other fungi. (17) A change in pH of the medium. (18) A change in the temperature of incubation. (19) Adequate aeration. (20) Exposure to light or alternate light and darkness. (21) Short exposures to ultraviolet radiation. (22) Variation in the intensity and wave length of light. (23) Mechanical injury to the mycelium. (24) Gradual desiccation of the cultures. (25) Allowing the cultures to age. (26) The proper combination of any two or more of the above factors. The secret of the sexual reproduction of many fungi no doubt lies in the proper combination of factors which singly are known to favor reproduction in other fungi.

REFERENCES

Ayres, T. T.: The distribution and association of *Gonatorrhodiella highlei* with *Nectria coccinea* in the United States, *Mycologia* **33**: 178–187, 1941.

★Barnett, H. L., and V. G. Lilly: The effects of biotin upon the formation and development of perithecia, asci and ascospores by *Sordaria fimicola* Ces. and de Not., *Am. Jour. Botany* **34**: 196–204, 1947.

Barnett, H. L., and V. G. Lilly: The relation of thiamin to the production of perithecia by *Ceratostomella fimbriata*, *Mycologia* **39**: 699–708, 1947*a*.

★Barnett, H. L., and V. G. Lilly: Nutritional and environmental factors influencing asexual sporulation of *Choanephora cucurbitarum* in culture, *Phytopathology* **40**: 80–89, 1950.

Benedek, T.: Unilateral stimulation of *Microsporum audouini* by a new species of *Bacillus*, *Mycologia* **35**: 222–242, 1943.

Bisby, G. R.: Zonation in cultures of *Fusarium discolor sulphureum*, *Mycologia* **17**: 89–97, 1925.

Bisby, G. R.: Geographical distribution of Fungi, *Botan. Rev.* **9**: 466–482, 1943.

Bose, S. R.: Moisture-relation as a determinant factor in the transformation of the basidia of certain Polyporaceae, *Mycologia* **35**: 33–46, 1943.

Brefeld, O.: Botanische Untersuchungen über Schimmelpilze, Heft III; 1–266, Verlag von Arthur Felix, Leipzig, 1877.

Brodie, H. J.: Variation in fruit bodies of *Cyathus stercoreus* produced in culture, *Mycologia* **40**: 614–626, 1948.

Buston, H. W., and S. N. Basu: Some factors affecting the growth and sporulation of *Chaetomium globosum* and *Memnoniella echinata*, *Jour. Gen. Microbiol.* **2**: 162–172, 1948.

Camp, W. G.: The fruiting of *Physarum polycephalum* in relation to nutrition, *Am. Jour. Botany* **24**: 300–303, 1937.

Christenberry, G. A.: A study of the effect of light of various periods and wave lengths on the growth and asexual reproduction of *Choanephora cucurbitarum* (Berk. and Rav.) Thaxter, *Jour. Elisha Mitchell Sci. Soc.* **54**: 297–310, 1938.

Conant, N. F., D. S. Martin, D. T. Smith, R. D. Baker, and J. L. Callaway: Manual of Clinical Mycology, W. B. Saunders Company, Philadelphia, 1944.

Coons, G. H.: Factors involved in the growth and the pycnidium formation of *Plenodomus fuscomaculans*, *Jour. Agr. Research* **5**: 713–769, 1916.

Denny, F. E.: Oxygen requirements of *Neurospora sitophila* for the formation of perithecia and growth of mycelium, *Contribs. Boyce Thompson Inst.* **5**: 95–102, 1933.

Doudoroff, M.: The utilization and synthesis of sucrose and related compounds by some microorganisms, *Fed. Proc.* **4**: 241–247, 1945.

★Drayton, F. L.: The perfect stage of *Botrytis convoluta*, *Mycologia* **29**: 305–318, 1937.

Elfving, F.: Studien über die Einwirking des Lichtes auf die Pilze, Helsingfors Central-Druckerei, Helsingfors, 1890.

Emerson, R., and E. C. Cantino. The isolation, growth, and metabolism of *Blastocladia* in pure culture, *Am. Jour. Botany* **35**: 157–171, 1948.

Emerson, R., and D. L. Fox: *Gamma*-carotene in the sexual phase of the aquatic fungus *Allomyces*, *Proc. Roy. Soc. (London)*, Ser. B, **128**: 275–293, 1940.

Hansen, H. N., and W. C. Snyder: Gaseous sterilization of biological materials for use as culture media, *Phytopathology* **37**: 369–371, 1947.

★Hawker, L. E.: The influence of various sources of carbon on the formation of perithecia by *Melanospora destruens* Shear in the presence of accessory growth factors, *Ann. Botany* (N.S.) **3**: 455–468, 1939.

Hawker, L. E.: Stimulation of the formation of perithecia of *Melanospora destruens* Shear by small quantities of certain phosphoric esters of glucose and fructose, *Ann. Botany* (N.S.) **12**: 77–79, 1948.

Hawker, L. E.: Physiology of Fungi, University of London Press, Ltd., Bickley, Kent, England, 1950.

Hazen, E. L.: *Microsporum audouini:* The effect of yeast extract, thiamine, pyridoxine, and *Bacillus weidmaniensis* on the colony characteristics and macroconidial formation, *Mycologia* **39**: 200–209, 1947.

★Herny, W. B., and A. L. Andersen: Sporulation by *Piricularia oryzae*, *Phytopathology* **38**: 265–278, 1948.

Houston, B. R., and J. W. Oswald: The effect of light and temperature on conidium production by *Helminthosporium gramineum* in culture, *Phytopathology* **36**: 1049–1055, 1946.

Jones, R. C.: Factors affecting the production of resistant sporangia of *Allomyces arbuscula*, *Mycologia* **38**: 91–102, 1946.

★Kauffman, C. H.: Klebs' theory of the control of developmental processes in

organisms and its application to fungi, *Proc. Intern. Congr. Plant Sci.*, 1st Congr., **2**: 1603–1611, 1929.

KLEBS, G.: Zur Physiologie der Fortflanzung einiger Pilze. II. *Saprolegnia mixta* de Bary, *Jahrb. wiss. Botan.* **33**: 519–593, 1899.

KLEBS, G.: Zur Physiologie der Fortflanzung einiger Pilze. III. Allegemeine Betrachtungen, *Jahrb. wiss. Botan.* **35**: 80–203, 1900.

KUNKEL, L. O.: A method of obtaining abundant sporulation in cultures of *Macrosporium solani*, *Brooklyn Botan. Garden Mem.* **1**: 306–312, 1918.

LEAVER, F. W., J. LEAL, and C. R. BREWER: Nutritional studies on *Piricularia oryzae*, *Jour. Bact.* **54**: 401–408, 1947.

LEONIAN, L. H.: The physiology and perithecial and pycnidial formation in *Valsa leucostoma*, *Phytopathology* **13**: 257–272, 1923.

★LEONIAN, L. H.: A study of factors promoting pycnidium formation in some Sphaeropsidales, *Am. Jour. Botany* **11**: 19–50, 1924.

LEONIAN, L. H.: Control of sexual reproduction of *Phytophthora cactorum*, *Am. Jour. Botany* **23**: 188–190, 1936.

LEONIAN, L. H., and V. G. LILLY: Partial purification of a vitamin-like substance which stimulates sexual reproduction in certain fungi, *Am. Jour. Botany* **24**: 700–702, 1937.

LILLY, V. G., and H. L. BARNETT: The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*, *Am. Jour. Botany* **34**: 131–138, 1947.

★LINDEGREN, C. C., and E. HAMILTON: Autolysis and sporulation in the yeast colony, *Botan. Gaz.* **105**: 316–321, 1944.

LINDEGREN, C. C., and G. LINDEGREN: Sporulation in *Saccharomyces cerevisiae*, *Botan. Gaz.* **105**: 304–316, 1944.

LOCKWOOD, L. B.: Hydrogen ion concentration and ascus formation, *Mycologia* **29**: 289–290, 1937.

LOCKWOOD, L. B., and G. E. WARD: The physiology of *Rhizopus oryzae*, *Jour. Agr. Research* **53**: 849–857, 1936.

MCCALLAN, S. E. A., and S. Y. CHAN: Inducing sporulation of *Alternaria solani* in culture, *Contribs. Boyce Thompson Inst.* **13**: 323–336, 1944.

MADER, E. O.: Some factors inhibiting the fructification and production of the cultivated mushroom, *Agaricus campestris L.*, *Phytopathology* **33**: 1134–1145, 1943.

MATHUR, R. S., H. L. BARNETT, and V. G. LILLY: Factors influencing growth and sporulation of *Colletotrichum lindemuthianum* in culture, *Phytopathology* **40**: 104–114, 1950.

MIX, A. J.: Factors affecting the sporulation of *Phyllosticta solitaria* in artificial culture, *Phytopathology* **23**: 503–524, 1933.

MRAK, E. M., and L. BONAR: The effect of temperature on asci and ascospores in the genus *Debaryomyces*, *Mycologia* **30**: 182–186, 1938.

NICKERSON, W. J., and K. V. THIMANN: The chemical control of conjugation in *Zygosaccharomyces*. I. *Am. Jour. Botany* **28**: 617–621, 1941.

NICKERSON, W. J., and K. V. THIMANN: The chemical control of conjugation in *Zygosaccharomyces*. II. *Am. Jour. Botany* **30**: 94–101, 1943.

PORTER, C. L.: Concerning the character of certain fungi as exhibited by their growth in the presence of other fungi, *Am. Jour. Botany* **11**: 168–188, 1924.

RAMSEY, G. B., and A. BAILEY: Effects of ultra-violet radiation upon sporulation in *Macrosporium* and *Fusarium*, *Botan. Gaz.* **89**: 113–136, 1930.

RANDS, R. D.: The production of spores by *Alternaria solani* in pure culture, *Phytopathology* **7**: 316–317, 1917.

RAPER, J. R.: Sexual hormones in *Achlya*. III. Hormone A and the initial male reaction, *Am. Jour. Botany* **29**: 159–166, 1942.

RAPER, J. R., and A. J. HAAGEN-SMIT: Sexual hormones in *Achlya*. IV. Properties of Hormone A of *Achlya bisexualis*, *Jour. Biol. Chem.* **143**: 311–320, 1942.

RICHARDS, M. C.: Ozone as a stimulant for fungus sporulation, *Phytopathology* **39**: 20, 1949.

RIKER, A. J., and R. S. RIKER: Introduction to Research on Plant Diseases, John S. Swift Co., St. Louis, 1936.

ROBBINS, W. J., and R. MA: Vitamin deficiencies of *Ceratostomella* and related fungi, *Am. Jour. Botany* **29**: 835–843, 1942.

ROBBINS, W. J., and M. B. SCHMITT: Factor Z_2 and gametic reproduction by *Phycomyces*, *Am. Jour. Botany* **32**: 320–326, 1945.

SHANOR, L.: The production of mature perithecia of *Cordyceps militaris* (L.) Link in laboratory culture, *Jour. Elisha Mitchell Sci. Soc.* **52**: 99–104, 1936.

SMITH, E. C.: Effects of ultraviolet radiation and temperature on *Fusarium*. II. Stimulation, *Bull. Torrey Botan. Club* **62**: 151–164, 1935.

SNYDER, W. C., and H. N. HANSEN: The effect of light on taxonomic characters in *Fusarium*, *Mycologia* **33**: 580–591, 1941.

SNYDER, W. C., and H. N. HANSEN: Advantages of natural media and environments in the culture of fungi, *Phytopathology* **37**: 420–421, 1947.

★STEVENS, F. L.: Effects of ultra-violet radiation on various fungi, *Botan. Gaz.* **86**: 210–225, 1928.

TERNETZ, C.: Protoplasmabewegung und Fruchtkörperbildung bei *Ascophanus carneus* Pers., *Jahrb. wiss. Botan.* **35**: 273–312, 1900.

TIMNICK, M. B., V. G. LILLY, and H. L. BARNETT: The influence of light and other factors upon sporulation of *Diaporthe phaseolorum* var. *batatatis* from soybean, *Phytopathology* **41**: 327–336, 1951.

WESTERGAARD, M., and H. K. MITCHELL: *Neurospora*. V. A synthetic medium favoring sexual reproduction, *Am. Jour. Botany* **34**: 573–577, 1947.

YARWOOD, C. E.: The diurnal cycle of the powdery mildew, *Erysiphe polygoni*, *Jour. Agr. Research* **52**: 645–657, 1936.

★YARWOOD, C. E.: Diurnal cycle of ascus maturation of *Taphrina deformans*, *Am. Jour. Botany* **28**: 355–357, 1941.

CHAPTER 15

SPORE DISCHARGE AND DISSEMINATION

While the production of mature viable spores may be considered the climax in the life of a fungus, the wide dissemination of these spores is often a requisite to the perpetuation of the species. Frequently, this is assured by the production of enormous numbers of spores, which increases their chances of falling into favorable environment. The chief agent of dissemination among the fungi is air currents. Water, insects, and other animals play lesser roles in the natural dispersal of spores. Light, dry spores are usually disseminated by air currents, which may be strong enough to loosen them from the fruiting structures on which they are produced. On the other hand, spores borne in a gelatinous matrix are better adapted to transmission by rain, by insects, or by other animals which come in contact with them. Of particular interest are those fungi which possess certain special mechanisms for discharging their spores away from the fruiting structures. A study of the functions of these special adaptations must of necessity be based upon a knowledge of the structure of the fruit bodies which produce the spores.

METHODS OF SPORE DISCHARGE

The discharge or liberation of spores from the reproductive structures which produce them may take place by (1) violent expulsion of the spores or sporangia, due to internal pressure, (2) motility, as in the zoospores of the aquatic Phycomycetes, and (3) external forces of the environment.

The violent discharge of spores, sporangia, or other reproductive bodies depends upon the development of considerable pressure within the fungus. The structure of the fungus cell is very similar to that of algae or the parenchyma of the higher plants. The vacuole is filled with water and its dissolved compounds, such as sugars, salts, and amino acids. Foods in the cell may be in the form of sugar, glycogen, or oil. If the amount of soluble materials, such as sugar, is increased, the cell has a tendency to absorb water. As a result, the cell becomes more distended and may continue to swell until the elasticity of the cell wall is exceeded. The increase in sugar concentration may be the result of the hydrolysis of glycogen.

Discharge of sporangia. The genus *Pilobolus* illustrates a remarkable combination of adaptations for the production, discharge, and subsequent

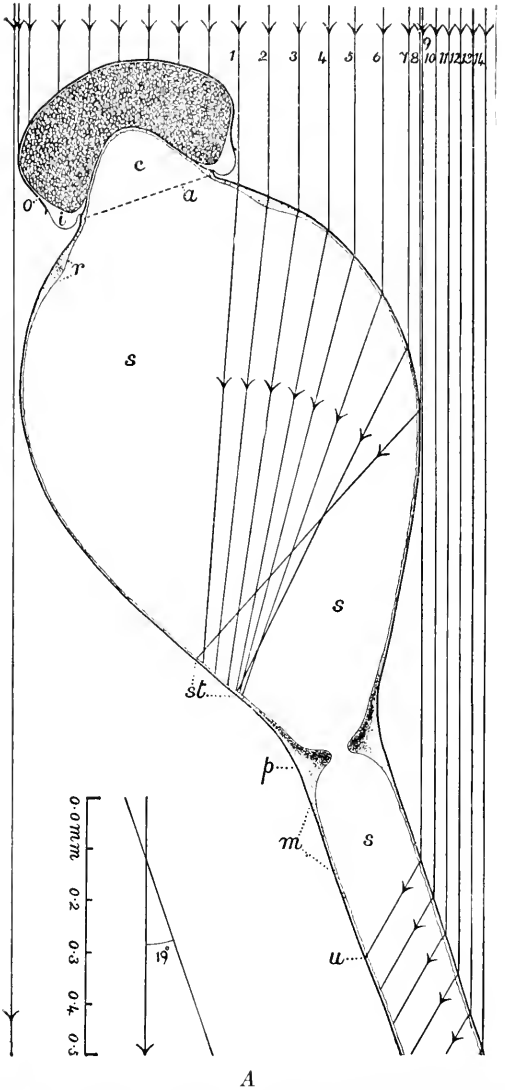
dissemination of its spores. These include (1) the dependence upon light for the production of sporangia, (2) the positively phototropic response of the sporangiophores (see frontispiece), (3) the violent discharge of the sporangium into the air toward the source of light, (4) the sticky nature and the heavy black wall of the sporangium, and (5) the dissemination of the spores by the passage through the digestive tract of animals which ingest them.

We owe much of our knowledge regarding the structure of *Pilobolus*, its physiology, and its life history to the careful study and comprehensive descriptions of Buller (1934). Much of his work was done with *P. kleinii* and *P. longipes*. Brefeld (1881) showed that, in the absence of light, no sporangia were formed, but that the sporangiophores continued to grow for 10 to 14 days and reached the length of 8 to 10 in. A 2-hr. exposure to light was sufficient for partially formed sporangiophores to complete their development in the dark. Sporangiophores and sporangia developed normally in blue light but did not develop in red-yellow light.

Under natural conditions, *Pilobolus* produces successive daily crops of sporangiophores and sporangia. Each crop requires approximately 24 hr. for its development. The sporangiophores begin to form near midday or early afternoon. By evening they have received enough light to allow the further development and production of the sporangia during the night. By the following morning, the sporangia are completely formed. During the morning the sporangiophores react phototropically, directing the sporangia toward the source of light. From midmorning to early afternoon the sporangia are discharged violently into the air for a considerable distance. The horizontal distance, according to Buller, may be as great as 8 ft. 7 in.

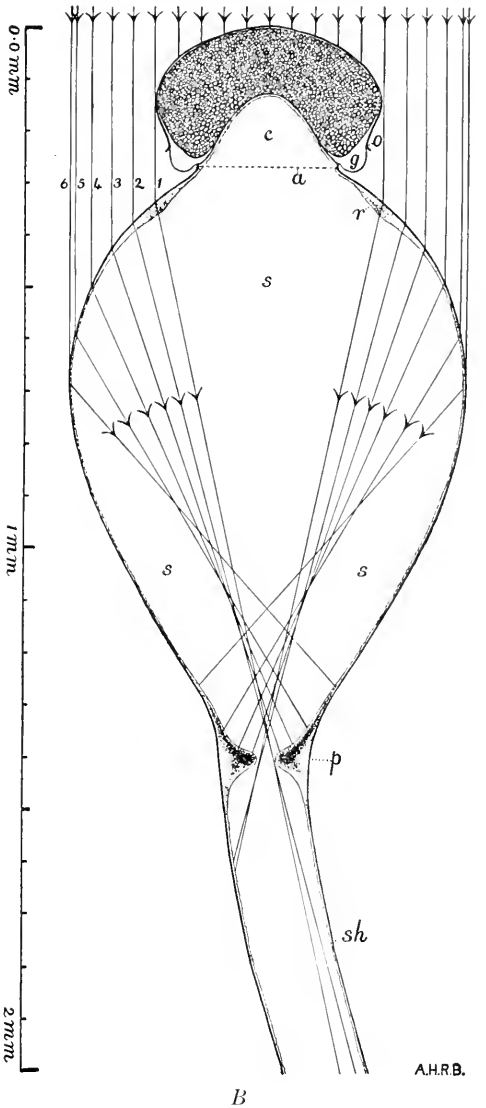
To understand the mechanism of sporangium discharge in *Pilobolus*, it is first necessary to know the structure of the sporangium and the sporangiophore (Fig. 69). The entire sporangiophore consists of a single large cell, with a rather slender lower portion, a subsporangial swelling, and a conical columella, which projects upward into the sporangium. A rather thin layer of cytoplasm lies next to the cell wall and surrounds a large central vacuole. At the base of the subsporangial swelling there is a thick perforated ring of protoplasm, which is reddish in color, containing carotene.

When the sporangiophore is pointed directly toward the source of light, the parallel rays of light which strike the black hemispherical sporangium are screened out. The subsporangial swelling acts as a lens, and the rays falling upon it are bent so that they converge on, or uniformly near, the red mass of protoplasm at the base of the swelling; this results in an equilibrium, *i.e.*, no bending occurs. When the sporangium is directed at an acute angle away from the source of light, the side of the sub-



A

FIG. 69. *A*, a median longitudinal section of *Pilobolus kleinii* just before discharge of sporangium. The gun is pointed at an acute angle away from the light source. The light rays that strike the sporangium are screened out. The rays that strike the side of the subsporangial swelling are bent and concentrated on the oposite side. This presumably gives a photochemical stimulus which is conducted to the motor region below the



B

subsporangial swelling. Bending toward the source of light then occurs until an equilibrium is reached and the light rays are concentrated at the base of the subsporangial swelling. *B*, at this time the gun is pointed directly toward the light source. (After Buller, Researches on Fungi, Vol. VI, pp. 91, 92, 1934. Reproduced by permission of Longmans, Roberts and Green.)

sporangial swelling, acting as a lens, causes the light rays to converge on the side of the swelling away from the source of light (Fig. 69*A*). Presumably, this causes a photochemical reaction in the protoplasm, and the stimulus is transmitted downward to the motor region, the portion of the sporangiophore just below the subsporangial swelling. The growth of this region is more rapid on the side away from the light source, which results in a bending of the sporangiophore until an equilibrium of light is again reached; *i.e.*, when the sporangium is pointing directly toward the source of light (Fig. 69*B*).

There is a thicker layer of protoplasm near the upper portion of the subsporangial swelling. This layer also contains some carotene. Buller believes that it is photochemically reactive and may serve to bring about chemical changes which result in the increase in the osmotic pressure of the cell. When a culture with nearly mature sporangia is placed in the dark, a much greater time is required for the discharge of the sporangia than when it is left in the light.

The weakest place in the wall of the *Pilobolus* structure is located just below the sporangium, and it is here that the wall of the subsporangial swelling breaks circularly as the sporangium is discharged. The increased osmotic pressure becomes too great for the resisting elastic wall, and the system is ruptured. A drop of cell sap is squirted out of the tip of the subsporangial swelling as discharge occurs. The conical columella is also carried away with the sporangium.

In nature the sporangia adhere to the surface of vegetation, where they may be ingested by herbivorous animals. The spores are released in the digestive tract and pass out in the feces unharmed. It seems probable that exposure to gastric juices helps to break dormancy of the spores and favors immediate germination. The sporangium adheres to the vegetation by the lower gelatinous part, with the black, hemispherical, nonwettable portion outward. Thus, the injurious ultraviolet rays are screened out, and the spores remain viable, although they may not be eaten for weeks or even months.

Basidiobolus ranarum shows a great many characters similar to those of *Pilobolus*, namely, the general structure of the sporangiophore, the mechanism of discharge of the sporangia, the coprophilous habit, and the general method of dissemination. The sporangiophore consists of a slender lower portion and cylindrical enlarged upper portion, which supports a spherical sporangium. The osmotic pressure in the sporangiophore increases to the point that it exceeds the tensile strength of the resisting wall, which is suddenly ruptured circularly near the base of the enlarged portion (Ingold, 1934). At this instant, the upper portion contracts and causes the cell sap to be squirted backward, giving a rocket-like effect. The dissemination of spores is accomplished after the spo-

rangia are eaten by beetles, which in turn are eaten by frogs or lizards. After the beetles are digested and the sporangia are released, the spores are formed. These spores are then capable of germination and production of mycelium on frog or lizard excreta.

A somewhat different method of discharge is described for the genus *Entomophthora* by Fitzpatrick (1930) and more specifically for *E. sphaerosperma* by Sawyer (1931). Instead of being due to a squirting action of the cell contents, as in the case of *Pilobolus* and *Basidiobolus*, the discharge of the sporangium in *Entomophthora* involves the opposing forces of osmotic pressure and the adhesive power between the two walls separating the sporangium and sporangiophore. As the sporangium matures, the pressure on both sides of the separating walls becomes so great that the outer wall is suddenly broken, and the sporangium is thrown into the air. The sporangia, being sticky, readily adhere to the objects which they strike.

The theory of violent discharge of sporangia in certain downy mildews was advanced by De Bary (1887), using *Peronospora parasitica* and *Phytophthora infestans* as examples. Later, Pinckard (1942) found the forcible adjection of sporangia in *Peronospora tabacina* to be the same as that described by De Bary. As the mature sporangiophore dries out, the entire crown, with its branches and sporangia, begins a counterclockwise rotation. Each portion of the sporangiophore, including the sterigma, rotates independently. The sporangiophore is hygroscopic, and as the air becomes more moist, the movement is reversed. The effect is a sudden release of the mature sporangia. During the course of rotation many of the branches become entangled with others, and the sporangia are dislodged by the spring-like action as the branches are disengaged. The discharge of sporangia was verified by observations on single isolated sporangiophores, showing that it is not dependent upon the intermingling of the sporangiophores. No sporangia were released in a saturated atmosphere, since no hygroscopic movement took place. Other species which were observed to react similarly were *Peronospora parasitica*, *P. geranii*, *P. halstedii*, and *P. effusa*. Similar rotation of conidiophores upon desiccation is apparently not uncommon among fungi of other groups, particularly those with long conidiophores.

Discharge of ascospores. In the majority of fungi (except those whose asci deliquesce), ascospore discharge is accomplished by the building up of osmotic pressure of the ascus to a point where it exceeds the resistance of the elastic ascus wall. In one type of expulsion, the ascus wall is suddenly ruptured, usually throwing the ascospores outward into the air simultaneously. In other species, the ascospores are discharged successively through an apical pore in the ascus. In the latter case, the ellipsoid or fusoid shape of the spore is apparently important. The spore pushes

part way through the pore to its broadest point and is then suddenly squeezed out by the contraction of the ascus tip (Ingold, 1933).

The increase in the osmotic pressure within the maturing ascus must be preceded by an increase in the soluble materials in the cell sap. This is believed to be accomplished by the digestion of glycogen, which is known to occur in the young ascus. In the majority of the Pyrenomycetes the asci are produced within a spherical or flask-shaped perithecium.

There are three general ways by which the ascospores are released through the ostiole of the perithecium, two of which depend upon the explosive rupture of the ascus wall. In the first type, which is the most common and believed to be the most primitive, the ascus wall remains attached at its base, while the spores are discharged. This is accomplished by the elongation of the elastic ascus, until the tip reaches or protrudes through the ostiole. The ascus then explodes, throwing the ascospores into the air. The wall of the empty ascus contracts to the base of the perithecium, and another ascus elongates. The process is repeated successively as the asci mature. This type is illustrated by *Sordaria*, *Pleurage*, and many other common fungi. An interesting parallelism exists between the method of dissemination of *Pilobolus* and that of *Pleurage*, *Sordaria*, and other coprophilous Pyrenomycetes. The short beaks of the perithecia are positively phototropic and, as they develop, are directed toward the source of light. In nature the ascospores fall upon vegetation and are subsequently eaten and disseminated by herbivorous animals. The vertical distances to which ascospores may be shot have been reported as 6 cm. for *S. fimicola* and 45 cm. for *P. curricolla* (Weimer, 1920).

A second general type of ascospore discharge occurs more commonly in species with long perithecial beaks. The asci become detached from the base of the perithecium and are pushed up through the beak to the ostiole, where the spores are released simultaneously or successively. This is a rapid method of spore discharge. Examples of this type are *Endothia parasitica*, *Gnomonia rubi*, *Guignardia bidwellii*, and *Ceratostomella ampullacea*. Most of these species are adapted to wind dissemination of ascospores. Some idea of the tremendous numbers of ascospores discharged is given by Heald and Walton (1914), who reported that some specimens of *E. parasitica* expelled ascospores every day for 168 days. The rate of spore discharge from one perithecium was found to be as high as one ascus explosion about every 2 sec. At this rate approximately 14,000 ascospores may be discharged per perithecium per hour. On the basis of these figures, it is little wonder that the fungus spread so rapidly among the American chestnuts.

A third group includes the nonexplosive type of ascus, in which the ascospores are released by the deliquescence of the ascus wall. They are

embedded in mucilage, and as they accumulate in the body of the perithecium, some spores ooze out through the ostiole, much like tooth paste from the tube. Examples of this type are *Chaetomium* spp., *Ceratostomella fimbriata*, and *C. ulmi*. These spores are not adapted to wind dissemination but may be carried in moist weather by insects (*C. ulmi*), by other contacts (such as *C. fimbriata* on stored sweet potatoes), or by rain.

The Discomycetes, in general, show a marked response to the stimulus of light in orienting the asci so that the ascospores may be discharged into the air away from the apothecium. The apothecium of *Ascobolus* is small, and only a few asci mature at one time. As an ascus matures, it enlarges greatly and extends well beyond the surface of the hymenium. It then reacts phototropically so that the tip is pointed directly toward the source of light. When the ascus bursts, the operculum at the tip is forced open, and the spores are expelled simultaneously. Most species of *Ascobolus* are coprophilous and are disseminated in much the same manner as *Pilobolus* and *Sordaria*.

The phenomenon of "puffing" in many of the larger Discomycetes is described in most textbooks of mycology and plant pathology. It is due to the simultaneous violent spore discharge from many asci, so that a cloud of spores may be seen to rise a few inches from the apothecium. This may be so violent that a faint hissing or fizzing sound can be heard. If the asci were to explode singly as they mature, the ascospores would be shot up into the air only by the initial force of the explosion. For most species, this distance would probably not exceed 1 or 2 in. However, when a great many asci explode simultaneously, an air blast is created which carries the ascospores vertically to a much greater height, as great as 5 to 7 in. (Buller, 1934). This additional distance above the fruit body, which is commonly located on or near the ground, increases the chances of dissemination by air currents.

In nature, the puffing of ascospores may be initiated by a sudden change from shade to open sun, by the passing of a cloud, or by swaying of a branch. Strong sunlight is not the only stimulus, for the phenomenon has been observed in the laboratory under uniform light conditions. A sudden jar of the fruit body, when it is tapped or picked up, may cause spore discharge in some species. Likewise, an instant's exposure to alcohol fumes may serve as the stimulus.

Buller (1934) has shown that in the cupulate or V-shaped apothecia, such as those of *Sarcoscypha protracta*, many of the asci are pointing directly toward the opposite side of the cup; yet the ascospores are discharged upward, free from the fruit body. The operculum of this species, instead of being centrally located at the tip of the ascus, is obliquely placed toward the upper side of the ascus. As the discharged ascospores

leave the ascus, they are directed vertically. Buller believes that the oblique position of the operculum is a physiological character formed as a response of the ascus end to the stimulus of light. Seaver (1928), however, believes that the position of the operculum is not determined by light.

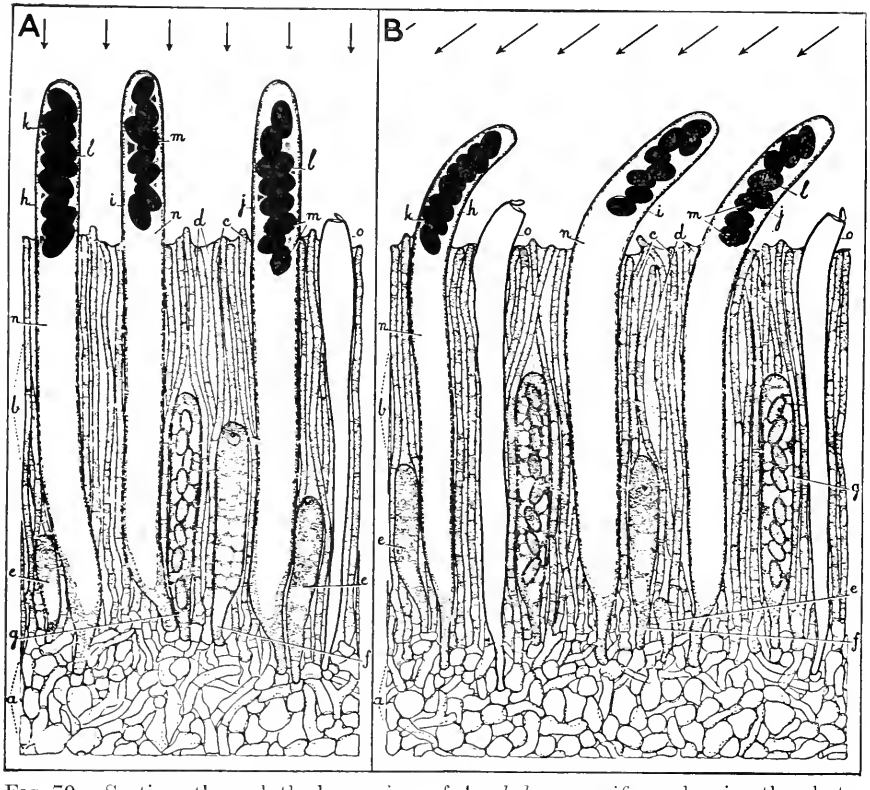


FIG. 70. Sections through the hymenium of *Ascobolus magnificus*, showing the phototropic response of the ascus tips to light. Discharge of the ascospores is then directly toward the source of light. (After Buller, Researches on Fungi, Vol. VI, p. 272, 1934. Reproduced by permission of Longmans, Roberts and Green.)

In the development of the apothecium the paraphyses are formed before the asci mature, and the developing asci push their way upward among the paraphyses. In some species (*Ascobolus* spp., *Lachnea scuicllata*) the paraphyses are straight, and only the portion of the ascus extending beyond the paraphyses tips responds phototropically (Fig. 70). The paraphyses of others (*Peziza badia*, *Aleuria vesiculosa*) bend toward the light, and the developing asci are likewise bent as they elongate. *Aleuria repanda* sometimes shows a coarse adjustment toward light, by the turning of the entire apothecium, and a fine adjustment, by the bending of the ascus tips in the same direction.

Discharge of peridioles. The discharge of the peridiole (gleba-containing basidiospores) of *Sphaerobolus* depends largely upon the unique structure of the fruit body (Fig. 71). The spherical fruit body measures but 2 to 3 mm. in diameter. The peridium is made up of six distinct layers. At maturity, the peridium breaks open at the top, in a stellate manner, through all but the sixth, or innermost, layer, which surrounds

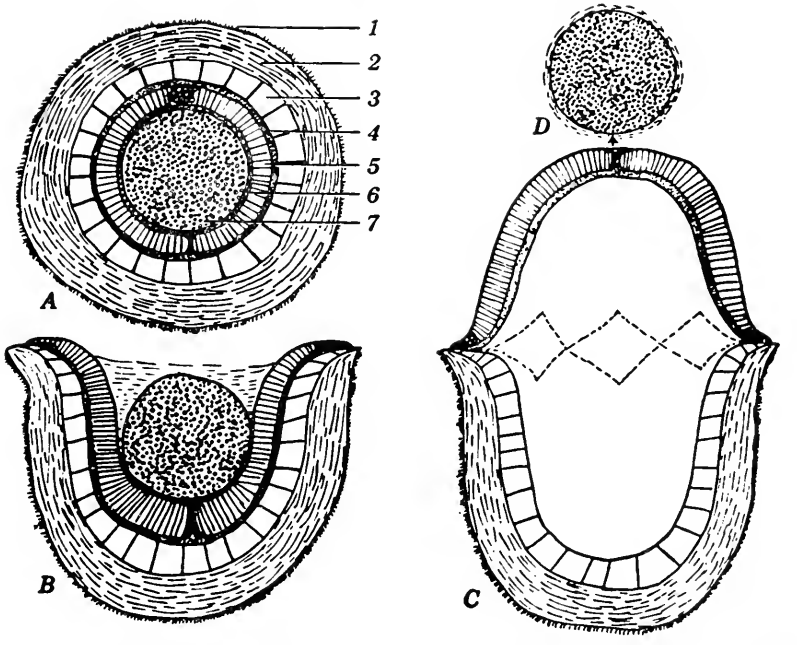


FIG. 71. Structure of fruit body and mechanism of discharge of peridiole of *Sphaerobolus stellatus*. *A*, section of mature sporocarp, with six layers (1–6) that invest the central peridiole (7). *B*, dehiscence of sporocarp at apex. The inner membrane is liquefied. *C*, eversion of the remaining two layers by which the peridiole, *D*, is suddenly discharged. (Reproduced by permission from Wolf and Wolf, The Fungi, Vol. II, p. 203, John Wiley & Sons, Inc., New York, 1947.)

the peridiole. This layer deliquesces, and the peridiole then rests in the watery substance produced. A split then occurs between layers 3 and 4, beginning at the base but not progressing to the very top. Layer 4, the fibrous layer, is composed of small, rather closely packed cells, while layer 5, the palisade layer, is made up of comparatively large, somewhat elongated cells. The cells of the palisade, which is on the concave surface, increase in turgor and in size and are held under great tension by the relatively inelastic fibrous layer. These inner layers are suddenly everted, acting as a catapult, throwing the peridiole violently upward. Walker (1927) has reported that the peridiole of *S. stellatus* may be thrown to a vertical distance of 14 ft., while Buller (1934) reports a maximum horizontal distance of 18 ft. 7 in.

The force which causes the discharge is apparently located in the palisade layer. It has been demonstrated by microchemical tests that the palisade cells of the unopened fruit body are densely filled with glycogen, which disappears before the discharge of the peridiole (Walker and Andersen, 1925). The glycogen is converted to reducing sugars, one of which is maltose, and this leads to the increase in osmotic pressure. Light hastens the opening of the fruit body and the discharge of the peridiole and is believed to speed up the conversion of glycogen into sugars. *S. stellatus* is a coprophilous or lignicolous species, and the peridioles may be eaten and disseminated by herbivorous animals.

Dodge (1941) reports his own observations as well as those of others upon the presence of peridioles of the bird's-nest fungi attached to leaves and branches as high as 10 to 15 ft. above the ground. Dodge describes the attachment of the peridioles of *Cyathus striatus* to the peridium but offers no theory to explain the mechanism of the peridiole discharge or the force which is responsible. The slender mucilaginous threads which attach the peridioles in the fruit bodies also serve to attach the discharged peridioles to certain objects.

Discharge of basidiospores. The mechanism and the force involved in the discharge of basidiospores in the Hymenomycetes and of the sporidia of the smuts and rusts have not been satisfactorily explained. There is no evidence that the explanation used for any of the types described above can be applied to the discharge of basidiospores. However, certain structural features are present which may be adaptations for this special method of spore discharge.

In all Basidiomycetes in which the spores are shot off forcibly, the sterigma is attached slightly to one side of the tip of the spore (Fig. 72). Just before a spore is discharged, a small drop of liquid appears at the tip of the sterigma. Its invariable presence is believed to be an important feature in the process of spore discharge. After discharge, there appears to be no pore present, either in the spore or in the tip of the sterigma. Buller (1922, 1924) suggests that the spore may be shot from the sterigma by hydrostatic pressure, but that the amount of liquid ejected as the explosion occurs at the sterigma may be so minute that it may not be detected by the microscope. He further suggests that the surface tension of the drop of water may in some way bring about the abjection of the spore. While neither theory is completely satisfactory, they are the best yet offered.

Discharge of aeciospores. Experimental work showing that the aeciospores of the rust fungi are violently discharged was reported by Buller (1924) and Dodge (1924, 1924*a*). They have reported this phenomenon in seven species, including *Puccinia coronota*, *P. graminis*, *P. podophylli*, *Gymnoconia peckiana*, and *Uromyces pisi*. The maximum

distance above the aecia to which the spores were shot varied from 6 to 15 mm., being about 8 mm. for *P. graminis*. The exact mechanism of aeciospore discharge is not known, but it probably depends largely upon turgor pressure of the mature aeciospores, which are formed in chains. The double wall between two aeciospores is at first flat, but near maturity of the spores the osmotic pressure increases and tends to make these walls convex. It is assumed that the adhesive force between the two walls is suddenly overcome by the increased osmotic pressure, and the terminal spore or group of spores is thrown outward into the air.

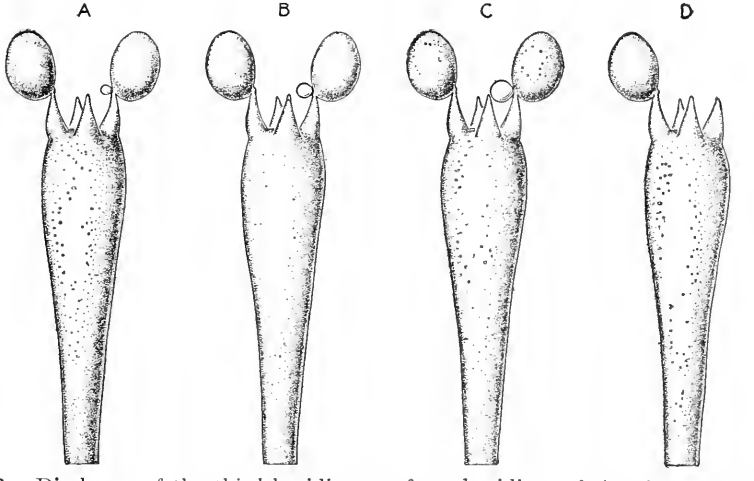


FIG. 72. Discharge of the third basidiospore from basidium of *Agaricus campestris*, following the formation of a drop of water at the tip of the sterigma. (After Buller, Researches on Fungi, Vol. II, p. 12, 1922. Reproduced by permission of Longmans, Roberts and Green.)

Liberation of zoospores. In most of the aquatic Phycomycetes and in some terrestrial forms which show definite aquatic affinity, zoospores are the primary means of reproduction. The characteristic motility of the zoospores may be more correctly considered as a means of local dissemination, but motility is also involved in the liberation from such large sporangia as those of *Saprolegnia* and related fungi.

INFLUENCE OF EXTERNAL CONDITIONS

The effect of light upon the discharge of spores by *Pilobolus*, *Ascobolus*, *Sordaria*, and *Sphaerobolus* has been discussed briefly. Light is necessary for the production of spores in a number of fungi in which it plays no direct part in spore discharge.

Favorable temperature is a prerequisite for all biological activity. Its effect upon spore formation and discharge is often not clearly defined. The maximum temperature permitting spore discharge is appreciably

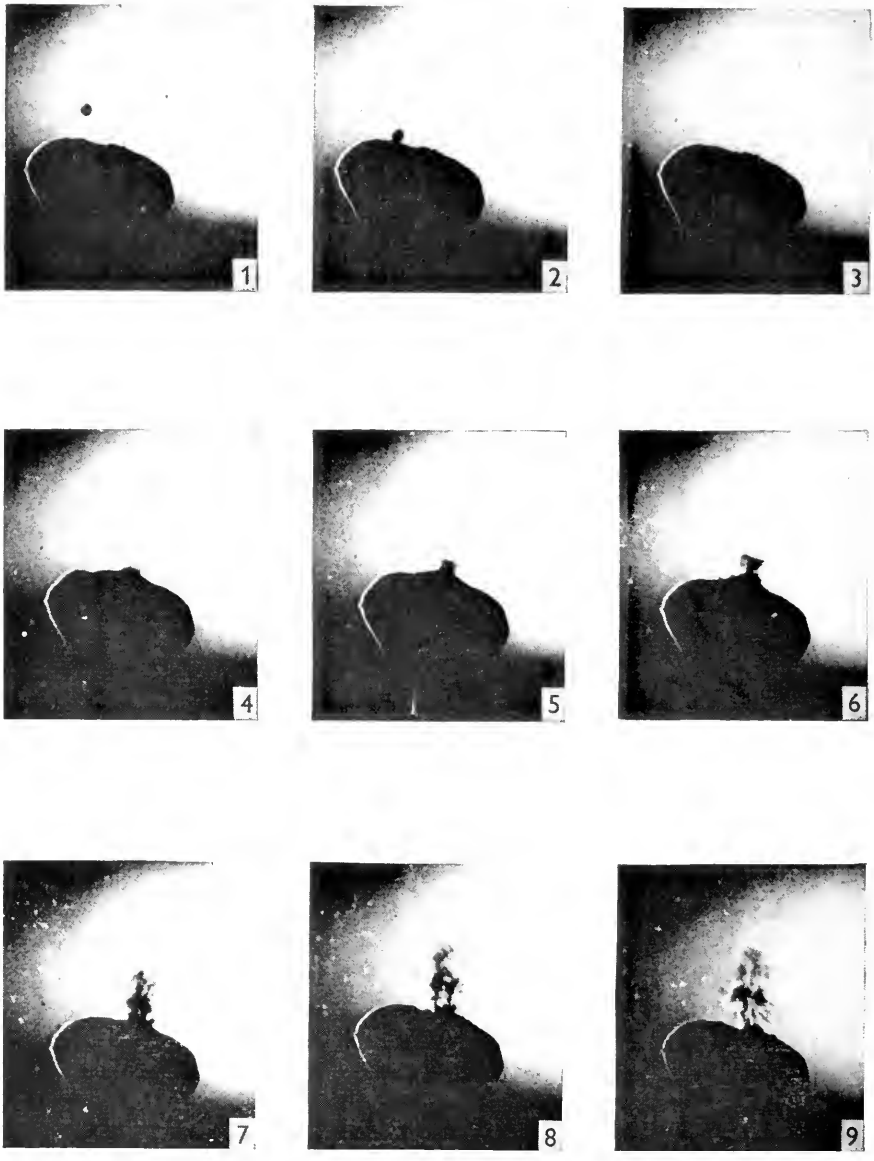


FIG. 73. Stills from ultra-high-speed film showing impact of drop of water with a fruit body of *Lycoperdon perlatum* and the subsequent puff of spores. The drop, 5.0 mm. in diameter, fell with a veloeity of 440 cm. per sec. The time elapsed between the contact of drop with peridium until the last photograph was 0.046 sec. (Courtesy of Gregory, *Trans. Brit. Mycol. Soc.* **32**: 14, 1949. Published by permission of Cambridge University Press.)

lower than that for viability of the fungus. Low temperatures usually merely slow down spore production and discharge. Buller (1909) found that *Daedalea unicolor*, *Lenzites betulinus*, *Polyporus versicolor*, and *P. hirsutus* discharged spores when the air temperature was 0°C. *Schizophyllum commune* shed spores vigorously at 5°C., but not at 0°C.

Andersen *et al.* (1947) showed that few conidia of *Piricularia oryzae* were liberated when the host plants were dry. Continued wetting greatly increased the secondary spread of the fungus in experimental trials. Sporidia of rusts are formed and discharged only during periods of high humidity. The need of the downy mildew fungi for changing conditions of humidity has been pointed out. The hygroscopic character of the capillitium of certain slime molds aids in pushing the spores to the surface, where they may be disseminated by various agents. Many other species of fungi appear to be independent of the air moisture, as long as there is sufficient moisture in the fruit body. Gravity is believed to have little effect upon spore discharge, except in the proper orientation of the fruit bodies.

The spores of some puffballs are enclosed within the nearly spherical peridium, which opens by an apical pore. When sudden pressure is applied to the peridium, the spores are puffed out of the pore in clouds of "smoke." Gregory (1949) has shown, by use of ultraspeed photography, that raindrops falling on the thin peridium of *Lycoperdon perlatum* cause a puffing of spores (Fig. 73). The velocity of the puff as the spores emerged from the ostiole was approximately 100 cm. per sec. Under the conditions of the experiment, it was estimated that a drop of water falling 130 cm. caused the ejection of approximately 15 million spores. The endoperidium and the spores inside remain dry, and the puffing is not hindered, even during a rain. The impact of raindrops is believed to be an important means of spore discharge from the ostiolate puffballs.

SPORE DISSEMINATION

Many other fungi do not have any special method of spore discharge and must depend upon physical or biotic agents in nature for getting their spores away from the fruit body where they are produced. Some of these possess some special adaptations for dissemination by certain agents. The most important agent of dissemination is air currents. The urediospores of the rusts are not violently discharged. They accumulate in the sorus and must be dislodged by the wind or movement of the host plant. Many of the Moniliales which produce dry conidia also depend upon air movement to shake them loose from the conidiophores. Convection currents are responsible for local spread of dry spores, while splashing rain is important in the dissemination of spores with matrix.

It is well known that spores of some of these fungi may be blown for hundreds of miles and remain viable.

Dissemination of the zoospores of the aquatic fungi may be accomplished locally by means of the flagella or for greater distances by the movement of water, which may carry the spores both in the motile and in the encysted stages. The condition of diplanetism, which involves two motile stages separated by an encysted stage, may be advantageous for the greater dissemination in water. The translocation of mud and moist soil by means other than water may also be important. Although the motility of zoospores of certain parasitic fungi, such as *Phytophthora infestans*, can scarcely be considered an important means of dissemination, it does enable the spore to move short distances in a drop of water on the host and facilitate penetration through stomata.

Insects are likewise important agents of dissemination of fungus spores. The insect may be attracted to spore masses by odor or color and feed upon the spores, or the insect may be merely an incidental carrier of spores adhering to the external parts of the body. Leach (1940) states that "in the majority of cases where the question has been investigated, spores have been found to pass through the intestinal tract of insects uninjured." The conidia of *Claviceps purpurea*, being produced in sweet droplets of liquid, also emit an odor which attracts insects. Similarly, the stinkhorn fungi produce their spores in a malodorous matrix and are frequently visited by flies. These adaptations ensure insect dissemination. The blue-stain fungi (*Ceratostomella* spp.) produce spores in sticky droplets in the tunnels of bark beetles, which act as the principal agents of dissemination.

The symbiotic relationship between species of *Septobasidium* and scale insects represents a highly evolved adaptation for the dissemination of the fungus spores. Although these fungi produce basidiospores, they are unlike most Basidiomycetes in that the spores are not forcibly discharged from the basidium. Couch (1938) has made a comprehensive study of this genus and has described the life history in detail. The fungus forms a layer over the bodies of scale insects, some of which are parasitized, while others are not. The uninfected female insects under the fungus may produce young, many of which crawl over the sporulating surface at the time of sporulation. These may become infected, crawl about, and settle down some distance away. Such infected young insects are solely responsible for the dissemination of the fungus.

Spore dissemination is also unique in the Tuberales (truffles), whose fruit bodies are formed entirely underground. There are no direct means of getting the spores up to the air for dissemination. These fruit bodies, which give off an odor, are dug up and eaten by rodents. In this process, pieces of the fruit bodies are dropped, and the spores are thus disseminated.

SUMMARY

Many fungi have no means of forcibly discharging their spores but must depend upon the physical and biotic factors in nature for liberation and dissemination of spores. Others possess special mechanisms for discharging their spores away from the fruiting structures which bear them. In most cases this violent discharge depends upon high osmotic pressure within certain cells of the fungus. Increased osmotic pressure usually is a result of the digestion of glycogen to soluble sugars.

Many coprophilous fungi, such as *Pilobolus, Ascobolus, Sordaria,* and *Pleurage,* forcibly discharge their spores for some distance toward the source of light. In nature this adaptation is of great advantage to the fungus in its dissemination by animals, which ingest the spores with the vegetation. Some Discomycetes exhibit a puffing of the spores when many asci discharge their spores simultaneously.

In *Pilobolus, Basidiobolus,* and *Entomophthora* the sporangia are forcibly abjected from the sporangiophores. Ascospores may be ejected either simultaneously or successively from the ascus. The ascus may elongate to reach the surface of the ascocarp and discharge its spores, or the asci may become detached in some Pyrenomycetes and, after being forced through the ostiole, may explode to release the spores. In other species the ascus walls are deliquescent, and the ascospores ooze out of the ostiole.

The basidiospores of most Basidiomycetes are forcibly discharged by a mechanism which is not well understood. A drop of liquid is extruded at the tip of the sterigma just prior to discharge and is believed to affect the process in some way. The peridioles of *Cyathus* and *Sphaerobolus* may be thrown several feet away from the fruit bodies. The latter fungus exhibits a unique catapult action by a portion of the fruit body. Other mechanisms act in the forcible discharge of the aeciospores of many rusts and the sporangia of *Peronospora.*

Air currents are the most common agent of dissemination of dry spores. Spores borne in a sticky, malodorous, or sweet matrix are well adapted to insect dissemination. Some fungi, such as *Septobasidium,* have established a symbiotic relationship with certain insects, which are the sole agents of dissemination.

REFERENCES

Andersen, A. L., W. B. Henry, and E. C. Tullis: Factors affecting infectivity, spread and persistence of *Piricularia oryzae, Phytopathology* **37**: 94–110, 1947.

Brefeld, O.: Botanische Untersuchungen über Schimmelpilze, Heft 4, Verlag von Arthur Felix, Leipzig, 1881.

★Buller, A. H. R.: Researches on Fungi, Longmans, Roberts and Green, London. Vol. I, 1909; Vol. II, 1922; Vol. III, 1924; Vol. VI, 1934.

Couch, J. N.: The Genus *Septobasidium,* The University of North Carolina Press, Chapel Hill, 1938.

De Bary, A.: Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria (trans. H. E. F. Garrney, rev. I. B. Balfour), Oxford University Press, New York, 1887.

Dodge, B. O.: Accidiospore discharge as related to the character of the spore wall, *Jour. Agr. Research* **27**: 749–756, 1924.

Dodge, B. O.: Expulsion of aeciospores by the may apple rust, *Puccinia podophylii* Schw., *Jour. Agr. Research* **28**: 923–926, 1924*a*.

Dodge, B. O.: Discharge of the sporangioles of bird's nest fungi, *Mycologia* **33**: 650–654, 1941.

Fitzpatrick, H. M.: The Lower Fungi, McGraw-Hill Book Company, Inc., New York, 1930.

Gregory, P. H.: The operation of the puff-ball mechanism of *Lycoperdon perlatum* by raindrops shown by ultra-high-speed Schlieren cinematography, *Trans. Brit. Mycol. Soc.* **32**: 11–15, 1949.

Heald, F. D., and R. C. Walton: The expulsion of ascospores from the perithecia of the chestnut blight fungus, *Endothia parasitica*, *Am. Jour. Botany* **1**: 499–522, 1914.

★Ingold, C. T.: Spore discharge in the ascomycetes, *New Phytologist* **32**: 178–196, 1933.

Ingold, C. T.: The spore-discharge mechanism in *Basidiobolus ranarum*, *New Phytologist* **33**: 274–277, 1934.

★Leach, J. G.: Insect Transmission of Plant Diseases, McGraw-Hill Book Company, Inc., New York, 1940.

★Pinckard, J. A.: The mechanism of spore dispersal in *Peronospora tabacina* and certain other downy mildew fungi, *Phytopathology* **32**: 505–511, 1942.

Sawyer, W. H.: Studies in the morphology and development of an insect-destroying fungus, *Entomophthora sphaerosperma*, *Mycologia* **23**: 411–432, 1931.

Seaver, F. J.: The North American Cup Fungi. Operculates, published by the author, New York, 1928.

★Walker, L. B.: The development and mechanism of the discharge in *Sphacrobolus iowensis* and *S. stellatus*, *Jour. Elisha Mitchell Sci. Soc.* **42**: 151–178, 1927.

Walker, L. B., and E. N. Andersen: Relation of glycogen to spore ejection, *Mycologia* **17**: 154–159, 1925.

Weimer, J. L.: Some observations on the spore discharge of *Pleurage curvicolla*, *Am. Jour. Botany* **7**: 75–77, 1920.

Wolf, F. A., and F. T. Wolf: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

CHAPTER 16

SPORE GERMINATION

Spore germination, in general, implies a change from an inactive to an actively growing condition. This is accomplished in most fungi by the formation of a germ tube, which continues to elongate and ultimately leads to the formation of the vegetative body of the fungus. In the Phycomycetes the germination of oospores and of some sporangia may take place by the internal formation of zoospores. Certain higher fungi also produce secondary spores externally without the formation of mycelium.

Among the universal requirements for the germination of spores are (1) suitable temperature, (2) adequate moisture supply, (3) adequate oxygen supply, (4) suitable hydrogen-ion concentration, and (5) viable spores.

Some of these factors may be measured quantitatively, and for each there is an optimum for germination for a given fungus. The maximum percentage of spore germination in the shortest time will occur when all the influencing factors are at or near the optimum. This is a situation which might seldom occur in nature. Germination will occur or not depending upon the number and relative importance of the favorable factors.

Clayton (1942) suggests that the differences in nutritional requirements for germination shown by the various species may be due to differences in the spore wall and in the composition and quantity of reserve foods. Some spores contain stored food in the form of oil, while others contain glycogen. It is believed that water enters the spore by imbibition or osmosis and activates the enzyme, glycogenase, which hydrolyzes glycogen to sugars.

Spores which do not germinate after being exposed to the usually favorable conditions for a reasonable length of time are said to have a period of dormancy, which may be broken by the presence of a special set of conditions. Among these are (1) exposure to high or low temperature, (2) the presence of certain nutrients or stimulants in natural products, (3) exposure to chemical stimulants, (4) alternate wetting and drying, and (5) aging. These same factors may also influence the percentage of spore germination of many species which do not have a definite dormant period. Dormancy may be due, at least in some cases, to the failure of

the usual favorable conditions to activate a certain enzyme. Some special stimulus may then be required to perform this function.

The present discussion deals primarily with the factors which influence some physiological phase of spore germination. The main emphasis will be placed upon a discussion of these factors and how they act, or what part they play in germination. Doran (1922) gives a good discussion of the more important factors affecting germination and lists many references to the earlier work. A more recent discussion of this subject is given in the excellent review by Gottlieb (1950).

PHYSICAL FACTORS

Temperature. Temperature is one of the most important external factors which influence germination. It not only affects the percentage of germination but also the length of time required for germination, and, in certain fungi, it often determines the method of germination. The literature contains a great many references to spore germination at different temperatures, but none of these have attempted to explain the intricate effects or responses within the spore. We may assume that a favorable temperature permits certain enzymatic activities essential to germination. Different species of fungi have different temperature requirements for germination. The cardinal temperatures (minimum, optimum, and maximum) for spore germination may be found for many fungi in scattered reports. These are based mainly on casual observations made during the study of other problems. Few comprehensive studies have been made of the effects of temperature upon spore germination.

The cardinal temperatures of a few selected species are presented as examples: *Alternaria solani*, 1 to 3°C., 26 to 28°C., 37 to 45°C.; *Cronartium ribicola* aeciospores, 5°C., 12°C., 19°C.; *Phyllosticta antirrhini*, 18°C., 25°C., 47°C.; *Phytophthora infestans*, 2 to 3°C., 12 to 13°C., 24 to 25°C.; *Puccinia graminis* teliospores, 5 to 9°C., 20 to 22°C., 23 to 25°C.; *Venturia inaequalis* conidia, 3°C., 14 to 15°C., 31°C. For other examples, see Doran (1922).

The general optimum temperature for spore germination of certain species of Agaricaceae and Nidulariaceae is near 30°C. (Kauffman, 1934). Walker and Wellman (1926) found that, when the soil temperature was above 25°C., there was low percentage of "chlamydospore" germination of *Urocystis cepulae*, while spore germination ceased entirely at 29 or 30°C. They attribute the low percentage of infection above 25°C. to the direct inhibitory effect of the higher temperature upon the parasite.

The presence of nutrients and the supply of oxygen caused variation in the minimum temperature for spore germination in *Colletotrichum lagenarium* (Gardner, 1918). The minimum was 14°C. in water with the

hanging-drop technique, 7°C. in exposed drops of water, and 4°C. in prune decoction. This may serve to explain, in part, the differences in cardinal temperatures reported by different authors.

The method of spore germination may be determined by temperature. Below 20°C. the sporangia of *Phytophthora infestans* germinate more frequently by the formation of zoospores, while above this temperature production of a germ tube is more common.

The optimum temperatures for germination for the various species of Myxomycetes were from 22 to 30°C., with an over-all range of 2 to 36°C. Certain species had narrower ranges. Below 10 and above 30°C. the rate of germination was greatly reduced. When the spores of *Enteridium rozeanum* were held for a time at a temperature above maximum and then returned to optimum, the spores germinated explosively through a thin area in the wall (Smart, 1937). Under uniform optimum temperature an irregular pore was formed and the protoplast slowly squeezed out.

It must be emphasized that temperature affects the time required for germination, as well as the percentage of germination and the growth rate of the germ tubes. All three have been used as measurements of spore germination. It seems likely that temperature might have a greater effect upon the time of germination than upon the percentage of germination.

Heat treatment and breaking of dormancy. The effectiveness of preheating ascospores of *Neurospora tetrasperma* in breaking their dormancy has been discussed by several authors. Heating the spores to 50°C. for a few minutes induced germination 2 or 3 hr. after they were returned to a favorable temperature. Goddard (1935) found that spores thus "activated" could be "inactivated" (returned to the dormant condition) by placing them under anaerobic conditions for a short time. They remained dormant when brought back into air. The respiration rate of the activated spores was greatly increased and germination occurred only after 2 to 3 hr. of continuous high respiration. Further work by Goddard and Smith (1938) led to the conclusion that the heat activated carboxylase, which is latent in the dormant spores, and that two different respiratory systems are in operation: one, the dormant system, functions in the absence of carboxylase; and the second, the active system, functions after the spores are heated. Similar stimulation of germination of *Ascobolus* ascospores by heat was found by Dodge (1912). Only a few spores germinated in water without being preheated. Heating the spores to 65 to 75°C. for approximately 15 min. and then returning them to favorable temperature allowed good germination of most species.

Moisture. The spores of many species of fungi will not germinate unless they are in contact with liquid water. Others are capable of germination on dry surfaces in an atmosphere of high humidity, usually

95 per cent or above. A third group is represented by some of the powdery mildews, whose spores are able to put out short germ tubes under conditions of extremely low relative humidity. Comparatively little careful work has been done to determine the moisture requirements for spore germination. Doran (1922) reviewed some of the earlier reports and gives the results of his own experiments. Among the species whose spores have been reported as requiring contact with liquid water for germination are the following: *Sclerotinia fructicola*, *Peronospora pygmaea*, *Phyllosticta antirrhini*, teliospores of *Gymnosporangium juniperi-virginianae*, *Cylindrocladium scoparium*, and *Plasmopara viticola*.

Numerous fungi whose spores may germinate in the absence of liquid water have been reported. Some of these have been germinated on a dry glass slide in a moist chamber, where the humidity is assumed to be at 100 per cent, the saturation point. However, Clayton (1942) showed that a humidity of 100 per cent sometimes gave visible condensation of water vapor, whereas a relative humidity below 99.85 per cent gave no condensation at constant temperature. The spores of this group of fungi usually show a much higher percentage of germination in liquid water if a plentiful supply of oxygen is present. Some representative fungi reported in this group with the approximate minimum humidity are *Puccinia glumarum* urediospores, 99 per cent; *Venturia inaequalis* ascospores and conidia, 98.7 per cent; *Ustilago nuda*, 95 per cent; and *Penicillium glaucum*, 84 per cent. The minimum relative humidity for *Aspergillus niger* is near 70 per cent (Bonner, 1948). Figure 74 shows germination curves.

The germination of the conidia of certain species of the Erysiphaceae in relative humidity at or near zero has been reported by several investigators (Brodie, 1945; Brodie and Neufeld, 1942; Yarwood, 1936; and Clayton, 1942). These species are *Erysiphe polygoni*, *E. graminis*, and *Microsphaera alni*. The mechanism for spore germination under these very dry conditions must be quite different from that of other spores, which require liquid water or high humidity for germination. The "apparent osmotic pressure" of the cell sap of the conidia is reported by Brodie (1945) as about 63 atm. for *E. polygoni* and 68 atm. for *E. graminis hordei*. It is likely that these high osmotic pressures may be an aid in absorbing moisture from a relatively dry atmosphere. Brodie believes that the conidia contain little free water but that imbibition may be partly brought about by hydrophilic colloids.

Brodie and Neufeld (1942) offer a tentative theory to explain "germination" under conditions of 0 per cent humidity. They believe that, as germination begins, free water is released by respiration and by changes in colloidal materials containing bound water. No changes in the length or width of the conidia could be detected during germination. The

formation of the germ tube was calculated to add approximately 2 or 3 per cent to the volume of the ungerminated conidium. It is believed possible that this slight increase in volume might be accounted for by one or both of the above factors. Yarwood (1936) offers no explanation of the process of germination at such a low humidity but reports a decrease

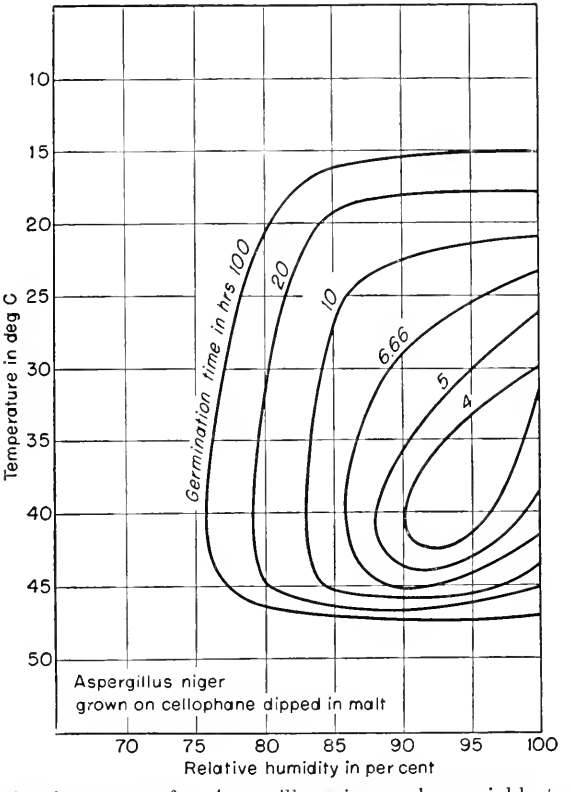


FIG. 74. Germination curves for *Aspergillus niger* under variable temperature and humidity. Note that the optimum temperature for germination varied with the relative humidity, being near 30°C. at relative humidity of 100 per cent and near 40°C. at 93 per cent. As the temperature or humidity digressed from the optimum, the time required for germination increased. (Courtesy of Bonner, *Mycologia* **40**: 733, 1948.)

of about 24 per cent in volume of the *Erysiphe* conidia during germination. Spores of all other fungi (except other powdery mildews) which he tested showed increases in volume during germination.

Dormancy of some spores may be broken by alternate wetting and drying. This treatment apparently makes the thick resistant wall more permeable to water.

Oxygen supply. Since respiration is greatly accelerated during spore germination, it follows that an adequate supply of oxygen is a prerequisite

for germination. Brief reports of a number of observers on oxygen requirements are given by Doran (1922). It is generally agreed that reduced oxygen supply decreases spore germination. Spores germinate better on or near the surface of a liquid than when submerged deep in the liquid. In some cases the spores may germinate under water, but only abnormal germ tubes are formed. Aerated water gives better germination than nonaerated water. The spore load in a drop of water, whether all of the same species or of mixed spores, influences greatly the percentage of germination. This is believed to be due primarily to the competition for the limited supply of oxygen, rather than to toxic substances produced by other germinating spores.

According to Jones (1923), spore germination of *Ustilago avenae* is greatest in soil with 30 per cent of water-holding capacity and is greatly reduced at 80 per cent. This was probably due to the amount of available oxygen. The spores failed to germinate in water when exposed to an oxygen-free atmosphere. The "chlamydospores" of *Ustilago zeae* do not germinate in the absence of oxygen, and at least 5 per cent oxygen must be present to allow germination as high as in the open air (Platz *et al.*, 1927).

The supply of oxygen may influence the method of spore germination (Uppal, 1926). Germination by zoospores was possible in the absence of oxygen for the sporangia of *Phytophthora infestans*, *P. colocasiae*, *P. palmivora*, and *P. parasitica*. Germination by germ tubes does not take place in these species in the absence of oxygen. However, the presence of oxygen is essential for zoospore formation by sporangia of *Albugo candida*, *Plasmopara viticola*, and *Sclerospora graminicola*. The two methods of germination are different processes, the direct method more nearly resembling vegetative growth.

Hydrogen-ion concentration. Under natural conditions acidity is not usually a limiting factor for spore germination. In general, spores will germinate within a wide pH range. It seems significant that, in most species of fungi, germination is favored by an acid medium, often at a pH considerably lower than the optimum for vegetative growth or sporulation. The effects of acidity of the medium upon a number of species, including *Botrytis cinerea*, *Aspergillus niger*, *Penicillium cyclopium*, *P. italicum*, *Puccinia graminis* urediospores, *Lenzites sacpiaria*, *Colletotrichum gossypii*, and *Fusarium* sp., are reported by Webb (1921). The spores of the *Fusarium* germinated equally well in alkaline and acid media, while *Colletotrichum gossypii* was the only species of the group studied in which germination was better in an alkaline medium. At pH 2.5 spore germination was prevented in all species, and the optimum for most species was 3.0 to 4.0. In sucrose-nitrate (Czapek's) solution, two maxima usually occurred, the primary one at pH 3.0 to 4.0 and a

secondary one between 6.0 and 7.0. Of all the media tested, beet decoction gave the maximum germination under the widest range of conditions. Webb also clearly demonstrated that the range of pH favoring germination is influenced by temperature and by the constituents of the medium.

All the species of Myxomycetes studied by Smart (1937) germinated within a pH range of 4.0 to 8.0. Spores of *Fuligo septica* germinated from pH 2.0 to 10.0. Optimum for all species ranged from 4.5 to 7.0, with some germinating better near 4.5 and others near 7.0. The spores of *Urocystis occulta* germinated between pH 5.0 and 8.9, with the optimum at 6.8 (Ling, 1940). This optimum is higher than those for most fungi. Kauffman (1934) found the range for spore germination of several species of Basidiomycetes (Agaricaceae and Nidulariaceae) to be pH 5.0 to 8.5 with the optimum near 7.5.

It is interesting that Doran (1922) in his review of spore germination makes no mention of acidity as a factor. It would appear that acidity is of more or less importance as a modifying factor, even though it is seldom a limiting factor for spore germination. This may explain, at least in part, the fact that we often find abundant ungerminated spores in fruiting liquid cultures. Some fungi sporulate only in neutral or alkaline media, which, in general, are not favorable to spore germination.

NUTRIENTS AND STIMULANTS

The constituents of the substrate are known to influence spore germination of some species of fungi. Some species germinate well in distilled or tap water, while others require certain special nutrients such as sugar, salts, or even a particular nitrogen source. No one medium has been found which will allow good germination of all fungi, although certain natural media, such as beet or bean decoction and soil infusion, seem to favor germination in a large number of fungi. When such media containing natural products are used, it is difficult to determine whether the higher percentage of spore germination is due to the nutrients or to some stimulant which is not used in the metabolism of the fungus.

Duggar was one of the foremost American workers interested in spore germination as a primary subject of experimentation. Prior to his work, most of the study on spore germination was only incidental to other problems. Duggar (1901) demonstrated that species differ in their nutrient requirements for germination by placing spores in water, bean decoction, nutrient-salt solution, and cane-sugar solution. A portion of his data showing the percentage of germination after 15 hr. is given in Table 59.

Among some of the compounds Duggar found to influence sporulation of *Aspergillus flavus* and *A. niger* were varying amounts of peptone, ammonium nitrate, and magnesium sulfate. Ammonium nitrate at a

particular concentration gave abundant germination of *A. flavus* but had no effect upon *A. niger*.

Brefeld (1905) was perhaps the first to observe the germination of the spores of various smuts in culture. He noted that the spores germinated poorly or not at all in water, while excellent germination occurred in nutrient solutions (probably dung infusion). Brefeld expressed surprise at the vigorous saprophytic development which followed, especially since the species had previously been known only as obligate parasites.

More recently it was noted that pretreatment with dung infusion markedly stimulated germination of spores of *Ustilago striiformis* (Cheo and Leach, 1950). Untreated spores in distilled water germinated only after 5 to 8 days, and the total germination was less than 1 per cent. Spores soaked in a concentrated horse-dung infusion for 15 days or more, then placed in distilled water, germinated within 5 hr., with a total germination of 50 per cent or higher. The exposure to the dung infusion is believed to increase the permeability of the spore wall, allowing the more rapid absorption of water. It might also be pointed out that the dung infusion evidently contains substances which prevent spore germination until highly diluted or removed entirely.

TABLE 59. PERCENTAGE OF SPORE GERMINATION AFTER 15 HOURS (Duggar, *Botan. Gaz.* **31**, 1901.)

Spores of	Water	Bean decoction	Nutrient-salt solution	Sucrose solution
Aspergillus niger	0	100	100	75
Penicillium glaucum	0	100	100	1
Monilia fructigena	75	100	100	100
Mucor spinosus	0	100	100	1
Phycomyces nitens	0	100	100	2–10
Coprinus fimetarius	0	5–10	0	0
C. comatus	0	0	0	0
C. micaceus	0	100	0	0
Uromyces caryophyllinus	100	75	-	100

Although the spores of the Myxomycetes germinate in distilled water, the percentage may be greater in weak decoctions of the natural substrate, such as rooting wood, bark, leaves, or humus (Smart, 1937). Similarly, the conidia of *Phyllosticta solitaria* germinate more profusely in apple-bark decoction and potato-dextrose broth than in distilled water (Burgert, 1934). While it is possible that increased spore germination is due primarily to some stimulating substance, it seems likely that certain nutrients are also involved.

The conidia of *Glomerella cingulata* apparently have special nutritional requirements for germination. There was little or no germination in

distilled water and in dextrose solution lacking minerals (Lin, 1945). From his experiments involving various inorganic compounds, Lin concluded that carbon, magnesium, nitrogen, and phosphorus, are required (Table 60). The need for sulfur was not so evident as that for the other elements, and sulfur was not essential. The minimum requirements of nitrogen and phosphorus were calculated to be of the order of 10^{-4} μg per spore. No evidence was found that an external supply of any organic substance, other than sugar, is necessary for spore germination.

TABLE 60. THE ESSENTIALITY OF VARIOUS IONS FOR THE GERMINATION OF THE CONIDIA OF *Glomerella cingulata*
(Lin, *Am. Jour. Botany* **32,** 1945.)

Chemical substance applied*	Element lacking	Germination, %
None (redistilled water)	Carbon and minerals	0.0
Glucose	Minerals	0.0
Glucose, KNO_3, KH_2PO_4, $MgSO_4$	None	80.4
Glucose, NH_4Cl, KH_2PO_4, $MgSO_4$	None	92.8
Glucose, KCl, KH_2PO_4, $MgSO_4$	Nitrogen	3.9
Glucose, $NaNO_3$, NaH_2PO_4, $MgSO_4$	Potassium	84.1
Glucose, KNO_3, KCl, $MgSO_4$	Phosphorus	1.5
Glucose, KNO_3, KH_2PO_4, $MgCl_2$	Sulfur	79.3
Glucose, KNO_3, KH_2PO_4, Na_2SO_4	Magnesium	0.9
KNO_3, KH_2PO_4, $MgSO_4$	Carbon	0.7

* In all cases, the concentration of glucose is 0.01 per cent, that of each of the mineral salts 1.0 millimole.

The constituents of the medium may modify the effects of pH on spore germination. This is illustrated in Fig. 75 by the germination of *Lenzites saepiaria* on 2 per cent bacto-peptone, in sucrose-nitrate (Czapek's) solution, and in beet decoction (Webb, 1921).

Emerson (1948) showed that D-xylose as a carbon source gave a high percentage of germination of ascospores of *Neurospora crassa* without heat treatment. Xylose was more effective when autoclaved than when filtered. This was believed to be due to the slight conversion to furfural, which was also shown to be active in increasing spore germination.

From this brief discussion it is evident that little is known about the effects of nutrition upon spore germination. This is no doubt due, in part, to the lack of planned experimental work along this line. Many of the favorable effects of natural products may in fact be due to the presence of stimulants rather than to the nutrients. At the present time we have no conclusive evidence that spores require an external source of vitamins for germination. In the light of the recent discovery of Ryan

(1948) that the amino acids leucine, lysine, and proline favored spore germination in mutants of *Neurospora* deficient for those amino acids, it also seems likely that spore germination in certain vitamin-deficient fungi may be aided by the addition of the vitamins in question. A careful study of the effects of vitamins is needed.

The spores of some fungi, such as *Botrytis cinerea*, germinate much better when in contact with plant tissue than in distilled water (Brown, 1922). It was concluded that certain substances diffuse out of the host plant into the infection drop containing the spores and stimulate germination and infection. Leach (1923) believes that a similar situation may

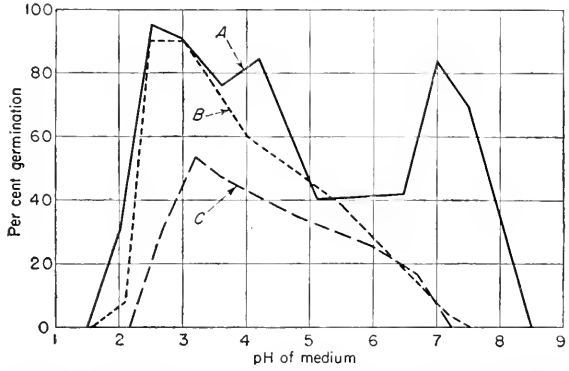


FIG. 75. The effect of the pH and kind of medium on the percentage of germination of spores of *Lenzites saepiaria* at 20 to 23°C. *A*, in sugar-beet decoction; *B*, in 2 per cent bacto-peptone solution; *C*, in Czapek's full nutrient solution. (Redrawn from Webb, *Ann. Missouri Botan. Garden* **8**: 325–327, 1921.)

exist with *Colletotrichum lindemuthianum*. The spores of this fungus germinated poorly in distilled water alone, but distilled water plus a piece of fresh bean tissue gave a high percentage of germination. Fresh bean juice was equally effective, but boiled bean decoction did not stimulate germination. However, green-bean agar made from a similar decoction gave excellent germination, as did potato-dextrose agar. These results led Leach to conclude that two distinct stimulating factors may be involved. A portion of Leach's data is summarized in Table 61.

Some known stimulants may eliminate the need for certain factors ordinarily supplied by natural media for the germination of spores of *Phycomyces* (Robbins *et al.*, 1942). Germination of spores was about 12 per cent or less on mineral-dextrose agar with thiamine. The addition of an extract of potatoes, or of other natural products, of hypoxanthine, acetate, or some other organic acids increased germination to nearly 100 per cent. Treatment of spores with aqueous pyridine had the same favorable effect. These authors believe that certain factors (called *Z* factors) are essential in spore germination. One of these (factor Z_1)

has been identified as hypoxanthine, while the identity of factor Z_2 is still unknown. An explanation of the effects of these stimuli is given by these authors:

The dormant spores are considered to lack sufficient available Z factors for germination. The extracts of natural products or the Z factors furnished in the medium supply this deficiency, which may also be met by treatment with heat, cold, acetate or pyridine. These treatments are thought to change the Z factors in the spores from an unavailable to an available form.

The effects of certain gases and volatile compounds upon germination have also been demonstrated. It has been observed that spores of numerous fungi germinate better in a container in which some living plant part is also present. This was demonstrated for *Basisporium gallarum* by Durrell (1925), who also found that the introduction of carbon dioxide

TABLE 61. THE EFFECT OF VARIOUS MEDIA AND PLANT TISSUES ON SPORE GERMINATION OF *Colletotrichum lindemuthianum*
(Leach, *Minn. Agr. Expt. Sta. Bull.* **14**, 1923.)

Medium	Germination, %
Distilled water	3–6
Sucrose-nitrate (Czapek's) solution	5–11
Sucrose-nitrate (Czapek's) solution plus bean decoction	10
Bean decoction	8
Distilled water plus fresh bean tissue	83–95
Distilled water plus sunflower tissue	5
Distilled water plus wheat tissue	12
Distilled water plus corn tissue	10
Distilled water plus tomato tissue	2
Sucrose-nitrate (Czapek's) solution plus bean tissue	95
Green-bean agar	97
Potato-glucose agar	98

into the container enclosing the spores gave the same increase in germination. The same effect was demonstrated for *Ustilago zeae* (Platz *et al.*, 1927). An atmosphere containing 15 per cent carbon dioxide was found to be optimum for spore germination. Such a condition gave a pH of the medium from 4.9 to 5.6. These authors conclude that the stimulating effect is apparently due to "a definite action of carbonic acid." Is it possible that this is an example of heterotrophic utilization of carbon dioxide?

While the release of carbon dioxide into the atmosphere by various living plant parts may explain the stimulation of spore germination in many cases, the presence of carbon dioxide alone will not explain certain results obtained by some workers. For instance, spore germination of *Botrytis cinerea* was stimulated by the presence of living tissues of apples or leaves of *Ruta* or *Eucalyptus* in the same container, while tissues of potato tuber and onion scales inhibited germination (Brown, 1922).

Distillates of these leaves increased germination four to ten times. Ethyl acetate likewise gave similar results. The possibility of specific activity was suggested by the fact that apple tissues distinctly stimulated germination of *B. cinerea* spores, while they inhibited germination of spores of *Colletotrichum lindemuthianum*. The stimulation was greater with old spores.

Presoaking and the subsequent addition of a stimulating volatile agent gave optimum germination of *Urocystis tritici* spores (Noble, 1923). The expressed sap of wheat placed in the same container with germinating spores, but in separate dishes, proved to be a good stimulating agent. Uninjured seedlings of certain nonsusceptible hosts likewise stimulated spore germination. Benzaldehyde, salicylaldehyde, butyric acid, and acetone in certain concentrations stimulated germination of presoaked spores. Noble believed that presoaking increased permeability of the spore and allowed the more rapid intake of the stimulatory volatile substance, which increased the permeability of the protoplasmic membrane by changing its physical condition.

Likewise, a solution of benzaldehyde (3/2,000,000) stimulated germination of *Urocystis occulta* spores, which germinated very poorly in water (Ling, 1940). Ethyl alcohol stimulated spore germination in *Aspergillus flavus;* methyl alcohol was slower and less effective (Duggar, 1901). *A. niger* was stimulated by oxalic acid, whereas *A. flavus* was not. It is understood that the stimulatory power of these chemicals depends upon the concentration.

An interesting situation exists in the germination response of some spores to the presence of other fungi, or even to the medium in which other fungi have grown. The few experiments conducted along this line suggest that the constant association with other organisms may be highly beneficial to spore germination as well as subsequent growth of some fungi in nature.

The germination of a number of species of Myxomycetes was increased by the addition of the filtrate of a medium in which spores had previously been germinated (Smart, 1937). Smart calls the stimulatory factor an "autocatalytic agent." A portion of Smart's data is presented in Table 62.

Fries (1941, 1943) obtained almost phenomenal results with spores of a number of Hymenomycetes, which previously had germinated poorly or not at all, by sowing the spores on malt agar with living cultures of *Torulopsis sanguinea*. Spores of ten species of *Tricholoma*, which germinated only with difficulty without the yeast, were found to germinate readily in its presence. One species of *Tricholoma* gave only negative results. In *Amanita mappa*, *A. porphyria*, and *A. rubescens* germination occurred only when *Torulopsis* was present. Germination of two other

species of *Amanita* was considerably improved by the presence of the yeast. None of the seven species of *Boletus* germinated on malt agar without the yeast. On the same medium and in the presence of *Torulopsis sanguinea*, germination was obtained with spores of *B. bovinus*, *B. elegans*, *B. flavidus*, *B. granulatus*, *B. luteus*, *B. variegatus*, and *B. viscidus*. Some germination of *Boletus* spores was also obtained in the presence of living colonies of certain other fungi, but none was so effective as *Torulopsis*. Spores of certain other fungi (*Hydnum repandum*, *H. imbricatum*, *Craterellus lutescens*, *Lycoperdon umbrinum*, *L. echinatum*, *L. nigrescens*, *L. pratense*, *L. pyriforme*, and *Scleroderma aurantium*) germinated in Fries's

TABLE 62. GERMINATION OF SINGLE MYXOMYCETE SPORES
(Smart, *Am. Jour. Botany* **24**, 1937.)

Species	Number of spores germinating	
	Lot 1 (10 spores) (fresh medium)	Lot 2 (10 spores) (previous germination medium)
Fuligo septica	9 after 3 hr.	10 in 45 min.
Physarum polycephalum	6 after 3 days	8 in 15 hr.
Stemonitis fusca	3 after 2 days	9 in 1 day
S. axifera	4 after 1 day	8 in 8 hr.
Enteridium rozeanum	0 in 2 weeks	10 in 30 min.
Reticularia lycoperdon	0 in 2 weeks	8 in 15 min.
Lycogala epidendrum	3 after 2 days	8 in 3 hr.
Areyria denudata	6 after 6 days	6 in 6 days
Dictydium cancellatum	2 after 18 days	2 after 18 days
Physarum cinereum	9 after 7 days	9 in 6 days

experiments only in the presence of *T. sanguinea*. He also tested the effects of mycelial extracts on spore germination and found that extracts of certain species of *Boletus* stimulated germination of spores of the same species. Many of the fungi studied by Fries are believed to be mycorhizal and may require the presence of a special set of conditions, perhaps the roots of certain plants (or conditions which simulate their presence), before germination will occur.

The time required for a spore to germinate after being subjected to favorable conditions is a reflection of the interaction and relative importance of all the various influencing factors. The nearer all these factors are to the optimum, the shorter will be the time required for germination. Time is an important factor for the subsequent infection of the host. In nature the near-optimum environmental conditions, principally temperature and moisture, may persist for but a short time, and a change in but one of these factors may inhibit spore germination.

LONGEVITY OF SPORES

The length of life of spores is usually measured by their ability to germinate after various periods of time. It is affected by environmental conditions, principally temperature and moisture. The greatest period of longevity reported for fungus spores appears to be among the Myxomycetes. Smith (1929) succeeded in germinating spores from herbarium specimens of Myxomycetes 5 to 32 years after they were collected. A few of the common species whose spores germinated after approximately 30 years are *Physarum cinereum*, *Fuligo septica*, *Hemitrichia clavata*, and *Stemonitis ferruginea*. Smut spores also have a long period of viability (Lowther, 1950). Spores of *Aspergillus oryzae* germinated after 22 years in a sealed tube at room temperature (McCrea, 1923).

In contrast to long periods of longevity, some fungus spores die very soon after they are liberated. The sporidia of *Cronartium ribicola* lived less than 10 min. at room temperature with a humidity of 90 per cent (Spaulding, cited by Doran, 1922). Sporidia of *Gymnosporangium juniperi-virginianae* lived no longer than 6 days in dry air. Eight weeks is reported as the maximum longevity of aeciospores of *C. ribicola*, with only 5 per cent germination after 7 weeks. In general, aeciospores of the rust fungi remain viable about 50 per cent longer than the urediospores, whose average longevity ranged from 30 to 60 days (Doran, 1922).

Other factors have been reported to influence longevity of spores. Ascospores of *Endothia parasitica* remained viable for a year when dried in the bark, but when removed from the bark, they lost the ability to germinate within 5 months (Anderson and Rankin, 1914). Similarly, conidia in dry spore horns retained viability for at least a year, but when placed in water, separated, and then dried, the time was less than 1 month. It seems likely that one of the functions of the gelatinous matrix of the conidia of certain fungi, such as *Glocosporium*, *Colletotrichum*, and *Cytospora*, is to increase the longevity of the spores through its water-holding capacity.

Light is apparently only of minor importance as a factor influencing longevity. No doubt ultraviolet light in nature plays an important part in reducing the period of viability and even in killing many of the hyaline spores. Spores having dark walls are protected somewhat against the penetration of the ultraviolet rays.

SUMMARY

Spore germination represents a change from an inactive to an active phase in the life cycle of a fungus. Since it involves the first stages of growth, it is reasonable to expect that many of the factors which influence vegetative growth also affect spore germination. On the other hand, the spore, being a resting cell, may contain stored materials not usually

present in appreciable quantities in vegetative cells. Since the metabolic activity of a resting spore is at a minimum in contrast with that of actively growing vegetative cells, the internal responses to the environmental factors may be quite different.

The variability of the needs of spores of different fungi for germination is adequately illustrated in the literature. Certain general conditions are essential for all spores, while some require a special set of conditions. Water is essential to activate certain enzyme systems, to initiate other internal chemical changes, and to increase the volume of the germinating spore. When the temperature is near the optimum, the enzymatic activity and the rate of spore germination are increased. The supply of oxygen must be adequate to meet the demands of the greatly increased rate of respiration. The acidity of the substrate must be favorable. Variability in the period of viability of spores is striking, but longevity is greatly influenced by the environment. Much information is yet to be gained regarding the longevity of spores, particularly of the plant pathogens.

Certain special conditions are required for germination of some spores. These may act as a stimulant in breaking dormancy or may supply needed nutrients. The effects of other living organisms, or even of the substrate upon which they have grown, are of particular interest, for such association is the usual condition under which germination occurs in nature One might suppose that the secretions of certain plants would exert a selective action on spore germination and affect the pathogenicity of certain fungi, but evidence on this point is lacking.

The production of short germ tubes by spores of some species of *Erysiphe* in an absolutely dry atmosphere is unusual. If this is to be considered as true germination, it must represent a unique method among fungi. The Erysiphales, however, are excellent examples of fungi whose spores germinate in atmospheres of lower relative humidity than most fungi can endure.

Under the changing conditions of nature, the period of time during which a factor is active is of utmost importance. Germination is the result of the action of all the influencing factors operating at the same time. Most of these factors vary in intensity or concentration, so that the combined optima of all factors are seldom, if ever, reached at any given time in nature. As a result, an extremely low percentage of the spores formed by a fungus ever germinate, while still fewer give rise to extensive mycelium.

REFERENCES

Anderson, P. J., and W. H. Rankin: Endothia canker of chestnut, *Cornell Univ. Agr. Expt. Sta. Bull.* 347, 1914.

Bonner, J. T.: A study of the temperature and humidity requirements of *Aspergillus niger*, *Mycologia* **40**: 728–738, 1948.

Brefeld, O.: Die Brandpilze IV. in Untersuchungen aus dem Gesammtgebiete der Mykologie, Heft 13, Muenster, Heinrich Schöningh, 1905.

★Brodie, H. J.: Further investigations on the mechanism of germination of the conidia of various species of powdery mildew at low humidity, *Can. Jour. Research* **23**: 198–211, 1945.

Brodie, H. J., and C. C. Neufeld: The development and structure of the conidia of *Erysiphe polygoni* DC. and their germination at low humidity, *Can. Jour. Research* **20**: 41–61, 1942.

★Brown, W.: Studies in the physiology of parasitism. IX. The effect on the germination of fungal spores of volatile substances arising from plant tissues, *Ann. Botany* **36**: 285–300, 1922.

Burgert, I. A.: Some factors influencing germination of spores of *Phyllostieta solitaria*, *Phytopathology* **24**: 384–396, 1934.

★Cheo, P. C., and J. G. Leach: The stimulating effect of dung infusion on the germination of spores of *Ustilago striiformis*, *Phytopathology* **40**: 584–589, 1950.

★Clayton, C. N.: The germination of fungous spores in relation to controlled humidity, *Phytopathology* **32**: 921–934, 1942.

Dodge, B. O.: Methods of culture and morphology of the archicarp in certain species of the Ascobolaceae, *Bull. Torrey Botan. Club* **39**: 139–197, 1912.

Doran, W. L.: Effect of external and internal factors on the germination of fungous spores, *Bull. Torrey Botan. Club* **49**: 313–336, 1922.

Duggar, B. M.: Physiological studies with special reference to germination of certain fungous spores, *Botan. Gaz.* **31**: 38–66, 1901.

Durrell, L. W.: Basisporium dry rot of corn, *Iowa Agr. Expt. Sta. Bull.* 84, 1925.

Emerson, M. A.: Chemical activation of ascospore germination in *Neurospora crassa*, *Jour. Bact.* **55**: 327–330, 1948.

Fries, N.: Ueber die Sporenkeimung bei einigen Gasterromyceten und mykorrhizabildenen Hymenomyceten, *Arch. Mikrobiol.* **12**: 266–284, 1941.

Fries, N.: Untersuchungen über Sporenkeimung und Myeelentwicklung bodenbewohnender Hymenomyceten, *Symbolae Botan. Upsaliensis* **6**: 1–81, 1943.

Gardner, M. W.: Anthracnose of cucurbits, *U.S. Dept. Agr. Bull.* 727, 1918.

Goddard, D. R.: The reversible heat activation inducing germination and increased respiration in the ascospores of *Neurospora tetrasperma*, *Jour. Gen. Physiol.* **19**: 45–60, 1935.

Goddard, D. R., and P. E. Smith: Respiratory block in the dormant spores of *Neurospora tetrasperma*, *Plant Physiol.* **13**: 241–264, 1938.

★Gottlieb, D.: The physiology of spore germination in fungi, *Botan. Rev.* **16**: 229–257, 1950.

Jones, E. S.: Influence of temperature, moisture and oxygen on spore germination of *Ustilago avenae*, *Jour. Agr. Research* **24**: 577–591, 1923.

Kauffman, F. H. O.: Studies on the germination of the spores of certain Basidiomycetes, *Botan. Gaz.* **96**: 282–297, 1934.

Leach, J. G.: The parasitism of *Colletotrichum lindemuthianum*, *Minn. Agr. Expt. Sta. Bull.* 14, 1923.

★Lin, C. K.: Nutrient requirements in the germination of the conidia of *Glomerella cingulata*, *Am. Jour. Botany* **32**: 296–298, 1945.

Ling, L.: Factors affecting spore germination and growth of *Urocystis occulta* in culture, *Phytopathology* **30**: 579–591, 1940.

Lowther, C. V.: Chlamydospore germination in physiologic races of *Tilletia caries* and *Tilletia foetida*, *Phytopathology* **40**: 590–603, 1950.

McCrea, A.: Spores of *Aspergillus oryzae* alive after 22 years stored in test tube, *Science* (N.S.) **58**: 426, 1923.

NOBLE, R. J.: Studies on the parasitism of *Urocystis tritici* Koern, the organism causing flag smut of wheat, *Jour. Agr. Research* **27**: 451–489, 1924.

PLATZ, G. A., L. W. DURRELL, and M. E. HOWE: Effect of carbon dioxide upon the germination of chlamydospores of *Ustilago zeae* (Beckm.) Ung., *Jour. Agr. Research* **34**: 137–147, 1927.

ROBBINS, W. J., V. W. KAVANAGH, and F. KAVANAGH: Growth substances and dormancy of spores of *Phycomyces*, *Botan. Gaz.* **104**: 224–242, 1942.

RYAN, F. J.: The application of *Neurospora* to bioassay, *Fed. Proc.* **3**: 365–369, 1946.

★RYAN, F. J.: The germination of conidia from biochemical mutants of *Neurospora*, *Am. Jour. Botany* **35**: 497–503, 1948.

SMART, R. F.: Influence of certain external factors on spore germination in the Myxomycetes, *Am. Jour. Botany* **24**: 145–159, 1937.

SMITH, E. C.: The longevity of Myxomycete spores, *Mycologia* **21**: 321–323, 1929.

UPPAL, B. N.: Relation of oxygen to spore germination in some species of Peronosporales, *Phytopathology* **16**: 285–292, 1926.

WALKER, J. C., and F. L. WELLMAN: Relation of temperature to spore germination and growth of *Urocystis cepulae*, *Jour. Agr. Research* **32**: 133–146, 1926.

★WEBB, R. W.: Studies in the physiology of fungi. XV. Germination of the spores of certain fungi in relation to hydrogen-ion concentration, *Ann. Missouri Botan. Garden* **8**: 282–341, 1921.

WOLF, F. A., and F. T. WOLF: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

YARWOOD, C. E.: The tolerance of *Erysiphe polygoni* and certain other powdery mildews to low humidity, *Phytopathology* **26**: 845–859, 1936.

CHAPTER 17

THE PHYSIOLOGY OF PARASITISM AND RESISTANCE

A discussion of parasitism occupies an important position in any treatise on the physiology of fungi, particularly for those students who are interested in plant diseases or the fungi which cause them. This phase of study offers many challenging unsolved problems. Parasitism involves primarily two living organisms, the parasite, whose actions are offensive, and the host, whose reactions are defensive. If the defenses of the host plant, either before or after penetration by the parasite, are successful, the plant is resistant; if not, it is susceptible. To be successful, a parasite must find the nutritional and environmental conditions favorable for its development. If even a single important factor is unfavorable to the parasite, the fungus may fail to establish a parasitic relationship with its proposed host. Such factors may exert their influence either before or after penetration by the fungus. Environmental factors acting before penetration may in reality bring about an escape from a disease rather than true resistance to it.

The present discussion is divided into three main parts: (1) penetration; (2) parasitism, the action of the parasite in becoming established and obtaining its food; (3) resistance of the host to penetration or against the parasite after penetration. The comprehensive reviews of the physiology of the host-parasite relationship given by Brown (1936, 1948) should be read by all students. Similar reference is made to Arthur *et al.* (1929), who give an excellent discussion of the parasitic relations of the rusts, and to the treatise of Gäumann (1946, 1950) on the principles of plant infection.

PENETRATION

A parasite may gain entrance into the host (1) through the natural openings, such as stomata or lenticels, (2) by direct penetration through the uninjured epidermis, or (3) through wounds.

Through stomata. Viable spores may fall upon a host plant and produce germ tubes, which by chance grow over or near stomata. The outer walls of the epidermal cells of aerial plant parts are covered with cutin, which is somewhat resistant to penetration by some fungi. The germ tube which enters through a stoma may then be favored by the moist atmosphere in the substomatal cavity. In some cases, the unspecialized hyphae may penetrate the host cells; in other fungi, haustoria, which

arise from the intercellular mycelium, penetrate the host cells and absorb food. Water vapor has been suggested as the stimulus which causes the germ tube to turn inward and enter a stoma. This, however, cannot be the case with zoospores which are immersed in water and which have been noted to cluster around stomata. The fungi which normally enter the host plant through stomata include the cereal rusts (aeciospore and urediospore stages), *Cercospora beticola*, *Phytophthora infestans* (zoospore stage), the Peronosporales, *Albugo candida*, and others.

The cereal rusts have received a great deal of attention in resistance studies. It has been reported (Hart, 1929) that *Puccinia graminis* apparently requires the open stomata of wheat plant for penetration. On the other hand, Caldwell and Stone (1936) have shown that the germ tubes of *Puccinia triticina* are able to force their way between the guard cells of closed stomata of wheat leaves. A germ tube from a urediospore may start to enter an open stoma, but as it forms an appressorium, the stoma closes. Further penetration is accomplished between the guard cells by a slender hypha. Allen (1926) believes that the appressorium probably secretes some toxin which harms or even kills the guard cells, causing the stoma to close. Caldwell and Stone, however, do not believe that this injury to the guard cells is necessary for entry of germ tubes. The appressorium seems to function as a special organ to apply the pressure needed for the forced entry between the closed guard cells.

Penetration through lenticels more often occurs in the underground parts of the host which are in a more or less moist situation. Potato tubers may become infected by *Actinomyces scabies* and by germ tubes from sporangia of *Phytophthora infestans*, chiefly through the lenticels.

Direct penetration. A large number of fungi are capable of penetrating the unbroken epidermis of a plant, directly through the cutinized outer walls. The spore may germinate on the surface of the plant in a drop of water. The germ tube grows over the epidermis and by some stimulus is caused to turn inward and penetrate the cell. Brown (1922) demonstrated that there is a certain amount of exosmosis of materials from host tissue into a drop of liquid on the surface. In some cases this may lead to a chemotropic response by the fungus. However, in most cases the stimulus of contact is believed to initiate appressorial formation and penetration. The formation of appressoria is common among many fungi when the germ tubes come in contact with the epidermal cells. The fact that the appressoria are often formed on a glass slide is further evidence that their formation is in response to contact with a solid surface. Appressoria are usually bulb-like or disk-like in shape and are believed to serve as an adhesive disk against which the slender infection hypha may push in penetrating the cell wall. Brown (1915, 1922) presents evidence that the host cells are not killed by *Botrytis cinerea*,

Sclerotinia sclerotiorum, and *Colletotrichum lindemuthianum* until after the fungus penetrates the cuticle of the host. In other words, there is little or no diffusion of the toxic materials through the cuticle. Direct penetration through cutinized walls is believed to be entirely by mechanical pressure, since no cutin-dissolving enzyme has been demonstrated in the fungi.

The rhizomorphs of *Armillaria mellea* usually gain entrance directly through the sound cork layer of comparatively old roots (Thomas, 1934). Penetration is believed to be accomplished partly by mechanical pressure and partly by chemical means. There is evidence that a suberin-dissolving enzyme aids in the destruction of some of the cork cells. Some fungi may enter the same host by more than one method. *Fusarium lini* may enter through young epidermal cells of the root, root hairs, stomata of seedlings, and perhaps through wounds.

The penetration of noncutinized cell walls may be either by mechanical pressure or by the dissolving action of enzymes secreted by the fungus. Hawkins and Harvey (1919) concluded that the hyphae of *Pythium debaryanum* penetrated the cell walls of susceptible potato tubers by mechanical pressure, and that the resistant varieties in general showed greater resistance to mechanical puncture. They found no evidence of cellulases which might aid in penetration by dissolving the cellulose cell wall. Using cane sugar as the plasmolyzing solution, they found that the hyphae of *P. debaryanum* were capable of exerting as high as 54 atm. osmotic pressure. These hyphae would have a strong tendency to absorb water, and as a result greater internal pressure would be exerted against the hyphal wall. Apparently the hyphal wall is capable of withstanding this pressure at all points except its tip, where growth occurs. The pressure exerted by the growing tip is believed to be sufficient to cause penetration of the host cell wall. By direct microscopic examination Hawkins and Harvey observed that, just after the hyphal tip came in contact with the host cell wall, it formed a swelling, back of which a bend developed. This was followed by penetration of the wall by a small tube.

Penetration through noncutinized cell walls by chemical means has been described for *Spongospora subterranea* by Kunkel (1915). It seems likely that other nonfilamentous fungi penetrate cell walls in the same way. Likewise, wood rot fungi penetrate the cellulose and lignified cell walls by enzymatic action, as evidenced by the boreholes in decaying wood. It may be significant that the hyphal walls of *Pythium*, as well as of other Oomycetes, contain cellulose, while the hyphal walls of other fungi are composed principally of chitin, which would not be acted upon by cellulases.

It must be emphasized that penetration of the host in itself does not necessarily lead to the establishment of the fungus in the host and the

production of a disease. In some cases it is known that a fungus may enter resistant or immune plants, as well as susceptible ones, but find the conditions unfavorable for its establishment and further development.

Through wounds. A number of fungi apparently are unable to penetrate a healthy plant except through wounds. These may be insect wounds, broken branches of trees, broken roots, etc. In addition, some fungi which are capable of entering the host by other means may also penetrate through wounds. *Phymatotrichum omnivorum*, the cause of numerous root rots, commonly enters roots through wounds, although these are not necessary. *Fusarium*, causing dry rot of potato, apparently enters the tubers only after they have been wounded. Likewise most of the wood-rotting Basidiomycetes enter the host only through wounds, principally at broken or dead branches and at pruning or lightning and fire scars. Here, the air-borne basidiospores must find suitable moisture for germination and for penetration of the wood. *Endothia parasitica* is said to enter the chestnut tree only through wounds that extend through the corky layer. *Ceratostomella ulmi* is transmitted by the European bark beetle, which introduces the spores into its feeding wounds. Bruises and wounds of fruits and vegetables are common ports of entry for numerous rot-producing fungi, such as *Rhizopus nigricans* on sweet potato. *Monilinia fructicola* on stone fruits, *Penicillium expansum* on apple, and *P. italicum* and *P. digitatum* on citrus fruits.

PARASITISM

A discussion of the action of the parasite after it enters the host is so closely correlated with the defense of the host that it is difficult to discuss each topic separately. For the sake of convenience, however, it seems desirable to discuss some of the outstanding effects of fungi upon their hosts and the methods by which the parasites obtain their food under a separate heading of parasitism.

Parasitism in plants. Parasitism may begin as soon as a fungus hypha enters the host. The primary consideration is the securing of suitable nutrients and water by the fungus. This may be accomplished by two general methods, (1) by killing the cells of the host and obtaining food from the dead cells, or (2) by establishing a close nutritional relationship with the living host cells and absorbing the soluble nutrients without causing necrosis. The fungi falling in the first group are the *destructive parasites*, while those belonging to the second group have been called the *balanced parasites* (Bessey, 1935). The latter group includes those fungi known at present as *obligate parasites* (such as the Uredinales, Erysiphales, and Peronosporaceae), and some other fungi (such as the Ustilaginales and *Taphrina*) which in their hosts obtain food only from living cells.

The destructive parasites, as a whole, are strong producers of enzymes

and toxins but may be weak in mechanical action. Some of these cause rapid rots of fruits or vegetables but are unable to penetrate the unbroken epidermis and must depend on wounds for their entrance. Others, which are seldom, if ever, found as pathogens in nature, may cause rot when artificially inoculated into succulent plant tissues.

Rotting of the tissue is due to two distinct effects of the fungus on the host: (1) death of the cells, and (2) dissolution of the middle lamellae. The separation of the cells is due to the action of the enzymes protopectinase, pectinase, and pectase on the middle lamella. These three enzymes are often collectively referred to as pectinase. There is some evidence that pectinase may also cause a change in permeability of the cell membranes and the death of the cells, but it is possible that some other toxic substance may be closely associated with pectinase. However, no such substance has been isolated. Extracts of rotted tissues have been shown to cause the same effects as the fungi themselves. These effects are described by De Bary (1886) for *Sclerotinia sclerotiorum* and by Brown (1915) for *Botrytis cinerea*. Higgins (1927) believes that oxalic acid produced by *Sclerotium rolfsii* is the principal agent of destruction. The death of the host cells well in advance of the invading hyphae indicates rapid diffusion of the toxic substance in the case of fungi producing soft rot. Brown (1948) believes that the enzyme pectinase acts as a cytolytic toxin. For a discussion of the identity of enzymes and toxins of species of *Clostridium*, see Smith (1949).

Thatcher (1942) has shown that *B. cinerea* and *S. sclerotiorum* cause a fourfold increase in the permeability to water of the host cells just beyond the discolored necrotic zone. Some substance other than pectinase may bring about this change in permeability and be a contributing factor to the "action in advance" of many fungi. *Phytophthora infestans* caused a change in permeability in host cells beyond the extent of the hyphae which penetrated the living tissue. The identity of the substance causing a change in permeability is unknown, but it is likely a weak toxin or an enzyme which alters the structure or activity of the plasma membrane. The increase in permeability may concern water alone or both nutrients and water.

An osmotic pressure higher in the fungus cells than in the surrounding host cells is apparently characteristic of the host-parasite relationship (Table 63). This is necessary before the parasite can absorb water from the host cells.

The production of pectinase and its activity under different conditions were studied by Vasudeva (1930) and Chona (1932), who showed that the amount produced by *Botrytis allii* varied with the medium in which the fungus was grown. *B. allii* did not secrete a demonstrable amount of pectinase when grown on apple extract, but when asparagine, potassium

nitrate, or ammonium sulfate was added to the apple extract, there was a decided increase in the amount produced. This is not surprising, for there are numerous reports that the available nutrients and the pH of the culture medium affect both the kind and amount of metabolic products of a fungus.

The activity of the enzymes produced by a pathogen varies with the conditions of the host cells. It seems probable that the inhibition of the fungus enzymes by the host cells is an important factor in resistance. Klotz (1927) proposed this hypothesis to explain the greater resistance of sour orange and the greater susceptibility of lemon to *Pythiacystis citrophthora* and *Phomopsis californica*, the causes of certain bark diseases.

TABLE 63. THE OSMOTIC PRESSURES OF HOST AND PARASITE
(Thatcher, *Can. Jour. Research* **20**, 1942.)

Fungus	Ave. osmotic pressure, atm.	Host	Ave. osmotic pressure, atm.
Uromyces fabae, germ tubes	44.25	*Pisum sativum*, leaf	9.15
haustoria	21.9	petiole	10.1
U. caryophyllinus, haustoria	18.6	*Dianthus*, leaf base	11.2
Botrytis cinerea, hyphae	29.8	*Apium graveolens*, petiole	8.3
Sclerotinia sclerotiorum, hyphae	23.5	*A. graveolens*, petiole	13.4
Puccinia graminis, haustoria (race 21)	18.9	Mindum wheat, leaf	9.4
Erysiphe polygoni, hyphae	18.0	*Brassica*, leaf	10.6
Phytophthora infestans, hyphae (aerial)	17.4	*Solanum tuberosum*, tuber	10.6
hyphae (intercellular)	15.5	petiole	8.9
sporangia	18.1		
Phoma lingam, hyphae	41.3	*Brassica*, root	11.3

The greater pathogenic action of a destructive fungus occurs in the host whose cells are favorable for the activity of the enzymes of the fungus.

Further evidence of enzyme inhibition of certain plant tissues was presented by Chona (1932), who studied the rotting action of *B. cinerea*, the cause of a soft rot of various plant tissues, and *Pythium* sp., a rot producer of potato tubers. Vigorous germination of spores of *Botrytis* and even some sporulation took place in artificial wounds in potato tubers, but no decomposition followed. The pectinase produced by *B. cinerea* was active against apple tissue, but the presence of potato tissue inhibited its activity. It was then found that the mineral salts, particularly KH_2PO_4 and $MgSO_4$, in the potato were the inhibiting factors. On the other hand, *Pythium* spores germinated well on apple tissue but failed

to rot it. The inhibition in this case was traced to the malic acid in the apple. The pectinase produced by *Pythium* was most active in an alkaline medium, near pH 8.0, while that of *B. cinerea* was more active in an acid medium, at pH 5.0 to 5.5.

In contrast with the destructive fungi which rot the host tissue are those which cause wilting and certain types of necrosis without disintegration of the host cells. These fungi produce little or no pectinase. Some common fungi which cause wilting of mature plants are species of *Fusarium*, *Verticillium*, *Cephalosporium*, and *Ceratostomella*. It is now generally believed that in most cases wilting caused by fungi is due to toxins or to the plugging of the vessels by polysaccharides or other similar metabolic products of the fungus, rather than to plugging by the excessive mycelial growth in the vessels. Extracts of the mycelium or the culture filtrate of a number of these fungi cause effects that are the same as or similar to those caused by the fungi themselves in their respective hosts.

A definite correlation between the pathogenecity of two strains of *Fusarium lycopersici* and the toxicity of their metabolic products was demonstrated by Haymaker (1928). There was similarity of symptoms and of the effect of temperature on wilting. The culture filtrate was more toxic when the fungus was grown at 28°C. than that obtained at any other temperature. The toxic substance was not identified. Other workers (Plattner and Clausson-Kaas, 1945; Woolley, 1946) have reported that the wilt-inducing compound produced by *F. lycopersici* is lycomarasmin, a peptide of aspartic acid. Gäumann and Jaag (1947) reported that clavacin exerted a wilting effect on detached tomato shoots similar to that of lycomarasmin. But, whereas lycomarasmin acted chiefly on the cells of the leaf blade, clavacin is toxic mainly to the phloem and parenchyma of wood and cortex of the stem and petiole. The action of both compounds is believed to be similar, destroying the semipermeability of the plasma membranes, thereby decreasing the water-holding capacity of the cells and inducing wilting.

Various polysaccharides have been shown to produce wilting in tomato cuttings (Hodgson *et al.*, 1949). Since there was a direct relationship between molecular weight and wilt-inducing action of these compounds, it was concluded that their action was mainly by mechanically interfering with the transportation of water. Dimond (1947) also reported wilting of elm leaves due in part to a polysaccharide produced by *Ceratostomella ulmi* in culture. Its action is believed to be similar in naturally infected elm trees.

More recently, Feldman *et al.* (1950) have presented evidence to show that the primary wilt-inducing agent produced by *C. ulmi* is not the polysaccharide, but a toxin. The production of toxin in liquid culture filtrate was greatly influenced by the pH of the medium, being greater in

buffered media at pH 4.25 than at 5.25, although growth was more rapid in the less acid medium (Fig. 76). The toxin was shown to be irreversibly inactivated at pH 6 or above. The introduction of calcium hydroxide into trees and the application of basic chemicals to the soil have been somewhat successful in retarding the disease. Presumably, these chemicals act by raising the pH of the sap of the tree.

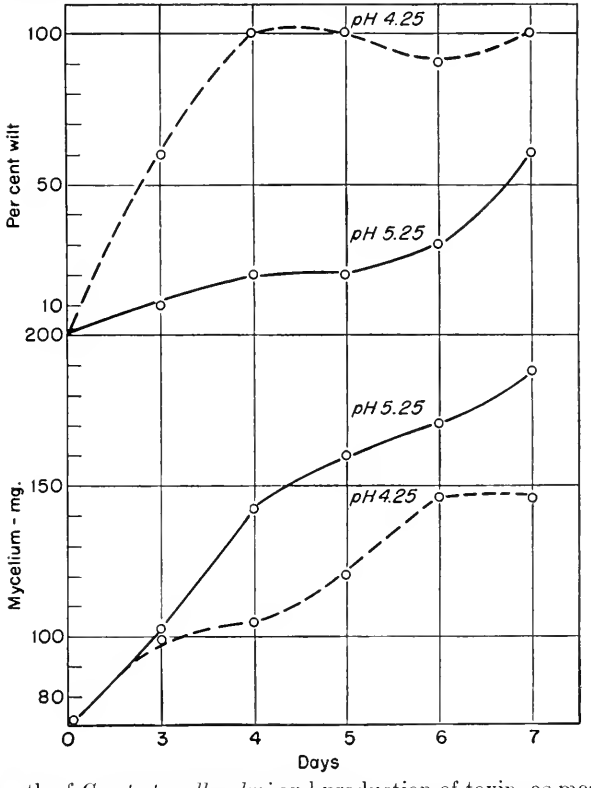


FIG. 76. Growth of *Ceratostomella ulmi* and production of toxin, as measured by wilt of tomato seedlings induced by culture filtrate, in buffered media at different pH levels. Note that toxin production is favored by the more acid medium, while growth is greater in the less acid medium. (Courtesy of Feldman, Caroselli, and Howard, *Phytopathology*. **40**: 348, 1950.)

The varieties of oats susceptible to toxic culture filtrates of *Helminthosporium victoriae* were the same that were susceptible to the fungus in nature (Meehan and Murphy, 1947). Plants of Boone variety were killed, but Clinton plants were unaffected when grown in the same concentrations of the filtrate. The toxic substance, which was not identified, was produced when the media contained either organic or inorganic nitrogen. This species differs from *H. sacchari*, which was reported by Lee (1929) to reduce nitrates to nitrites, which were toxic to sugar cane.

The toxicity of the metabolic products of *Fusarium vasinfectum* was found to be dependent upon the medium on which the fungus was cultured (Rosen, 1926). Filtrates of cultures grown in a medium containing potassium nitrate and sucrose were highly toxic to cotton plants, while filtrates from cultures grown in a medium containing ammonium lactate, sodium asparaginate, and glycerin were not toxic. The filtrate of the nitrate-sucrose medium contained nitrites. Solutions of chemically pure sodium nitrite were also decidedly toxic to cotton plants. We may assume that the action of this fungus in converting nitrates to nitrites is the same within the host plant as it is in the culture vessel.

Thus, there seems to be abundant evidence that the metabolic products, including enzymes and toxins, of a given fungus vary both in kind and in amount with the pH and composition of the culture medium. On the other hand, the evidence that the same situation exists in nature is extremely scarce. One may speculate, however, that the types of nutrients furnished by the host cells and the pH of the cell sap may also influence the metabolic products of the fungus in the host plant. If this is true, a given fungus may find the nutrients and environment supplied by one host particularly favorable for the production of a disease-inducing toxin or enzyme. If the host is unable to inhibit the action of these substances, disease may result. The natures of both the pathogen and the host determine the severity of the disease. This hypothesis may help to explain, in part, the variation in intensity of parasitism of a fungus on its different hosts. While there is little evidence to support this idea at present, it is hoped that experimental work will be conducted to test its merits.

The possibility that the presence of vitamins may affect pathogenicity has been suggested (Pehrson, 1948; Prasad, 1949). There is no evidence that deficiencies for vitamins are correlated with either parasitism or pathogenicity, and vitamin deficiency may be excluded as a factor leading to the parasitic habit. Likewise, there seems to be little or no correlation between the nitrogen requirements of fungi and the parasitic habit. Nonliving organic materials in nature are sources of vitamins and organic nitrogen just as are the living plants. For example, *Ustilago striiformis*, a highly parasitic fungus, is self-sufficient with respect to vitamins, and some isolates are capable of utilizing nitrate nitrogen, while *Phycomyces blakesleeanus*, an obligate saprophyte, is deficient for thiamine and is unable to utilize nitrate nitrogen.

Opposed to the destructive parasites discussed above are the balanced parasites, which, in general, have a strong power to penetrate mechanically but whose chemical actions on the host are relatively weak. Most of the filamentous balanced parasites produce intercellular mycelium, sending haustoria into the host cells. These serve as food-absorbing

structures, but the exact mechanism of the transfer of food is not so well understood. The haustorium of the filamentous parasite is very similar in its behavior to the intracellular nonfilamentous parasite, being surrounded by the protoplasm of the host cell. Haustoria may be of several forms, simple and nearly spherical, coiled, and branched in various ways. Most cytologists agree that there is a cellulose wall, or sheath, around the older haustoria. It is presumably formed by the host cell and suggests a weak mechanism of defense against the invading parasite, yet it does not prevent the diffusion of soluble food into the haustorium.

The haustorium commonly comes into contact with the nucleus of the host cell. In 23 of the 35 cases (host-parasite combinations) reported (Rice, 1927, 1935), habitual contact was observed between haustorium and nucleus. Two theories as to the meaning of this contact have been suggested. One is that the haustorium seeks out the region of the cell nucleus in order to facilitate the absorption of food from the cell. The second theory is that the action of the cell nucleus is defensive and that in some cases it may cause the death and degeneration of the haustorium.

In the case of *Synchytrium* (*Chrysophylyctis*) *endobioticum* the swarm cells migrate into close proximity with the nucleus of the host cell (Orton and Kern, 1919). In the majority of cases the nucleus is engulfed at the time or soon after the swarm cells unite to form the vegetative body of the parasite. The host nucleus disappears as the sporangia develop. The exact significance of this close relationship between parasite and host nucleus is not clear, but it apparently represents a more or less unique method of parasitism among the fungi.

It is generally believed that the balanced parasite causes harm to a susceptible host primarily through its demand upon food and water. There is little or no evidence that the protoplast is attacked chemically, although host cells may be killed by growth pressure. There are numerous reports of the disappearance of food in the region of haustoria. Butler (1918) reported that starch is absent in cells containing haustoria of *Sclerospora graminicola*, and at the time of sporulation the host cells collapse and die. The only abnormal effect observed by Mains (1917) on the cells of corn parasitized by *Puccinia sorghi* was the absence of starch in the bundle sheaths near the rust pustules. He interpreted this to mean that the parasite uses the food materials before they reach the bundle sheath where they are normally stored. Similar disappearance of starch in the host cells near infection by *Synchytrium endobioticum* has been reported by Orton and Kern (1919).

On the other hand, starch may accumulate in the infected tissues during early stages of development of rusts but usually disappears in later stages of development. This may be due to some disruption of the host's physiology. The physiological reactions of the host are known to involve

translocation of food, transpiration, respiration, and photosynthesis. Increased respiration has been reported for some hosts, while a decrease has been found for others. The rate of transpiration is usually increased. An early infection of orange rust on *Rubus* may even cause the formation of stomata on the upper epidermis, where they are normally lacking (Dodge, 1923).

The reactions of the chloroplasts of the host cells are believed to indicate the degree of adjustment between the host and parasite (Rice, 1935). Local chlorosis and streaking are common symptoms of a number of diseases caused by haustoria-forming parasites.

Thatcher (1939, 1942) has shown that certain obligate parasites cause an increase in permeability of the cell membranes of susceptible hosts. There was a decided reduction in osmotic pressure of the tissues of *Pisum* surrounding the rust hyphae. If the fungus is unable to bring about an increase in permeability so that it can obtain its necessary nutrients, the host is resistant. Thatcher found evidence that the plasma membranes of some resistant varieties of wheat may actually become less permeable as a reaction to the rust hyphae, and starvation of the fungus may result.

The change in permeability incited by the balanced parasites seems to be similar to the action of the destructive parasites, except for the matter of degree. Thatcher (1939) believes that parasitism in the rusts has become highly specialized, and the intensity of the effect on permeability of the cell membranes has been reduced. The substance involved is apparently a metabolic product of the fungus.

If the conditions afforded by a certain variety of host are favorable for the production of a comparatively large amount of toxin (assuming that this substance is a toxin), the host cells may be killed and the further development of the obligate parasite would be prevented. The sudden death of the host cells is the condition described by Stakman (1914) as *hypersensitiveness*. Hypersensitive hosts are highly resistant or immune to the pathogenic action of the obligate parasites. Stakman reported that, in varieties of wheat resistant to *Puccinia graminis tritici*, when the hyphae of the fungus come in contact with the host cells, the latter often show plasmolysis, disintegration, and finally death. After the death of a few surrounding cells the tips of the hyphae die. However, it was discovered that in some cases the hyphae may die before the host cells are killed. Stakman concluded that the problem of resistance to rusts is one of toxins of the parasite or the host, or both, and can best be explained by what he terms the "toxin or enzyme theory." Brooks (1948) also concluded that the death of the parasite is due to the lethal action of the host rather than to starvation.

Opponents of the toxin (or enzyme) theory of parasitism in the rusts

point out that no toxin has ever been demonstrated experimentally. Leach (1919) believes that each physiologic race of *Puccinia graminis* has its own characteristic food requirements which are met by only a few varieties of the host. According to this hypothesis, if a race of rust enters a host which does not meet its specific nutritional requirements, it dies, and enzymes which are injurious to the host cells are released. This hypothesis is supported by Wellensiek (1927) who worked with *Puccinia sorghi*.

While it is evident that the food supply varies with the varieties of the host, it seems equally possible that the difference in nutrients may have a more indirect effect in determining whether the fungus survives. Is it merely that the fungus starves if the host does not provide the appropriate food, or are the conditions in the host unfavorable for the production of certain metabolic products which are essential to the pathogenic actions of the fungus?

The type of host-parasite relationship found in *Phyllachora graminis* seems to be unique (Orton, 1924). This fungus apparently has the power of digesting and absorbing the tissues within the leaf, producing cavities in which the ascocarps later form. The hyphae bore their way through the cell walls of any of these tissues and, in doing so, absorb a portion of the wall. The parenchyma cells become disorganized, and their contents disintegrate. The vascular cells may be invaded and partially absorbed and become filled with hyphae. The most striking physiological characteristic of this fungus is its ability to absorb, replace, and engulf the tissues of the host leaf without any external evidence of necrosis of the host. This would seem to indicate the presence of highly active cellulolytic enzymes (and perhaps others) confined to the area near the fungus, without the presence of toxic substances, which would cause necrosis of the leaf tissue.

Actually, comparatively little is known about the activities which lead to parasitism, particularly of the balanced parasites. It is hoped that more planned experiments will be conducted in an attempt to gain more knowledge regarding the mode of parasitism of plant pathogens. Only by understanding the action of the parasite can we understand the basic facts underlying resistance and susceptibility.

Parasitism and symbiosis with insects. There are numerous reports of the parasitic and symbiotic relations of fungi with insects. For a more complete discussion than this text offers, see Leach (1940) and Steinhaus (1946). In many cases the relationship is solely to the advantage of the fungus (true parasitism), but a number of cases of mutualistic symbiosis do exist. The fungi may be disseminated by the insects which serve as their hosts. One can only speculate regarding the basic nutri-

tional requirements of these fungi, since very little is known. We may assume that rather specific nutritional needs, either for growth or for reproduction, are satisfied by the relation with insects.

Among the fungi parasitic on insects the genus *Entomophthora* is the most common. Various common species attack houseflies, grasshoppers, and other insects. A direct correlation between the amount of precipitation and the number of infections on houseflies was reported by Yeager (1939). *Massospora cicadina* infects the seventeen-year cicada and produces spores inside the abdomen. The posterior portion of the abdomen sloughs away, exposing the spores while the insect is still able to crawl about. This is apparently the chief method of dissemination of the spores. The mode and time of infection are unknown. Species of *Cordyceps* are common on pupae and larvae of certain insects. The fact that *C. militaris* produces abundant mycelial growth on a variety of synthetic media in the laboratory suggests the possibility that in nature this fungus may grow on other substrata, requiring the insect association only to fruit.

Fawcett (1910) described the use of a fungus, which he named *Aegerita webberi*, in controlling whitefly in the orange groves of Florida. *Aschersonia aleyrodis* has also been used for the same purpose. A chytrid, *Myrophagus ucrainicus*, is reported (Karling, 1948) as a parasite on scale insects in Bermuda, Louisiana, and Ontario. In severe outbreaks as many as 45 per cent of the female insects may be killed. It has also been transmitted to mealy bugs. Another group of fungi parasitic on insects is the Laboulbeniales. These are minute fungi developing almost entirely on the surface, sending short haustoria into the insect to obtain food.

The symbiotic relationship between *Septobasidium* and scale insects is interesting because of the high degree of specialization on the part of the fungus (Couch, 1938). The dependence of the fungus for its distribution upon the migrating young scale insects was previously mentioned in Chap. 15 under Spore Dissemination. The fungus forms a crust over scale insects, some of which are parasitized while others are not. The uninfected females give rise to young insects, which may remain under the fungus crust, crawl out through tunnels under the fungus, or crawl out over the sporulation surface of the fungus. The young insects are infected only by the bud cells from the basidiospores, never by the older fungus hyphae. The bud cells germinate on the surface of the insect and apparently enter principally through the natural openings. The fungus then produces coiled haustoria, which absorb food directly from the circulatory system of the insect, which in turn sucks its food from the host tree. Some infected insects may settle down on the bark, while others crawl under a nearby protective fungus colony. Only the former are responsible for distributing the fungus, while the latter are responsible

for the survival of the already formed fungus colonies. Connections are then made by anastomoses of the hyphae from the insect and the hyphae of the fungus crust under which the insect has come to rest. Thus, the fungus colony does not originate from one individual but from the aggregation of several individuals by anastomosis, or grafting. The parasitized insects are dwarfed and do not reproduce but may live as long as the uninfected insects. The fungus covers the insect's body but is in contact with it only by the numerous coiled haustoria. The insect in turn receives protection from severe weather conditions, from parasitic wasps, other insects, and birds. Certain species, particularly *S. burtii*, are easily cultivated on liquid or agar media. Couch believes that failure of the fungus to fruit in culture may be due to lack of proper nutrition, which is furnished by insects in nature, or to a complicated heterothallic condition of the fungus.

Fungi parasitic on other fungi. The parasitic habit of many of the chytrids upon other aquatic or semiaquatic fungi and algae is apparently quite common. A number of these genera are described and illustrated by Fitzpatrick (1930) and Karling (1942). Practically nothing is known regarding their nutritional requirements. There appear to be fewer filamentous fungi parasitic upon fungi. The mention of only a few of these will serve as examples. Species of *Piptocephalis*, *Chaetocladium*, and *Syncephalis* are parasitic on other Mucorales. A number of fungi are reported to be parasitic on members of the Agaricaceae and other higher fungi. Among these are species of *Spinellus*, *Mycogone*, *Hypomyces*, *Nyctalis*, and some Myxomycetes. A species of *Penicillium* is parasitic upon an *Aspergillus* (Thom and Raper, 1945). Of particular interest are the hyperparasites, fungi parasitic upon other parasitic fungi. *Cicinnobolus cesatii* is parasitic on the Erysiphales, and *Darluca filum* is parasitic on Uredinales. So far as is known, no study of the basic nutritional requirements of these fungi has been attempted.

Fungi parasitic on man and animals. Many of the fungi which cause disease of man and animals show distinct differences in morphology when grown under different conditions. The spore forms produced on artificial media may be quite different from those developed in the host. This may be a response to certain nutritional factors, to temperature differences, or to the presence of certain chemical substances which inhibit or limit the production of certain spore forms.

In general, the pathogens of man and animals have no unique nutritional requirements. Some are able to utilize inorganic nitrogen, while others are not; some are deficient for certain vitamins. Nickerson (1947) points out that there is no direct correlation between nutritional requirements and pathogenicity. In fact, there is little concrete evidence regarding the mode of action of these fungi in causing disease.

In the case of the dermatophytes, Nickerson has suggested that growth and sporulation in vivo may be affected by a chemical supplied to the hair and scales of the skin by diffusion from the adjacent resistant tissues. There is some evidence that resistance of skin to fungus infection may be influenced by the nutrition of the individual.

For more complete discussions of the fungus diseases of man and animals, the student is referred to Nickerson (1947), Wolf and Wolf (1947), Conant *et al.* (1944), Emmons (1940), and Dodge (1935). The pioneering work of Sabouraud (1910) should also be consulted.

Cultural characteristics and pathogenicity. Studies of numerous isolates of a given species or genus have indicated a possible correlation between pathogenicity and some particular cultural characteristic. The recognition of such relations and definite knowledge regarding them would be of great value to plant pathologists. One such study was made by Houston (1945) on 52 isolates of *Corticium solani* from various hosts. These isolates fell into three culture types based upon the characteristics of the mycelium and sclerotia. There was a certain degree of correlation between culture type and pathogenicity and symptoms on certain hosts. He concluded that the culture type of *C. solani* is more important in predicting the pathogenicity of an isolate than the host from which it was isolated.

During a study of the physiological characteristics and pathogenicity of 143 isolates of *Actinomyces*, mostly from scabbed potato tubers, it was found (Taylor and Decker, 1947) that certain isolates produced a dark ring of growth at the surface of separated milk. This characteristic was correlated perfectly with the ability to produce typical scab lesions on potato tubers. No attempt was made to explain the basic relation of these two apparently unrelated physiological characteristics.

RESISTANCE

Resistance is the ability of a host to prevent or oppose the entrance or subsequent growth and development of a parasite. It may be effective either before or after penetration of the host. A host which cannot successfully prevent such actions of the parasite is susceptible. Studies in the nature of resistance have been only partially enlightening, and in many cases the nature of disease resistance is still obscure. Some of the present theories are based on what might be termed "circumstantial evidence," such as a general correlation between resistance and some characteristic of the host. There is sufficient evidence, however, that it is dangerous to generalize about the nature of resistance. It seems likely that in many cases the cause of resistance may be specific, being common perhaps to but one or only a few host-parasite combinations.

The types of resistance may be placed for convenience into three

groups: (1) mechanical, the prevention of penetration or of unlimited spread by the structure of the host; (2) functional, the prevention of penetration by stomatal action of the host; (3) physiological, chemical action against the parasite or incompatible food relations. The relative importance of these factors is difficult to determine, but Butler (1918) states that physiological characters are much more important as a factor for resistance than the anatomical characters of the host.

Mechanical resistance might be considered as the first line of defense by the host. According to Melander and Craigie (1927) resistance of species of *Berberis* to infection by sporidia of *Puccinia graminis* is due to the thickness of the cuticle. *B. thunbergii*, which is immune, has a heavy layer of cutin, while in general the susceptible species have a thin layer. These conclusions were reached after anatomical studies and after using a mechanical device to measure the resistance of the epidermis to puncture. The thickness of the cuticle increases with age, as does the resistance to mechanical puncture and to infection. The same is true with the apple scab fungus and powdery mildew of barley; young leaves are susceptible but become more resistant with age.

Resistance in some cases is apparently due to layers of cork cells formed by the host in advance of the invading parasite. Varieties of flax resistant to wilt (*Fusarium lini*) and of cotton to black root rot (*Thielaviopsis basicola*) seem to be successful in walling off the parasite by forming such a layer of cork which it cannot penetrate. Varieties of potatoes resistant to scab (*Actinomyces scabies*) form cork more quickly when wounded than do susceptible varieties and are believed to owe their resistance to this characteristic. Thomas (1934) found that the newly formed layer of cork cells was penetrated by invading hyphae of *Armillaria mellea* and that the cork layer did not successfully stop the advance of this fungus. Brown (1936) states that there is some doubt as to whether the cork layer really functions at all or whether it is formed after the fungus has been stopped by some chemical means.

Lignified tissues offer more mechanical resistance than nonlignified cells. Certain varieties of wheat resistant to stem rust have a comparatively greater amount of sclerenchyma and a correspondingly lesser amount of collenchyma and parenchyma in the stem, as compared with susceptible varieties. The maturity of host tissue may be a factor in resistance, even though the tissue does not become lignified or suberized. Some of the systemic smut fungi in cereals are able to grow and penetrate the cell walls in meristematic tissue but are apparently unable to penetrate the cellulose walls of mature parenchyma cells. After infection in the embryo or seedling stage, the fungus must continue to grow in the growing tip of the shoot if it is to reach the flower parts. Conditions which favor slow growth and delay the maturity of the host favor the

fungus, while conditions which favor rapid maturity of the host cells may cause the fungus to be left behind in the mature tissues which it cannot penetrate.

Hart (1929) studied the nature of resistance of wheat varieties to stem rust and described a type of resistance that she terms *functional resistance*, which is dependent upon the stomatal movements of the host, and concluded that the parasite enters the wheat only through open stomata.

There has been frequent discussion regarding the importance of the acidity of the cell sap of the host and its effect upon resistance. The effects of cell-sap acidity may be threefold: (1) an increase in the hydrogen ions; (2) the toxicity of the organic acids; (3) the influence upon the chemical changes and the possible formation of toxic products by the host cells. In some cases these effects have not been satisfactorily distinguished. Numerous examples may be found in the literature in which resistance has been attributed to the acidity of the host or host part. Butler (1918) refers to investigations showing that the leaves of varieties of grape resistant to powdery mildew contain three to five times as much acid as the nonresistant varieties. He also showed that the red rot fungus of sugar cane, *Colletotrichum falcatum*, was present in infected canes from sowing time but usually did not develop severely until maturity of the canes. He attributed this to either the increase in sugar or the decrease in acid. The more acid lemons are less attacked by the fruit-rotting fungi. The amount of acid in the fruit, as indicated by chemical analysis, may be greater than the amount necessary to check the growth of the fungus in culture (Cook and Taubenhaus, 1911). A number of workers have considered cell-sap acidity as a possible cause for resistance of wheat to stem rust, but this factor now is believed to be of little importance. No correlation was found between resistance and acidity of the expressed sap (Hurd, 1924). Similarly, there was no correlation between resistance and hydrogen-ion values or the titratable-acid values of the juice of wheat plants resistant to *Ustilago tritici* (Tapke, 1929).

Some of the most complete experimental evidence showing the correlation between acidity and resistance is presented by Reddy (1933) for different inbred lines of corn in relation to *Basisporium gallarum*. Briefly, he found that when the pH of the cob was below 5.0, resistance to cob infection was high. Resistance was notably lower at high pH values. Table 64 gives a summary of some of Reddy's experiments. Reddy also believes that the influence of pH may explain why the seedlings, which are acid, are resistant to infection by *B. gallarum*, while the dry kernels, which are neutral or alkaline, are susceptible. On the basis of evidence previously discussed, it is likely that the pathogenic activities of certain enzymes produced by *B. gallarum* are inhibited in media having pH of 5.0 or less.

On the other hand, greater acidity of the cell sap may favor the development of some diseases. The susceptibility of certain varieties of grape to *Guignardia bidwellii* has been correlated with a greater amount of tartaric acid (Butler, 1918). This author points out that leaves are susceptible only while they are young and rich in tartaric acid.

TABLE 64. HYDROGEN-ION READINGS OF APPARENTLY HEALTHY COBS OF 75 INBRED LINES OF CORN AND INCIDENCE OF *Basisporium* EAR INFECTION FOLLOWING BOTH NATURAL AND ARTIFICIAL INOCULATION
(Reddy, *Iowa Agr. Expt. Sta. Research Bull.* 167, 1933.)

Cob pH	No. of inbreds in class interval	No. of inbreds infected	No. of ears observed	Ears infected, %
4.4–4.7	5	0	116	0
4.8	6	1	121	2.5
4.9–5.0	14	7	312	7.4
5.1–5.2	16	12	313	22.7
5.3–5.4	12	11	258	38.0
5.5–5.6	7	7	175	41.7
5.7–5.8	8	7	185	33.5
5.9–6.3	7	6	173	48.6

According to Smith *et al.* (1946), there is evidence that slight variation in pH may have a greater influence upon disease resistance of a plant than is generally believed. Such resistance is not due directly to the number of hydrogen ions. These authors state:

> The observed behavior of hydroquinone and catechol, representatives of the widely occurring ortho- and para-dihydroxyphenolic compounds, suggested that hydrogen ion differences also may influence toxicity by affecting the rate or extent of conversion to the more toxic quinones on invasion by pathogens or by other injury.

The possibility that the presence of the pathogen may alter the pH of the host cells, making it more favorable to extensive invasion, should not be overlooked. Apparently this situation exists in the relation of *Erwinia carnegieana* to its host, the giant cactus of Arizona. Boyle (1949) reported that the freshly expressed sap from uninfected plants gave pH readings of 5.0 to 5.5, while the healthy-appearing tissue from infected plants had pH values of 7.0 to 7.4, and the pH of discolored tissue not yet broken down was 8.7 to 9.0. These differences could not be attributed to genetic variation and were believed to be a result of the pathogen. The possibility that similar relations exist between fungus pathogens and their hosts seems to merit greater consideration than it has received.

That resistance is due to the presence of some toxic substance, perhaps an organic acid or some related compound, in the living host cell is one of the most popular theories. However, detailed proof of the effectiveness of such a compound, even though present in the plant, is often difficult to obtain. Cook and Taubenhaus (1911) list some organic acids in order of their toxicity as follows: tannic, gallic, malic, tartaric, and citric. They state that vegetable juices contain an enzyme which acts upon gallic acid to produce tannin or a tannin-like compound which is toxic to fungi. The amount of the enzyme decreases with maturity and ripening of the fruits (apples, pears, persimmons, etc.), which accordingly become more susceptible to rot fungi.

An outstanding example of chemical resistance is that described by Link and Walker (1933) for onion smudge caused by *Colletotrichum circinans*. The cell sap of the colored varieties (resistant) is much more toxic to the fungus than the cell sap of the white-skinned varieties (susceptible). Furthermore, the sap of the colored varieties contains catechol and protocatechuic acid in amounts that would account for the resistance of these varieties to the fungus. The action of volatile and nonvolatile antibiotics in the fleshy scales of the onion is believed to be a definite factor in relative resistance of onion varieties to *C. circinans*, *Aspergillus niger*, and *Botrytis allii* (Hatfield *et al.*, 1948). Reynolds (1931) explains resistance of flax varieties to *Fusarium lini* as being due to the higher amounts of glucosides, which upon hydrolysis yield hydrocyanic acid. Similarly, the resistance of species of *Solanum* to *Cladosporium fulvum* is believed to be due to the presence of higher amounts of solanine (Schmidt, 1933; cited by Brown, 1936). Rochlin (1933) believes that there is a direct connection between resistance of crucifers to clubroot and the amount of glucosides, which on fermentation give rise to pungent mustard oils. The isolation of 2-methoxy-1,4-naphthoquinone from *Impatiens balsamina* was reported by Little *et al.* (1948). This substance had a high antibiotic activity against several fungi and was nontoxic to tomato and bean plants. This may be an example of a naturally occurring antibiotic as a factor in resistance. Fontaine *et al.* (1947) suggest that tomatin may be a factor in the resistance of certain tomato varieties to *Fusarium lycopersici*.

An interesting theory of resistance to obligate parasites is presented by Dufrenoy (1936). He divides the hosts into three groups: (1) highly resistant, (2) moderately susceptible, (3) extremely susceptible. He believes that, when a fungus enters the highly resistant host, it kills the cells it penetrates and that the death of these cells alters the metabolism of the surrounding cells, so that their cell sap becomes rich in phenolic compounds, which prevents the further invasion by the pathogen. In the moderately susceptible host the host cells and their living contents

are so modified that they revert to the embryonic condition and may even divide. When the obligate parasite enters the extremely susceptible host, it causes so little disturbance that, at least in the first stages of infection, the metabolism of the host is affected but little or not at all.

Walker and Link (1935) caution against jumping at conclusions regarding the importance of phenolic compounds as factors in resistance. They point out that

> . . . the mere presence of phenolic substances in a host plant does not warrant the conclusion that they play a role in the resistance of that host to a given parasite or parasites. Toxic phenolic substances might be present in concentrations so low that their inhibitory effects are negligible, or they might also be present in concentrations that have a stimulative effect. When a phenolic substance with a specific toxicity toward a given organism is present in the host in an appropriate concentration, it may be regarded as a part of the disease resisting mechanism of that host.

The four fungi studied by Walker and Link (*Colletotrichum circinans*, *Gibberella saubinetii*, *Botrytis allii*, and *Aspergillus niger*) reacted quite differently to the various phenolic compounds. Protocatechuic acid inhibited *C. circinans* at 1/800 and retarded growth at 1/12,800, while it did not affect *A. niger* at 1/200. Colored onions containing this acid are resistant to *C. circinans* but quite susceptible to *A. niger*.

The immunity of monocotyledonous plants to *Phymatotrichum omnivorum* is due to certain unidentified toxic materials present in monocots but apparently absent in most or all dicots (Ezekiel and Fudge, 1938). Growth of the pathogen was prevented by the expressed juices from a number of monocots but not by juices of susceptible dicots. Ether fractions of monocot roots, or other underground parts, were highly potent against the pathogen, while similar extracts from susceptible dicot plants were uniformly nonpotent.

In some other highly parasitic fungi the action of the fungus causes the death of the surrounding cells, which then prevents the further spread of the parasite. Leach (1923) found that in a highly resistant variety of bean the hyphae of *Colletotrichum lindemuthianum* seldom attack more than one or two cells of the host. Both the host cells and the fungus hyphae then die, and the entire cell contents are stained a reddish brown. In less resistant varieties the parasite attacks more host cells, but sooner or later the mycelium disintegrates. Leach interprets this as "a nutritional phenomenon," which results in death of the fungus by starvation, and the products of autolysis then kill and stain the host cells.

It has been pointed out previously that certain fungi are able to penetrate some plants but are then unable to establish themselves (Stakman, 1914; Jones, 1919; Salmon, 1905). These plants may be either closely

related or unrelated to those which serve as the natural host of the fungus. In such cases the failure to cause disease may be due to unfavorable nutritional relations. The theory of a toxin-antitoxin, or toxin-countertoxin, between parasite and host has been suggested by a number of investigators (Ward, 1905; Marryat, 1907; Stakman, 1914; Allen, 1923; Walker, 1924) as a possible explanation for resistance to the rusts.

Cytological studies of *Puccinia graminis tritici* infections of both susceptible and resistant varieties of wheat were made by Allen (1923), who concluded that secretions from the fungus stimulate the metabolic activities of the susceptible host to produce more food, while in the resistant host the same secretions cause disintegration and death of the host cells near the infection. More distant cells may be stimulated. The haustoria usually die soon after the host cells are killed. Leach (1919) believes resistance to *P. graminis tritici* and *P. graminis tritici-compacti* can best be explained on the basis of specific food requirements of the parasite and specific food production by the host. It was suggested that the injury to the host cells might be due to an excess in amount of enzymes stimulated by a limited supply of food in resistant hosts. Similarly, Wellensiek (1927) believes that this theory best explains the resistance of corn to strains of *P. sorghi*. He suggests that the difference between susceptibility and resistance is of a quantitative nature and that the amount of the specific nutrient determines resistance or susceptibility.

Walker (1924) points out that resistance may be due to the action of a number of factors and that a clear understanding of resistance must be based upon a thorough understanding of parasitism. Walker's excellent discussion of the nature of disease resistance gives many references to the literature on this subject.

Host nutrition and its effect on the development and severity of disease is a relatively new phase of study, and much more investigation is necessary before general conclusions can be drawn. The fungi vary widely in their reactions to differences in host nutrition, the type of parasitism apparently being a determining factor. The action on the pathogen is believed to be principally indirectly through the effects of nutrition on the host, although it is possible that some of the vascular parasites may be directly affected by the nutrients which pass through the xylem. An increase in the salt concentration of the nutrient solution increased the development of clubroot, while it decreased the severity of cabbage yellows (Walker, 1946). The development of Fusarium wilt of tomato was affected in a way similar to cabbage yellows. More recently, Gallegly (1949) reported that the development of Verticillium wilt of tomato was reduced with a reduction in salt concentration of the balanced solution used to grow the tomato plants. Stakman (1914) and Ward (1902) came to the conclusion that deficiencies in nitrogen and phosphorus salts avail-

able to the host had no appreciable direct effect upon the resistance to rusts. A summary of the work on the effect of soil nutrients and environment upon resistance to disease has been presented by Wingard (1941).

The carbon metabolism of a plant likewise influences resistance to certain rusts. Waters (1926) found that urediospores of *Uromyces fabae* developed on detached leaves floating on 5 per cent sucrose solution in the dark, while none formed when leaves were floated on water. These observations were confirmed by Yarwood (1934) for rust and powdery mildew of clover. It follows that active carbon assimilation increases susceptibility of the host to the obligate parasites.

Although the environmental factors are of great importance in determining the resistance or susceptibility to a disease, their effects are usually upon the host and only indirectly upon the parasite. Abundant references on this subject can be found in the literature. The effect of temperature upon the metabolism and resistance of certain hosts may be illustrated by *Gibberella zeae* on wheat and corn (Dickson, 1923). Seedling infection of wheat was found to occur at high temperatures and of corn at low temperatures; *i.e.*, the temperatures unfavorable to host development. In the germination of wheat at low soil temperatures the starch of the endosperm is hydrolyzed more rapidly than the proteins, which results in abundant sugar but little available nitrogen for seedling growth. Thus, the cell walls are thickened and more resistant. At higher temperatures both starch and proteins are rapidly hydrolyzed; there is a greater supply of available nitrogen, and growth is more rapid. The cell walls remain longer in the pectic condition and are more susceptible to attack. In corn the situation is reversed. At high temperatures, which favor the corn, the cell walls develop more rapidly and are more resistant.

Sharvelle (1936) concludes that the resistance of flax to flax rust cannot be attributed to any single factor but probably results from a number of factors operating together. Doubtless, the same statement could be applied to many other diseases to which the nature of resistance is not well understood.

SUMMARY

Some of the different types of parasitism may be summarized as follows: (1) The parasite produces extracellular enzymes, particularly pectinase, which dissolves the middle lamellae of the host cells, allowing the cells to separate (rotting). This may or may not be accompanied by toxic substances but results in the death of the cells. The soluble food materials are then free to be absorbed by the fungus. The insoluble foods stored in the host cells may be digested by other extracellular enzymes. This type is illustrated by the rots of fruits and vegetables.

(2) The parasite may produce toxic materials or other substances which may be active at some distance from the fungus, but it usually does not cause the rotting of the tissue. This is illustrated by a number of wilt diseases and by some others. (3) The third type depends upon a congenial nutritional relationship between the parasite and the host cells. In susceptible hosts of this type there is little or no apparent effect upon the host cells. The resistant hosts may show a high degree of sensitivity to the parasite, which may result in the death of the invaded cells and starvation of the parasite. This type of parasitism is characteristic of the balanced parasites. The balanced parasite enters the susceptible host cell and establishes a compatible food relationship, absorbing the soluble nutrients elaborated by the host, without disturbing the metabolic activity of the host in the early stages. In this respect, the relationship of parasite and susceptible host represents the most highly specialized type of parasitism.

The destructive parasites, as a rule, are strong producers of toxins and exoenzymes, while the balanced parasites must be quite weak in this respect. In many host-parasite relations studied, there is a change in the permeability of the host cells surrounding the invading hyphae. This is believed to be a direct response to substances secreted by the parasite. Increased permeability would allow greater diffusion of water and nutrients from the host cells to the parasite. The metabolic products of the fungi involved in parasitism are for the most part undetermined, but they are known to include toxins, enzymes, and polysaccharides. Since the kind and amounts of such products are known to vary with the composition of the medium in the laboratory, it is believed that like variation may occur in different hosts in nature.

The basis of resistance to disease may be mechanical, functional, or physiological. Some of the known or proposed causes of physiological resistance are (1) cell-sap acidity; (2) toxic substances of the host; (3) inhibition of the activity of certain enzymes of the parasite by the host; (4) hypersensitiveness; (5) incompatible nutritional relationship; (6) decreased permeability of the cell membranes of the host, resulting in partial or complete starvation of the parasite; (7) a combination of various factors acting together.

The obligate parasites, principally the rusts, offer some challenging unsolved problems for the future students of parasitism. Probably the principal one involves the culturing of such fungi under controlled conditions on media of known composition. All of the many attempts to solve this problem have met with failure, yet few investigators doubt that it can be solved. The phenomenon of heteroecism among the rusts is of great interest from the standpoint of food relationships. For instance, we must either assume that the wheat and the barberry furnish the same

nutrients for *Puccinia graminis tritici*, and the white pine and *Ribes* for *Cronartium ribicola*, or that the nutrient requirements of the haploid mycelium are different from those of the diploid mycelium.

Much more investigation is needed to increase our knowledge of possible correlations between pathogenicity and metabolic products. This should lead to a better understanding of parasitism. The possible role of antibiotics occurring naturally in host plants as a factor in disease resistance has received some attention recently, but much more knowledge of this type is desired. Many of the problems of today may come nearer to solution with a clearer understanding of the enzyme systems of the parasitic fungi and the basic principles of specific enzymatic action.

REFERENCES

Allen, R. F.: Cytological studies of infection of Baart, Kanred and Mindum wheats by *Puccinia graminis tritici* forms III and XIX, *Jour. Agr. Research* **26**: 571–604, 1923.

Allen, R. F.: A cytological study of *Puccinia triticina* physiologic form 11 on Little Club Wheat, *Jour. Agr. Research* **33**: 201–222, 1926.

★Arthur, J. C., F. D. Kern, C. R. Orton, F. D. Fromme, H. S. Jackson, E. B. Mains, and G. R. Bisby: The Plant Rusts, John Wiley & Sons, Inc., New York, 1929.

Bessey, E. A.: A Textbook of Mycology, The Blakiston Company, Philadelphia, 1935.

Boyle, A. M.: Further studies of the bacterial necrosis of the giant cactus, *Phytopathology* **39**: 1029–1052, 1949.

★Brooks, F. T.: Host resistance to fungi, chiefly in relation to obligate parasites, *Proc. Roy. Soc.* (*London*), Ser. B, **135**: 180–186, 1948.

Brown, W.: Studies in the physiology of parasitism. I. The action of *Botrytis cinerea*, *Ann. Botany* **29**: 313–348, 1915.

Brown, W.: Studies in the physiology of parasitism. VIII. On the exosmosis of nutrient substances from the host tissues into the infection drop, *Ann. Botany* **36**: 101–119, 1922.

★Brown, W.: The physiology of the host-parasite relation, *Botan. Rev.* **2**: 236–281, 1936.

Brown, W.: Physiology of the facultative type of parasite, *Proc. Roy. Soc.* (*London*), Ser. B, **135**: 171–179, 1948.

Butler, E. J.: Immunity and disease in plants, *Agr. Jour. India*, Special Indian Sci. Congr. Issue, **1918**: 10–28.

Caldwell, R. M., and G. M. Stone: Relation of stomatal function of wheat to invasion and infection by leaf rust (*Puccinia triticina*), *Jour. Agr. Research* **52**: 917–932, 1936.

★Chona, B. L.: Studies in the physiology of parasitism. XIII. An analysis of factors underlying specialization of parasitism, with special reference to certain fungi parasitic on apple and potato, *Ann. Botany* **46**: 1033–1050, 1932.

Conant, N. F., D. S. Martin, D. T. Smith, R. D. Baker, and J. M. Callaway: Manual of Clinical Mycology, W. B. Saunders Company, Philadelphia, 1944.

Cook, M. T., and J. J. Taubenhaus: The relation of parasitic fungi to the contents of the cells of the host plants, *Delaware Agr. Expt. Sta. Bull.* 91, 1911.

Coucu, J. N.: The Genus *Septobasidium*, The University of North Carolina Press, Chapel Hill, 1938.

De Bary, A.: Ueber einige Sclerotinien und Sclerotien Krankheiten, *Botan. Ztg.* **44**: 377–474, 1886.

Dickson, J. G.: Influence of soil temperature and moisture on the development of the seedling-blight of wheat and corn caused by *Gibberella saubinettii*, *Jour. Agr. Research*. **23**: 837–870 1923.

Dimond, A. E.: Symptoms of Dutch elm disease reproduced by toxins of *Graphium ulmi* in culture, *Phytopathology* **37**: 7, 1947.

Dodge, B. O.: Effect of the orange-rusts of *Rubus* on the development and distribution of stomata, *Jour. Agr. Research* **25**: 495–500, 1923.

Dodge, C. W.: Medical Mycology, The C. V. Mosby Company, St. Louis, 1935.

Dufrenoy, J.: Cellular immunity, *Am. Jour. Botany* **23**: 70–79, 1936.

Emmons, C. W.: Medical mycology, *Botan. Rev.* **6**: 474–514, 1940.

Ezekiel, W. N., and J. F. Fudge: Studies on the cause of immunity of monocotyledonous plants to *Phymatotrichum* root rot, *Jour. Agr. Research* **56**: 773–786, 1938.

Fawcett, H. S.: An important entomogenous fungus, *Mycologia* **2**: 164–168, 1910.

★Feldman, A. W., N. E. Caroselli, and F. L. Howard: Physiology of toxin production by *Ceratostomella ulmi*, *Phytopathology* **40**: 341–354, 1950.

Fitzpatrick, H. M.: The Lower Fungi, Phycomycetes, McGraw-Hill Book Company, Inc., New York, 1930.

Fontaine, T. D., G. W. Irving, Jr., and S. P. Doolittle: Partial purification and properties of tomatin, an antibiotic agent from the tomato plant, *Arch. Biochem.* **12**: 395–404, 1947.

Gallegly, M. E.: Host nutrition in relation to development of *Verticillium* wilt of tomato, *Phytopathology* **39**: 7, 1949.

Gäumann, E.: Pflanzliche Infektionslehre, Verlag Birkhäuser, Basel, 1946. Trans. by W. B. Brierly as Principles of Plant Infection, Hafner Publishing Co., New York, 1950.

Gäumann, E., and O. Jaag: Die physiologischen Grundlagen des parasitogenen Welkens. II, III. *Ber. schweiz. botan. Ges.* **52**: 132–148, 227–241, 1947.

Hart, H.: Relation of stomatal behavior to stem-rust resistance in wheat, *Jour. Agr. Research* **39**: 929–948, 1929.

★Hatfield, W. C., J. C. Walker, and J. H. Owen: Antibiotic substances in onion in relation to disease resistance, *Jour. Agr. Research* **77**: 115–135, 1948.

Hawkins, L. A., and L. B. Harvey: Physiological study of the parasitism of *Pythium debaryanum* on potato tuber, *Jour. Agr. Research* **18**: 275–297, 1919.

Haymaker, H. H.: Relation of toxic excretory products from two strains of *Fusarium lycopersici* to tomato wilt, *Jour. Agr. Research* **36**: 697–719, 1928.

Higgins, B. B.: Physiology and parasitism of *Sclerotium rolfsii*, *Phytopathology* **17**: 417–448, 1927.

Hodgson, R., W. H. Peterson, and A. J. Riker: The toxicity of polysaccharides and other large molecules to tomato cuttings, *Phytopathology* **39**: 47–62, 1949.

Houston, B. R.: Culture types and pathogenicity of isolates of *Corticium solani*, *Phytopathology* **35**: 371–393, 1945.

Hurd, A. M.: The course of acidity changes during the growth period of wheat with special reference to stem-rust resistance, *Jour. Agr. Research* **27**: 725–735, 1924.

Jones, F. R.: The leaf-spot disease of alfalfa and red clover caused by the fungi *Pseudopeziza medicaginis* and *Pseudopeziza trifolii*, respectively, *U.S. Dept. Agr. Bull.* 759, 1919.

Karling, J. S.: Plasmodiophorales, published by the author, New York, 1942.

KARLING, J. S.: Chytridiosis of scale insects, *Am. Jour. Botany* **35**: 246–254, 1948.

KLOTZ, L. J.: Inhibition of enzymatic action as a possible factor in the resistance of plants to disease, *Science* **66**: 631–632, 1927.

KUNKEL, L. O.: A contribution to the life history of *Spongospora subterranea*, *Jour. Agr. Research* **4**: 265–278, 1915.

LEACH, J. G.: The parasitism of *Puccinia graminis tritici* Erikss. and Henn. and *Puccinia graminis tritici-compacti* Stak. and Piem., *Phytopathology* **9**: 59–88, 1919.

★LEACH, J. G.: The parasitism of *Colletotrichum lindemuthianum*, *Minn. Agr. Expt. Sta. Bull.* 14, 1923.

LEACH, J. G.: Insect Transmission of Plant Diseases, McGraw-Hill Book Company, Inc., New York, 1940.

LEE, A.: The toxic substance produced by the eye-spot fungus of sugar cane, *Helminthosporium sacchari*, *Plant Physiol.* **4**: 193–212, 1929.

★LINK, K. P., and J. C. WALKER: The isolation of catechol from pigmented onion scales and its significance in relation to disease resistance in onions, *Jour. Biol. Chem.* **100**: 379–383, 1933.

LITTLE, J. E., T. J. SPROSTON, and M. W. FOOTE: Isolation and antifungal action of naturally occurring 2-methoxy-1,4-naphthoquinone, *Jour. Biol. Chem.* **174**: 335–342, 1948.

MAINS, E. B.: The relations of some rusts to the physiology of their hosts, *Am. Jour. Botany* **4**: 179–220, 1917.

MARRYAT, D. C.: Notes on the infection and histology of two wheats immune to the attacks of *Puccinia glumarum*, yellow rust, *Jour. Agr. Sci.* **2**: 129–138, 1907.

MEEHAN, F., and H. E. MURPHY: Differential phytotoxicity of metabolic by-products of *Helminthosporium victoriae*, *Science* **106**: 270–271, 1947.

MELANDER, L. W., and J. H. CRAIGIE: Nature of resistance of *Berberis* spp. to *Puccinia graminis*, *Phytopathology* **17**: 95–114, 1927.

NICKERSON, W. J.: Biology of Pathogenic Fungi, Chronica Botanica Co., Waltham, 1947.

ORTON, C. R.: Studies in the morphology of the Ascomycetes. I. The stroma and the compound fructification of the Dothideaceae and other groups, *Mycologia* **16**: 49–95, 1924.

ORTON, C. R., and F. D. KERN: The potato wart disease, *Penna. Agr. Expt. Sta. Bull.* 156, 1919.

PEIRSON, S. O.: Studies of the growth physiology of *Phacidium infestans* Karst., *Physiologia Plantarum* **1**: 38–56, 1948.

PLATTNER, P. A., and N. CLAUSSON-KAAS: Ueber ein Welke erzeugendes Stoffwechselprodukt von *Fusarium lycopersici* Sacc., *Helv. Chim. Acta* **28**: 188–195, 1945.

PRASAD, N.: Variability of the cucurbit root-rot fungus, *Fusarium* (*Hypomyces*) *solani* f. *cucurbitae*, *Phytopathology* **39**: 133–141, 1949.

★REDDY, C. S.: Resistance of dent corn to *Basisporium gallarum* Moll., *Iowa Agr. Expt. Sta. Research Bull.* 167, 1933.

REYNOLDS, E. S.: Studies on the physiology of plant disease, *Ann. Missouri Botan. Garden* **18**: 57–95, 1931.

RICE, M. A.: The haustoria of certain rusts and the relation between host and pathogenes, *Bull. Torrey Botan. Club* **54**: 63–153, 1927.

★RICE, M. A.: The cytology of the host-parasite relations, *Botan. Rev.* **1**: 327–353, 1935.

ROCHLIN, E. J.: On the question of non-susceptibility of Cruciferae to *Plasmodiophora brassicae* Wor., *Bull. Plant Protection*, (*U.S.S.R.*), Ser. II, *Phytopathology*, Leningrad, 1933. (Abst. in *Rev. Applied Mycol.* **13**: 140–141, 1934.)

Rosen, H. R.: Efforts to determine the means by which the cotton wilt fungus, *Fusarium vasinfectum*, produces wilting, *Jour. Agr. Research* **33**: 1143–1162, 1926.

Sabouraud, R.: Maladies du cuir chevelu. Vol. III, Les Maladies cryptogamiques, Les Teignes, Masson et Cie, Paris, 1910.

Salmon, E. S.: On the stages of development reached by certain biological forms of *Erysiphe* in cases of non-infection, *New Phytologist* **4**: 217–222, 1905.

Schmidt, M.: Zur Entwicklungsphysiologie von *Cladosporium fulvum* und über die Widerstandfähigkeit von *Solanum racemigerum* gegen diesen Parasiten, *Planta* **20**: 407–439, 1933.

Sharvelle, E. J.: The nature of resistance of flax to *Melampsora lini*, *Jour. Agr. Research* **53**: 81–127, 1936.

★Smith, F. G., J. C. Walker, and W. J. Hooker: Effect of hydrogen-ion concentration on the toxicity to *Colletotrichum circinans* (Berk.) Vogl. of some carboxylic acids, phenols, and crucifer extracts, *Am. Jour. Botany* **33**: 351–356, 1946.

Smith, L. D. S.: Clostridia in gas gangrene, *Bact. Revs.* **13**: 233–254, 1949.

Stakman, E. C.: A study in cereal rusts. Physiological races, *Minn. Agr. Expt. Sta. Bull.* 138, 1914.

Steinhaus, E. A.: Insect Microbiology, Comstock Publishing Company, Inc., Ithaca. 1946.

Tapke, V. F.: Influence of varietal resistance, sap acidity and certain environmental factors on the occurrence of loose smut of wheat, *Jour. Agr. Research* **39**: 313–319, 1929.

Taylor, C. F., and P. Decker: A correlation between pathogenicity and cultural characteristics in the genus *Actinomyces*, *Phytopathology* **37**: 49–58, 1947.

Thatcher, F. S.: Osmotic and permeability studies in the nutrition of fungus parasites, *Am. Jour. Botany* **26**: 449–458, 1939.

★Thatcher, F. S.: Further studies of osmotic and permeability relations in parasitism, *Can. Jour. Research* **20**: 283–311, 1942.

Thom, C., and K. B. Raper: A Manual of the Aspergilli, The Williams & Wilkins Company, Baltimore, 1945.

Thomas, H. E.: Studies on *Armillaria mellea* (Vahl.) Quel., infection, parasitism and host resistance, *Jour. Agr. Research* **48**: 187–218, 1934.

Vasudeva, R. S.: Studies in the physiology of parasitism. XI. An analysis of the factors underlying specialism of parasitism, with special reference to the fungi *Botrytis allii* and *Monilia fructigena*, *Ann. Botany* **44**: 469–493, 1930.

Walker, J. C.: On the nature of disease resistance in plants, *Trans. Wisconsin Acad. Sci.* **21**: 225–247, 1924.

★Walker, J. C.: Soil management and plant nutrition in relation to disease development, *Soil Sci.* **61**: 47–54, 1946.

Walker, J. C., and K. P. Link: Toxicity of phenolic compounds to certain onion bulb parasites, *Botan. Gaz.* **96**: 468–484, 1935.

Ward, M.: On the relations between host and parasite in the bromes and their brown rust, *Puccinia dispersa* Erikss., *Ann. Botany* **16**: 233–315, 1902.

Ward, M.: Recent researches on the parasitism of fungi, *Ann. Botany* **19**: 1–54, 1905.

Waters, C. W.: The relations of bean rust grown on leaves in solution, *Papers Mich. Acad. Sci.* **5**: 163–177, 1926.

Wellensiek, S. J.: The nature of resistance in *Zea mays* L. to *Puccinia sorghi* Schw., *Phytopathology* **17**: 815–825, 1927.

★Wingard, S. A.: The nature of disease resistance in plants, *Botan. Rev.* **7**: 59–109, 1941.

Wolf, F. A., and F. T. Wolf: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

Woolley, D. W.: Strepogenin activity of seryl glycyl glutamic acid, *Jour. Biol. Chem.* **166**: 783–784, 1946.

Yarwood, C. E.: The comparative behavior of four clover-leaf parasites on excised leaves, *Phytopatholgoy* **24**: 797–806, 1934.

Yeager, C. C.: Empusa infections of the house-fly in relation to moisture conditions of northern Idaho, *Mycologia* **31**: 154–156, 1939.

CHAPTER 18

PHYSIOLOGICAL VARIATIONS AND INHERITANCE OF PHYSIOLOGICAL CHARACTERS

Variation in the results of experimental work with fungi is of frequent occurrence; it is perhaps even more frequent than uniformity. Different investigators conducting the same experiments with the same species of fungus have often failed to obtain the same results. Such variation may be attributed to (1) genetic differences in the strains or isolates used, (2) slight nutritional differences in the experiments, or (3) differences in the physical environment. Examples of the second and third groups of factors have been pointed out frequently in the earlier chapters. A brief discussion of the genetic differences involving physiological expression and the general mode of inheritance of these factors (in so far as they are known) will be given.

PHYSIOLOGICAL VARIATION

Variation in physiological behavior of different species of fungi has been noted in the preceding chapters. The present discussion emphasizes the physiological variation within a species, *i.e.*, between different isolates, strains, or races, which show little or no morphological difference.

Nutritional requirements. Variations in the nutritional requirements of different isolates of the same species are numerous. Differences in vitamin requirements or in carbon and nitrogen utilization may serve as examples.

Differences in deficiencies for one or more vitamins have been reported for different isolates of *Fusarium avenaceum* (Robbins and Ma, 1941), *Sclerotinia minor* (Barnett and Lilly, 1947), *Saccharomyces cerevisiae* (Leonian and Lilly, 1942; Burkholder and Moyer, 1942), *Sordaria fimicola* (Hawker, 1939; Barnett and Lilly, 1947*a*), and numerous others. For example, certain isolates of *Sordaria fimicola* from nature are totally deficient for biotin alone, while others are deficient for both biotin and thiamine. A somewhat different type of variation is reported by Thren (1941) for *Ustilago nuda*. The haploid mycelium showed no deficiency for vitamins, while the diploid mycelium required an external supply of thiamine or pyrimidine. The plus and minus strains were also found to differ in their nutritional requirements.

Different isolates of *Ustilago striiformis* have shown strikingly different responses to sources of carbon and nitrogen (Cheo, 1949). The isolates from bluegrass segregated into two groups based on mycelial type, "fragmenting" and "mycelial." The "fragmenting" type grew well only on media containing sucrose and organic nitrogen, while the "mycelial" type could utilize a number of sugars and nitrate nitrogen. Single-spore (haploid) isolates from the same fruit body of *Lenzites trabea* collected in nature varied nearly fourfold in their ability to synthesize thiamine (Lilly and Barnett, 1948).

Induced deficiencies for a number of vitamins and amino acids have been demonstrated by Beadle (1946) in mutants of *Neurospora* and by Bonner (1946) in mutants of *Penicillium*. The mutations were induced by exposure of spores of these fungi to ultraviolet and X-ray radiation. Mutants that showed deficiencies for thiamine and differences in nitrogen requirements were also reported for *Aspergillus terreus* (Thom and Raper, 1945). One mutant differed from most species of *Aspergillus* in its inability to utilize nitrate nitrogen. Fries (1948) describes spontaneous mutations of *Ophiostoma* which yield the same strains and in the same proportion as those induced by X rays. These results lead us to conclude that similar mutations are the principal cause of variation in the isolates obtained from nature.

Response to environment. Isolates of the same species frequently vary in their physiological responses to some environmental factors, among which are temperature and light. For example, isolates of *Phytophthora infestans* were found to vary in their resistance to high temperature (Martin, 1949). Of the eight isolates studied, four from Louisiana withstood exposure to 36°C. for 6 days, while three isolates from Minnesota were killed after 4 days and one isolate from New York was killed in less than 6 hr. at the same temperature. The presence of the high-temperature strain is believed to be responsible for the prevalence of late blight in Louisiana during the past few years. Houston (1945) found that, for one group of isolates of *Corticium solani*, the optimum temperature for growth was 24 to 25°C. and the maximum was 33°C. For two other groups the optimum and maximum temperatures were 28 to 29°C. and 40°C., respectively. The three groups also varied in growth rates.

Variation in response to light is illustrated by *Choanephora cucurbitarum*. This was indicated first by Wolf (1917) for two isolates. The isolate used by Christenberry (1938) produced conidia in continuous total darkness, while two isolates used in our laboratory failed to produce conidia in continuous darkness (Barnett and Lilly, 1950).

Metabolic products. Both qualitative and quantitative variations in the metabolic products of different isolates of the same species are com-

mon. Industries involved in the commercial production of alcohols, certain organic acids, and antibiotics are in constant search for higher yielding "strains" of the species in present use, as well as of other species of fungi. Such a search led to the discovery of *Penicillium chrysogenum* Q176 and its variants, which are high producers of penicillin. Brewer's yeast is said to grow in media with an alcohol content as high as 14 to 17 per cent, while the baker's yeast is checked in about 4 per cent alcohol (Wolf and Wolf, 1947). Both yeasts belong to the species *Saccharomyces cerevisiae*.

A different type of variation, apparently linked with sexuality, is reported in *Mucor racemosus* (Harris, 1948). Here, the production of an undetermined antibiotic seems to be confined to the minus strain. Variation in bioluminescence is reported for *Panus stypticus* (Macrae, 1942). The fruit bodies and mycelium of the strain found in North America are luminescent, while those found in Europe are not (Fig. 77). Variants of the same species commonly differ in pigment production (Christensen and Graham, 1934; Leonian, 1929). Mutants, or *saltants*, are commonly lighter in color than the parent type.

Sporulating ability. Many investigators have noted the spontaneous development of nonsporulating cultures or sectors from a sporulating mycelium. Variation in abundance of spores produced by different isolates from nature is also common. For example, some of the species which illustrate this variability are *Fusarium* spp., *Phytophthora* spp., *Phoma terrestris*, *Gibberella zeae*, *Glomerella cingulata*, *Lenzites trabea*, *Monilinia fructicola*, and *Ustilago striiformis*. Variations in fruit bodies of *Cyathus stercoreus* produced in culture are described by Brodie (1948). Variation in production of sclerotia has been observed in isolates of *Sclerotinia trifoliorum* by Kreitlow (1949) and of *S. sclerotiorum* in our laboratory.

Pathogenicity. Variability in the metabolic products such as enzymes and toxins and in the ability to establish compatible food relations with the host may be of great importance in determining pathogenicity. Das Gupta (1936) discusses the pathogenicity as well as other characteristics of "saltants." Such soil-inhabiting fungi as *Fusarium* spp. are notorious for their variability in pathogenicity within a species. Species of *Helminthosporium* (Christensen, 1922; Dickinson, 1932) and *Corticium solani* (Houston, 1945) are likewise highly variable. In the highly parasitic fungi, such as the smuts, rusts, and powdery mildews, there is a high degree of physiologic specialization of races.

The determination of physiologic races is based on infection types of several varieties or species of the host. Dickson (1947) reports the existance of 189 known physiologic races of *Puccinia graminis tritici* and 128 physiologic races of *P. rubigo-vera tritici*. Genetic studies indicate

that the physiologic races may vary in but a single gene and that they may arise by hybridization or by mutation.

There is abundant evidence that the haploid and diploid stages of some fungi may differ in pathogenicity. The haploid phase of a number of

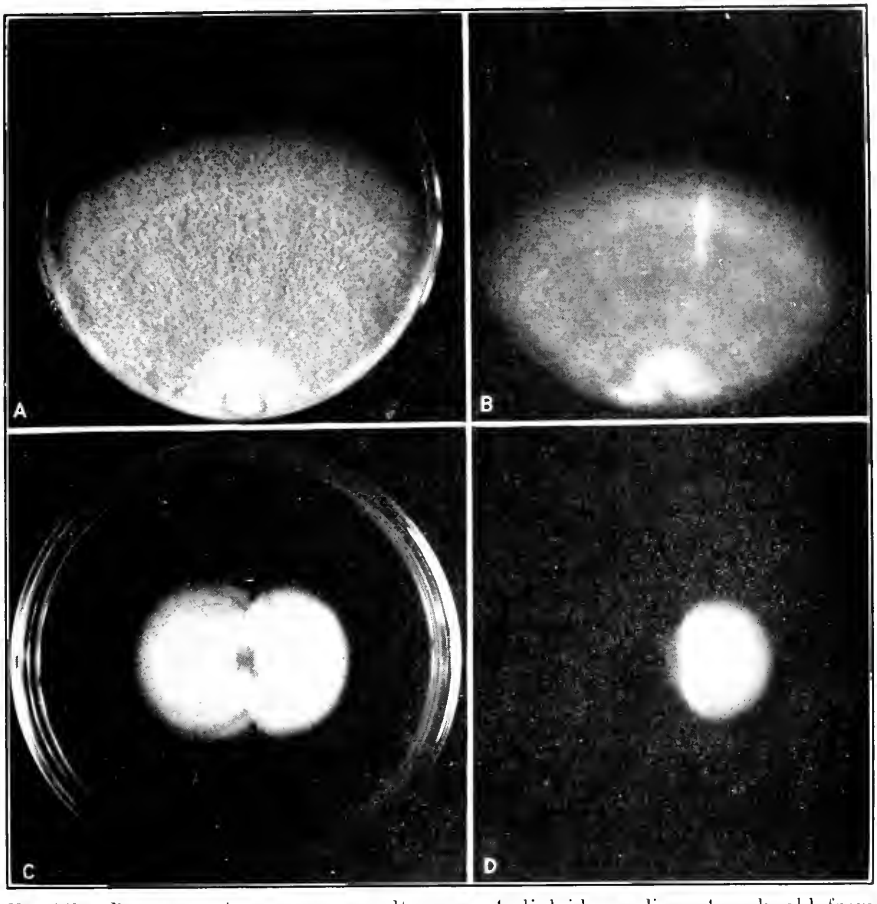


FIG. 77. *Panus stypticus* grown on malt agar. *A*, diploid mycelium, 4 weeks old, from a pairing between a haplont of the luminous American form and a haplont of the nonluminous European form, photographed by reflected light; *B*, the same culture as *A* photographed by its own light; *C*, a 2-weeks-old pairing between a nonluminous haplont, on the left, and a luminous haplont, on the right, photographed by reflected light; *D*, the same pairing as *C* photographed by its own light. (Courtesy of Macrae, *Can. Jour. Research*, Sec. C, **20**: 424, 1942.)

smuts is apparently unable to cause infection, while the diploid mycelium is pathogenic. Since the haploid and diploid mycelia of the heteroecious rusts parasitize different hosts, we must conclude that they also differ in pathogenicity.

INHERITANCE OF PHYSIOLOGICAL CHARACTERS

The genetics of the fungi has been, in general, a neglected study. Numerous papers have appeared on the sexuality of the fungi, particularly with regard to the various sexual or compatibility groups in the Basidiomycetes. The sexuality of the Mucorales has been studied to a lesser extent. Genetic studies of morphological characters have been decidedly fewer. Perhaps this is due to the failure to recognize definite morphological differences between individuals of opposite sex but of the same species. An equally great handicap to such studies lies in the difficulty in obtaining the perfect stage of many of the fungi which otherwise might be suitable. Studies dealing with inheritance of physiological characters (if sexuality is excluded) are comparatively few and recent.

The basis of inheritance. The physical basis of inheritance is the gene, located at a specific position on a certain chromosome. In mitosis the chromosomes and their genes divide, and half of each goes to each daughter nucleus. With the exception of parthenogenesis, all perfect stages of the fungi arise as a result of the union of two nuclei. These two nuclei may arise from the same haploid individual (homothallism) or from separate haploid thalli (heterothallism). The union of the two haploid nuclei, each with a single set of chromosomes, initiates the diploid nucleus, or the syncaryotic stage, in which the chromosomes are paired. The syncaryotic stage in fungi is usually short in duration, being followed closely by meiosis, which involves the separation of the two chromosomes (and genes) of each pair. Certain pairs of chromosomes may separate in the first division, while others separate in the second. Therefore, two successive nuclear divisions are necessary to complete the reduction of all pairs of chromosomes (and likewise all the pairs of genes). In the Ascomycetes and the Eubasidiomycetes karyogamy and meiosis occur in the same cell, the ascus and the basidium, respectively. In the smuts and rusts, meiosis typically takes place in a promycelium, while karyogamy occurs in the teliospore. When a single pair of genes is considered, half the haploid ascospores or basidiospores carry one gene and half carry the other gene.

Inheritance in the Ascomycetes. Some of the outstanding genetic work has been done by Dodge (1927, 1928) and others with *Neurospora*, by Ames (1934) and Dowding (1931) with *Pleurage anserina*, by Edgerton *et al.* (1945), Chilton and Wheeler (1949), and their associates with *Glomerella*, and by Lindegren (1945, 1948) and his colleagues with yeasts. Most of these investigations have been concerned primarily with sexual or morphological characters. The life cycle of *Neurospora* is shown diagrammatically in Fig. 78.

Beadle and his associates have contributed much to our knowledge

of the inheritance of physiological characters in the Ascomycetes. Beadle (1946) believed that, if the ability to synthesize a certain amino acid or growth factor were due to the action of a single gene, it should be possible to modify the gene in such a way that the fungus could no longer synthesize that compound. Previous work of other geneticists with corn, *Drosophila*, and other organisms had shown that exposure to X rays or ultraviolet radiation caused mutations by either destroying the gene or modifying it so that it could no longer function normally. Beadle found that exposure of conidia of *Neurospora crassa* and *N. sitophila* to X rays or ultraviolet rays had the similar effect of causing mutations that were

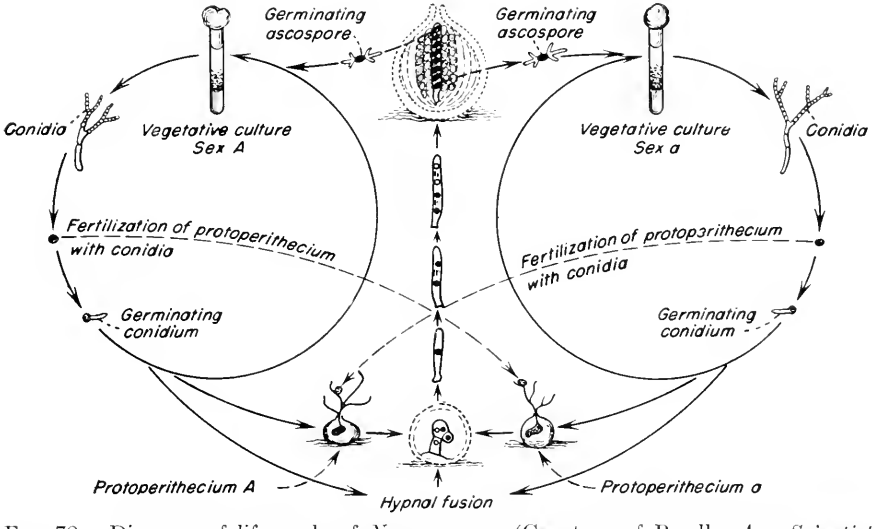


FIG. 78. Diagram of life cycle of *Neurospora*. (Courtesy of Beadle, *Am. Scientist* **34**: 36, 1946, and *Science in Progress*, 1947. Published by permission of the Society of the Sigma Xi.)

expressed in the inability of the fungus to synthesize vitamins, amino acids, and other essential metabolites.

The "wild type" of *Neurospora* is deficient for biotin but is self-sufficient for all other vitamins and for its necessary amino acids. The conidia were exposed to the ultraviolet rays of a Sterilamp for such a time that most of the spores were killed. The spores were then sown over the surface of agar plates in such concentration as to give individual "colonies," which were isolated and allowed to grow. When these were transferred to a minimal medium, containing sucrose, nitrate, mineral salts, and biotin, the failure of an isolate to grow showed an induced variation from the wild type in its capacity to synthesize essential metabolites.

The variant cultures were then selected and crossed with the wild

strain of the opposite sex to determine if the changes were inherited. The ascospores from these crosses were planted on both the minimal medium and a complete medium. The appearance of the deficiency in half of the cultures was considered as evidence that the change was of genetic origin; *i.e.*, a mutation. Transfers of the mutant to four different media (minimal, with amino acids, with vitamins, and complete) then determined whether the deficiency was for an amino acid or a vitamin. All media contained biotin. For a diagrammatic scheme of the procedure see Fig. 79.

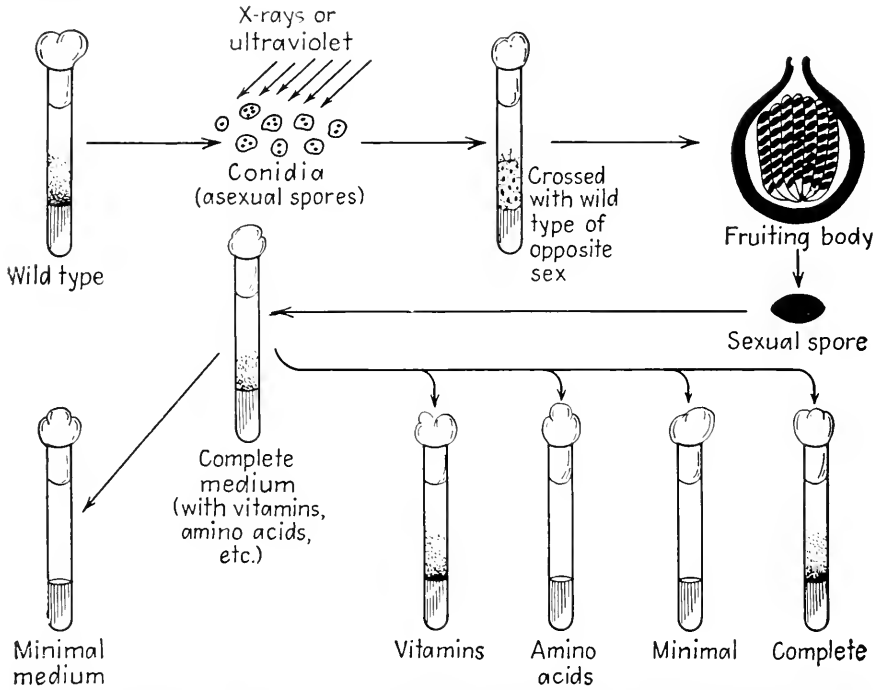


FIG. 79. Experimental procedure by which biochemical mutants are produced and detected in *Neurospora*. (Courtesy of Beadle, *Am. Scientist* **34**: 37, 1946, and *Science in Progress*, 1947. Published by permission of the Society of the Sigma Xi.)

The identification of the specific deficiency involved the growth of the mutant upon the minimal medium plus each of the amino acids and vitamins added singly. This procedure is shown in Fig. 80. Figure 81 shows the proof of inheritance of the deficiency for pantothenic acid.

Mutations involving the following vitamins have been described: thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, *p*-aminobenzoic acid, inositol, and choline. All mutants, as well as the wild type, are deficient for biotin. In addition, mutations have appeared which cannot synthesize the following amino acids: arginine, isoleucine,

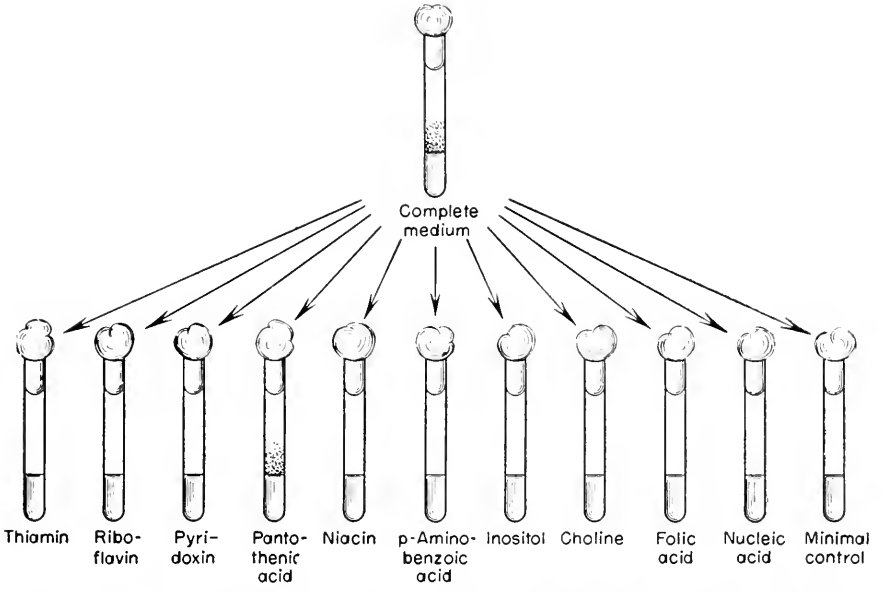


FIG. 80. Tests of mutant on individual vitamins or growth factors. Growth only on pantothenic acid indicates a single deficiency for this vitamin. (Courtesy of Beadle, *Am. Scientist* **34**: 39, 1946, and *Science in Progress*, 1947. Published by permission of the Society of the Sigma Xi.)

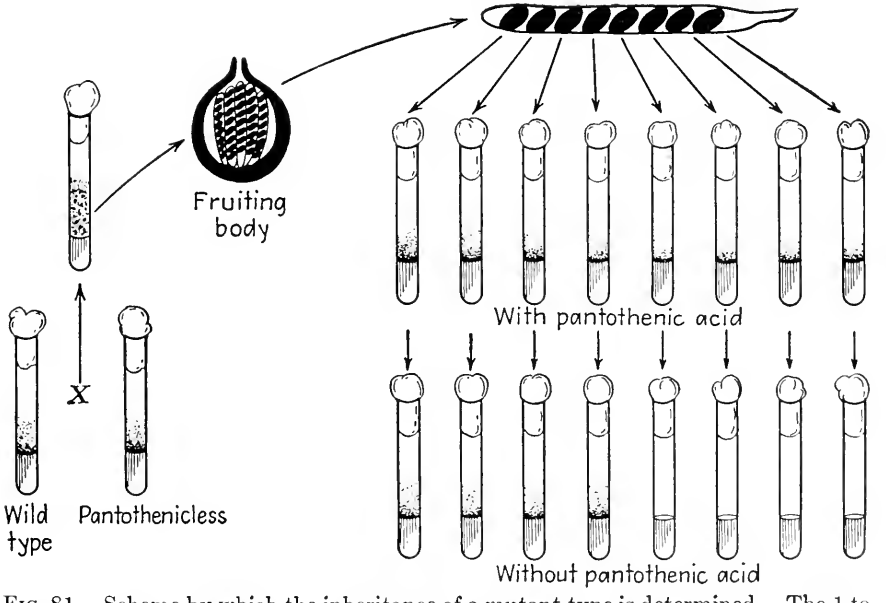


FIG. 81. Scheme by which the inheritance of a mutant type is determined. The 1 to 1 ratio with regard to need for pantothenic acid indicates simple Mendelian inheritance. (Courtesy of Beadle, *Am. Scientist* **34**: 40, 1946, and *Science in Progress*, 1947. Published by permission of the Society of the Sigma Xi.)

leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophane, and valine.

Beadle (1946) states:

> The list of compounds that *Neurospora* can be made to require from an external source is remarkably similar to a list of chemicals that we cannot make and require in our food supply. It is clear, therefore, that the substances the bread mold needs in its metabolism are very much the same as those we need. The difference is only an apparent one and results from the fact that bread mold makes them whereas we let some other organism make them for us. By inactivating the right genes the bread mold can be made very similar to man in its nutritional requirements.

Using a technique similar to that described above for Beadle's work, Bonner (1946) exposed conidia of *Penicillium notatum* and *P. chrysogenum* to X rays and ultraviolet rays. Of a total of 85,595 "strains" tested, 398 were found to be deficient in synthetic ability. Since these species of *Penicillium* are imperfect, the genetic basis for the biochemical changes cannot be proved, but it seems likely that this is the case, just as in *Neurospora*.

Inheritance in the Basidiomycetes. In the life cycle of the Basidiomycetes there exists a distinct diploid (dicaryotic) vegetative phase of extended duration, in which the cells usually contain two haploid conjugate nuclei. Buller (1941) cites the results of numerous experiments by himself and others to furnish ample proof that one nucleus of the conjugate haploid pair in the diploid mycelium may affect the expression of the other nucleus and thus exhibit dominance. This is true for physiological as well as morphological characters. Experimental evidence indicates that the genetic behavior of a cell containing two conjugate haploid nuclei is similar to that of the diploid nucleus, if the two were fused. Buller believes that the term "diploid cell" can apply equally well to a cell containing two conjugate haploid nuclei and to a cell containing one diploid nucleus. He prefers to use the terms "haploid" and "diploid" in describing mycelium or cells to the terms "monocaryotic" and "dicaryotic" which are also in use. We prefer to follow Buller in the use of these terms.

In the higher Basidiomycetes, principally the Agaricales, genetic studies have been chiefly limited to the inheritance of sex factors or compatibility factors. Fewer studies have dealt with the more strictly physiological characters. In a heterothallic species, two compatible haploid mycelia unite to initiate the diploid mycelium, which in many species is recognized by the presence of clamp connections. Usually, the formation of the diploid mycelium is a prerequisite to the production of fruit bodies.

Exidia, in the Tremellales, will serve as an example of the higher Basidiomycetes. In four species studied (Barnett, 1937) the single-

spore haploid cultures from the same fruit body fell into two compatibility groups. Such a condition is described as *bipolar*. Diploid mycelium was formed only when two haploid mycelia of different compatibility groups were paired. If *A* and *a* represent the genes for compatibility, the combination of *Aa* would be necessary for the formation of diploid mycelium. *AA* and *aa* would be incompatible.

While the single-spore cultures of a single fruit body of *Exidia glandulosa* give rise to two compatibility groups *A* and *a*, a second fruit body collected at some distance away may give rise to haploid mycelia which apparently fall into the same groups (*A* and *a*). Yet we may find that all the haploid mycelia of the first fruit body are compatible with all the haploid mycelia of the second fruit body. In other words, the two groups of the second fruit body are slightly different from the two groups of the first fruit body. It is, therefore, likely that numerous compatibility groups exist, even though only two occur in any one fruit body. Compatibility, in this case, is apparently determined by multiple alleles. The existence of geographic races has been described for a number of fungi by Buller (1941) and others.

A somewhat different situation exists in *Collybia velutipes* and a number of other species. It has been found that each spore on a basidium may differ in its compatibility factors. Compatibility in this case is determined by two pairs of genes on different chromosomes. These groups are usually designated as *AB*, *Ab*, *aB*, and *ab*. The combination of *AaBb* is then necessary for compatibility and formation of diploid mycelium.

Compatibility in itself does not necessarily indicate that fertile fruit bodies will be formed. For instance, some of the single-spore isolates of *Lenzites trabea* were found to produce fertile fruit bodies, while other cultures failed to do so (Barnett and Lilly, 1949). By pairing compatible fruiting isolates and also the compatible nonfruiting isolates, it was possible to establish a correlation between the fertility of the diploid mycelia with that of the haploid "parents." It seems probable, therefore, that the ability to produce fertile fruit bodies has a genetic basis, in addition to that of compatibility.

In *Schizophyllum commune* the ability to produce normal fruit bodies is dominant over the formation of abnormal, knot-like fruit bodies (Zatler, in Buller, 1941). If *G* represents the factor for normal fruit bodies and *g* the factor for knot-like fruit bodies, the results could be expressed as follows: *G* crossed with *G* or *G* crossed with *g* gives normal fruit bodies, while *g* crossed with *g* gives knot-like fruit bodies. Zatler also showed that in *Collybia velutipes* inheritance of pigmentation of his cultures was due to two genes located on different chromosomes. A combination of the two dominant factors in the haploid mycelium resulted in a deep

brown color; one dominant and one recessive factor gave lighter shades of brown; while a combination of the two recessive factors gave pure white mycelium.

The normal haplont of *Peniophora allescheri* is reported (Nobles, 1935) as slow-growing with scant mycelium bearing conidia. A mutant grew rapidly with abundant mycelium but bore no conidia. The combination of normal haplont and mutant haplont yielded diploid mycelium which grew rapidly and abundantly and produced conidia. Thus, rapid growth and conidial production were dominant over slow growth and nonproduction of conidia.

Bioluminescence of the North American race of *Panus stypticus* was found (Macrae, 1942) to be dominant over nonluminescence of the European race when the two haplonts were crossed (Fig. 77).

The single-spore isolates from a single fruit body of *Lenzites trabea* were found (Lilly and Barnett, 1948) to vary nearly fourfold in their ability to synthesize thiamine. When a haplont of low synthetic ability was crossed with one of high synthetic ability, no definite evidence of dominance was observed. By making other types of crosses it was found that, in general, the synthetic ability of the F_1 haplonts was similar to that of the "parent" haplonts. Yet, when the "parent" haplonts differed widely in synthetic ability, the F_1 haplonts did not segregate into the 1 to 1 ratio, as would be the case if inheritance were due to a single gene. Single-spore cultures from haploid fruit bodies produced by certain haplonts were more uniform in their ability to synthesize thiamine than were single-spore cultures from diploid fruit bodies of known origin. It was concluded that the ability to synthesize thiamine by *L. trabea* is genetically controlled, and that the mode of inheritance is complex and not due to a single gene.

The smuts have received much attention in genetic studies by Stakman, Christensen, and their associates at the University of Minnesota. Such characters as sex, pigmentation, pathogenicity, and morphological features of the mycelium and spores have been included in the study. Little is known regarding the factors governing the more strictly physiological or nutritional processes. An excellent review of the genetics of the smuts is given by Christensen and Rodenhiser (1940). These authors discuss the work of Goldschmidt, who found that the diploid mycelium derived from two haplonts of different races of *Ustilago violaceae* was able to attack the hosts which were susceptible to each parent race. This indicates that the diplont contained the combined pathogenic characters of the two haplonts. Hanna (1932) made an interspecific cross between *Tilletia levis*, with smooth spores and an odor of trimethylamine, and *T. tritici*, which had rough spores and no odor of trimethylamine. The F_1 "chlamydospores" had smooth walls and emitted an odor of trimethylamine, showing that both characters were dominant.

Numerous articles on the inheritance of the rusts may be cited. Among the characters commonly studied are color of urediospores and pathogenicity. One striking example of inheritance of pathogenicity of races of *Puccinia graminis tritici* is reported by Johnson and Newton (1940). Using pathogenically homozygous mycelia of race 9 and race 36, it was found that Kanred wheat was not attacked by race 9 but was highly susceptible to race 36. Urediospores were obtained from a hybrid of these two races and were sown on different varieties of wheat. No infection occurred on Kanred, indicating that the nonpathogenicity of race 9 was dominant over the pathogenicity of race 36. In the F_2 urediospores the pathogenicity to Kanred wheat segregated in a 1 to 3 ratio, indicating true Mendelian inheritance.

The basis of variation in the imperfect fungi. Any change or variation in the imperfect fungi may be either temporary or permanent. Temporary variations do not involve gene changes, while the permanent variations are believed to have their basis in the gene, or at least in the nucleus. The Mendelian inheritance of these variations cannot be proved in those fungi with no sexual stage.

Most investigators studying the permanent variations which arise in culture or which are recognized in different isolates of many of the imperfect fungi would explain the origin of these variants as mutations. For example, Dickinson (1932) studied "saltation" in the genera *Fusarium* and *Helminthosporium* and noted frequent anastomoses between hyphae of different "saltants." This author discussed the possibility of cytoplasmic inheritance but concluded that the permanent variations were due to actual mutations.

Hansen (1938) would explain many such variations in the imperfect fungi on a different basis and presents abundant evidence to substantiate his argument. Only the essential features of Hansen's "dual phenomenon" will be presented below. The conidia and mycelial cells of many of the imperfect fungi contain two or more nuclei. These nuclei may not all be alike. Considering the nucleus rather than the cell as the basic unit of the individual, an isolate may be composed of two culturally distinct individuals. This condition is referred to as the *dual phenomenon*. A heterocaryotic fungus, when single-spored, would give rise to homotypes of each of the individuals and the heterotype like the parent isolate. One homotype is characterized by abundant mycelium and few or no conidia and is called the *M* (mycelial) type. The other is characterized by abundant conidia and often a lesser amount of mycelium and is called the *C* (conidial) type. The heterotype is, in general, intermediate between the *M* and *C* types and is called the *MC* type. Cultures of the *M* and *C* types give only the parent type when single-spored. The frequency with which the dual phenomenon is encountered in the imperfect fungi suggests that this is the natural condition for many fungi.

In a later paper, Hansen and Snyder (1943) state that the change from the *C* to the *M* type is a true mutation. In *Hypomyces solani* f. *cucurbitae* this change also involved the change from the hermaphroditic phase to the unisexual male phase. They conclude that the *M* and *C* genes are alleles and are inherited independently of the factors for compatibility. On the contrary, Robbins and McVeigh (1949) have presented evidence that variants of *Trichophyton mentagrophytes* arise as mutations and that the dual phenomenon does not exist in this fungus.

Nutritional adaptations. Most of our present knowledge regarding nutritional adaptations has come from experiments with yeasts, and to a lesser extent with *Neurospora* and bacteria. Leonian and Lilly (1942) were able to "train" eight strains of *Saccharomyces cerevisiae* so that they grew without the addition of one or more of the vitamins which they formerly required. The technique employed consisted in increasing the amount of initial inoculum, prolongation of the incubation period, and repeated subculturing on media deficient for one of the necessary vitamins. Reversions occurred in most of the strains after being cultured continuously on media containing all the vitamins. Such adaptations as these may or may not involve gene changes.

Lindegren and his associates have written numerous articles on the genetics and adaptations of yeasts. From over 400 isolates of *Saccharomyces cerevisiae*, Skoog and Lindegren (1947) found 12 which could not utilize glucose. Eleven of these isolates reverted to glucose utilization within a period of a few days. One isolate remained glucose-negative for a period of 3 months when grown on lactate medium. They believe that the reversion to glucose utilization involves more than a single-step change.

Spiegelman (1950) points out that "a basic assumption of modern biology is that genes function by controlling enzyme synthesis. From this point of view it is obvious that enzymatic adaptation has profound implications for one of the central themes of biological thinking." This does not mean that the presence of the gene is always accompanied by the presence of the enzyme in the cell, but merely that the potentialities for the production of the enzyme are present. The synthesis of the specific enzyme, as well as its subsequent activity, depends upon other factors, a major one being the type of substrate. From numerous experiments it is evident that the specific enzyme either is produced, or becomes detectable, only when its corresponding substrate is present in the medium. It appears, however, that the specific enzyme may be formed even when the corresponding substrate is not present but that, under such conditions, the adaptive enzymes are usually not detected. In the course of a "long-term adaptation," there is not only synthesis of the specific enzyme but also an increase in the rate of enzyme formation. In

this respect, Spiegelman states, "In particular, the rate of formation of a given enzyme is an autocatalytic function of the amount of that particular enzyme present in the cytoplasm."

Ryan (1946) found that certain adaptations of the "prolineless" and "thiamineless" mutants of *Neurospora* are not inherited. For example, the "thiamineless" mutant may not grow for several days after being placed on a thiamine-free agar medium and may finally begin to grow. This is explained on the basis of adaptive enzyme formation. For further discussion of adaptive enzymes, see Chap. 4. For a more complete discussion of cytoplasmic inheritance and adaptive enzymes in yeast, the reader is referred to Lindegren (1945, 1949) and Spiegelman (1950).

Back mutations. The mutations studied by Beadle and others were those involving deficiencies for growth factors which the wild type was able to synthesize. It has been shown that, under certain conditions, there may be a reversion from the deficient type to the wild type. This may be due to a noninherited condition (an adaptation), such as that described above, or it may involve a gene change (a back mutation). Ryan (1946) has discussed at some length the topic of back mutation and adaptation in certain organisms. Only a few of his ideas will be presented here, omitting the detailed results of his experiments. He points out that the change from a deficient to a self-sufficient habit for growth factors may be induced experimentally in both the fungi and the bacteria. In some cases these nutritional changes are inherited, indicating gene changes. In the case of the "leucineless" mutant of *Neurospora* the adaptation back to the autotrophic habit was determined to be due to a reverse mutation of the leucineless factor to the wild type. Ryan believes that the ultraviolet rays, in causing the original "leucineless" mutation, caused a change in the wild-type gene so that it still retained the ability to reproduce but was unable to act in the synthesis of leucine. He found that the "adaptation frequency" varied inversely with the amount of leucine present in the medium. This theory assumes merely the inactivation and reactivation rather than the destruction of a gene by the ultraviolet rays.

Lindegren (1949) found that a mutation from pantothenate deficiency to pantothenate independence was at a different locus, and that the synthesis of pantothenate by the mutant was by a different route than in the original wild type.

Giles and Lederberg (1948) have recently studied the effects of various mutagenic agents in inducing adaptations (reversions) of deficient mutants of *Neurospora crassa*. They found that the frequency of adaptation of certain mutants may be greatly increased by ultraviolet radiation. This was true with the "inositolless," "cholineless," "methionineless," and "riboflavinless" mutants. The "pantothenicless" mutants remained

unchanged by the same treatment. Indications are that these changes represent mutations to the wild type. These adaptations may also be initiated by X rays, nitrogen mustard, and radiophosphorus.

Chemically induced mutations. Nitrogen and sulfur mustard gases have been used to induce mutations in various fungi. The method of treatment is simple and consists in exposing spores or mycelium to a buffered solution of the chemical for 30 min. or longer. The spores or mycelium are then washed and plated out. The methods used in detecting mutants are then the same as when X rays or ultraviolet irradiations are used. Treatment of young conidia or germinating conidia of *Neurospora crassa* with nitrogen mustard produced more mutants than treatment of old or ungerminated conidia (McElroy *et al.*, 1947). The technique of using the vapor of mustard gas to induce mutation is described by Hockenhull (1948). Mustard gas in a buffered solution (pH 6.9 to 7.0) was used by Hockenhull (1949) to produce mutants of *Aspergillus nidulans.*

The mustard gases, in common with nitrous acid, react with proteins. For example, casein which has been treated with mustard gas no longer supports the growth of the chick or rat. This is due to the inactivation of certain essential amino acids (Kinsey and Grant, 1946).

Mutation-inducing chemicals may be encountered by fungi under natural conditions. It was shown that toxic metabolic products of *Bacillus mesentericus* affected the production of mutants by certain strains of *Helminthosporium sativum* (Christensen and Davis, 1940). The filtrate of *B. mesentericus* cultures induced sectoring of *H. sativum.* These mutants differed from the parent in morphology, pathogenicity, and other physiological characters.

Among the chemical compounds which induce mutations in fungi, the action of nitrous acid has been especially studied (Thom and Steinberg, 1939; Steinberg and Thom, 1940, 1942). Mutants of *Aspergillus niger, A. amstelodami, A. varicolor, A. fumigatus, A. fischeri, A. flavus, A. alliaceus,* and *A. nidulans* were produced with ease by growing fungi on mannitol-nitrite medium. Some of these mutants were stable in culture for over 20 years. In addition to morphological changes, these nitrous acid–induced mutants were characterized by reduced ability to sporulate and other physiological changes, especially ability to utilize certain amino acids and a reduced rate of growth. It was postulated that nitrous acid reacted with free amino groups of the proteins of the genes. Evidence supporting this hypothesis was obtained when it was found that other chemicals which also react with free amino groups (ninhydrin, chloramine-T, potassium iodide, and hexamethylenetetramine) induced similar mutations in *A. niger.*

Certain amino acids, when added to the medium on which these

mutants were cultured, induced partial or complete reversion to the morphology of the parent type. Of the single amino acids tested, only lysine, cystine, β-phenyl-β-alanine, threonine, and valine induced complete reversion with the mutant of *A. niger* studied. Nicotinic acid, lysine, and valine in combination gave the best results. Complete reversion of a mutant of *A. amstelodami* was obtained only with a mixture of lysine and threonine.

It should be pointed out that, although the mutants of *Aspergillus* have been apparently stable for a long period of time, proof of the inheritance of these characters by crossing the "mutants" with the parent type is not available.

SUMMARY

Some knowledge of physiological variation and of the inheritance of the underlying factors is of great importance to the investigator who cultures fungi. The plant pathologist is greatly interested in knowing the stability of the pathogenicity of the plant pathogenic fungi and in learning whether the variations which he finds are apt to be permanent or only temporary. Considerable effort has been made toward an understanding of the genetics of the smuts, the rusts, and the yeasts. A few other fungi, such as *Neurospora*, *Glomerella*, and *Phycomyces*, have received attention because they are particularly adapted to genetic studies. However, the work up to the present leaves much to be desired regarding the relation between genetics and physiology.

The genetics of sex and compatibility has been most frequently studied. It is only natural that such is the case, for much of the understanding of a fungus depends upon the completion of its sexual life cycle and a clear knowledge of the various stages. Studies on the inheritance of morphological characters are represented by numerous isolated reports on characters more or less clearly defined. The reasons for the limited number of investigations on the genetics of physiological characters are numerous. Perhaps the principal reason is our meager knowledge of the intricate physiological processes of the fungi. The difficulty in obtaining strains of the different sexes of suitable fungi which have clear-cut physiological differences is great. The actual difficulty in carrying out the physiological tests has no doubt discouraged work along this line.

Studies on the inheritance of induced deficiencies for a number of vitamins and amino acids have indicated that such deficiencies may be inherited in a simple Mendelian fashion. On the other hand, the inheritance of partial thiamine deficiency in *Lenzites trabea* is complex and does not follow the simple Mendelian pattern. Studies of the yeasts have indicated that some physiological characters are inherited through, or influenced by, the cytoplasm. This possibility should not be overlooked in the filamentous fungi, where anastomoses of hyphae are common.

The basis for permanent variation in imperfect fungi (as well as in other groups) is believed by many to be true mutation. There is circumstantial evidence that many such sudden changes are mutations, but the inheritance of such a change in the imperfect fungi cannot be proved. Other variations in the imperfect fungi are explained on the basis of heterocaryosis. Separation of such nuclei into different spores would result in the segregation into different mycelial or sporulating types. Thus, the dual phenomenon would explain many of the variations in the imperfect fungi.

The type of variation known as physiological specialization is of practical interest and importance among the pathogenic fungi. Pathogenicity on a number of varieties of hosts offers a rigorous test to distinguish races which differ perhaps only slightly in their physiology. The future of the work dealing with physiology and genetics of the fungi lies principally in the hands of competent, well-trained investigators who may be interested enough to spend much time and effort in this narrow field of investigation. Many problems in this field are waiting to be solved.

REFERENCES

Ames, L. M.: Hermaphroditism involving self-sterility and cross-fertility in the ascomycete *Pleurage anserina*, *Mycologia* **26**: 392–414, 1934.

Barnett, H. L.: Studies in the sexuality of the Heterobasidiae, *Mycologia* **29**: 626–649, 1937.

Barnett, H. L., and V. G. Lilly: Vitamin deficiencies in the Sclerotiniaceae, *Phytopathology* **37**: 2, 1947.

Barnett, H. L., and V. G. Lilly: The effects of biotin upon the formation and development of perithecia, asci and ascospores by *Sordaria fimicola*, *Am. Jour. Botany* **34**: 196–204, 1947*a*.

Barnett, H. L., and V. G. Lilly: The production of haploid and diploid fruit bodies of *Lenzites trabea* in culture, *Proc. West Va. Acad. Sci.* **19**: 34–39, 1949.

Barnett, H. L., and V. G. Lilly: The influence of nutritional and environmental factors upon asexual reproduction of *Choanephora cucurbitarum* in culture, *Phytopathology* **40**: 80–89, 1950.

★Beadle, G. W.: Genes and the chemistry of the organism, *Am. Scientist* **34**: 31–53, 1946.

★Bonner, D.: Production of biochemical mutants in *Penicillium*, *Am. Jour. Botany* **33**: 788–791, 1946.

Brodie, H. J.: Variation in fruit bodies of *Cyathus stercoreus* produced in culture, *Mycologia* **40**: 614–626, 1948.

★Buller, A. H. R.: The diploid cell and the diploidization process in plants and animals, with special reference to the higher fungi. I, II. *Botan. Rev.* **7**: 335–431, 1941.

Burkholder, P. R., and D. Moyer: Vitamin deficiencies of fifty yeasts and molds, *Bull. Torrey Botan. Club* **70**: 372–377, 1942.

Cheo, P. C.: Stripe smut of blue grass (*Ustilago striiformis* forma *poae-pratensis*): Spore germination, artificial inoculation, pathological histology, and growth in artificial media, thesis, West Virginia University, 1949.

CHILTON, S. J. P., and H. E. WHEELER: Genetics of Glomerella. VI. Linkage, *Am. Jour. Botany* **36**: 270–276, 1949.

CHRISTENBERRY, G. A.: A study of the effect of light of various periods and wave lengths on the growth and asexual reproduction of *Choanephora cucurbitarum* (Berk. and Rav.) Thaxter., *Jour. Elisha Mitchell Sci. Soc.* **54**: 297–310, 1938.

CHRISTENSEN, J. J.: Studies on the parasitism of *Helminthosporium sativum*, *Minn. Agr. Expt. Sta. Bull.* 11, 1922.

CHRISTENSEN, J. J., and F. R. DAVIS: Variation in *Helminthosporium sativum* induced by a toxic substance produced by *Bacillus mesentericus*, *Phytopathology* **30**: 1017–1033, 1940.

CHRISTENSEN, J. J., and T. W. GRAHAM: Physiologic specialization and variation in *Helminthosporium gramineum* Rab., *Minn. Agr. Expt. Sta. Bull.* 95, 1934.

★CHRISTENSEN, J. J., and H. A. RODENHISER: Physiologic specialization and genetics of the smut fungi, *Botan. Rev.* **6**: 398–425, 1940.

DAS GUPTA, S. N.: Saltation in fungi, *Lucknow Univ. Studies* 5, 1936.

★DICKINSON, S.: The nature of saltation in *Fusarium* and *Helminthosporium*, *Minn. Agr. Expt. Sta. Bull.* 88, 1932.

DICKSON, J. G.: Diseases of Field Crops, McGraw-Hill Book Company, Inc., New York, 1947.

DODGE, B. O.: Nuclear phenomena associated with heterothallism and homothallism in the ascomycete *Neurospora*, *Jour. Agr. Research* **35**: 289–305, 1927.

DODGE, B. O.: Production of fertile hybrids in the ascomycete *Neurospora*, *Jour. Agr. Research* **36**: 1–14, 1928.

DODGE, B. O.: Some problems in the genetics of the fungi, *Science* **90**: 379–385, 1939.

DOWDING, E. S.: The sexuality of the normal, giant and dwarf spores of *Pleurage anserina* (Ces.) Kuntze., *Ann. Botany* **45**: 1–15, 1931.

EDGERTON, C. W., S. J. P. CHILTON, and G. B. LUCAS: Genetics of Glomerella. II. Fertilization between strains, *Am. Jour. Botany* **32**: 115–118, 1945.

FRIES, N.: Spontaneous physiological mutations in *Ophiostoma*, *Hereditas* **34**: 338–350, 1948.

★GILES, N. H., and E. Z. LEDERBERG: Induced reversions of biochemical mutants in *Neurospora crassa*, *Am. Jour. Botany* **35**: 150–157, 1948.

HANNA, W. F.: The odor of bunt spores, *Phytopathology* **22**: 978–979, 1932.

HANSEN, H. N.: The dual phenomenon in imperfect fungi, *Mycologia* **30**: 442–455, 1938.

★HANSEN, H. N., and W. C. SNYDER: The dual phenomenon and sex in *Hypomyces solani* f. *cucurbitae*, *Am. Jour. Botany* **30**: 419–422, 1943.

HARRIS, H. A.: Heterothallic antibiosis in *Mucor racemosus*, *Mycologia* **40**: 347–351, 1948.

HAWKER, L. E.: The nature of the accessory growth factors influencing growth and fruiting of *Melanospora destruens* Shear and of some other fungi, *Ann. Bot.* (N.S.) **3**: 657–676, 1939.

HOCKENHULL, D. J. D.: Mustard-gas mutations in *Aspergillus nidulans*, *Nature* **161**: 100, 1948.

HOCKENHULL, D. J. D.: The sulphur metabolism of mould fungi: The use of "biochemical mutant" strains of *Aspergillus nidulans* in elucidating the biosynthesis of cystine, *Biochim. et Biophys. Acta* **3**: 326–335, 1949.

HOUSTON, B. R.: Culture types and pathogenicity of isolates of *Corticium solani*, *Phytopathology* **35**: 371–393, 1945.

JOHNSON, T., and M. NEWTON: Mendelian inheritance of certain pathogenic characters of *Puccinia graminis tritici*, *Can. Jour. Research* **18**: 599–611, 1940.

KINSEY, V. E., and W. M. GRANT: The reaction of mustard gas with proteins. II. Biological assay of amino acids affected, *Arch. Biochem.* **10**: 311–322, 1946.

KREITLOW, K. W.: *Sclerotinia trifoliorum*, a pathogen of ladino clover, *Phytopathology* **39**: 158–166, 1949.

LEONIAN, L. H.: Studies in the variability and dissociations in the genus *Fusarium*, *Phytopathology* **19**: 753–868, 1929.

LEONIAN, L. H., and V. G. LILLY: The effects of vitamins on ten strains of *Saccharomyces cerevisiae*, *Am. Jour. Botany* **29**: 459–464, 1942.

LILLY, V. G., and H. L. BARNETT: The inheritance of partial thiamine deficiency in *Lenzites trabea*, *Jour. Agr. Research* **77**: 287–300, 1948.

LINDEGREN, C. C.: Mendelian and cytoplasmic inheritance in yeasts, *Ann. Missouri Botan. Garden* **32**: 107–123, 1945.

LINDEGREN, C. C.: Genetics of the fungi, *Ann. Rev. Microbiol.* **1948**: 47–70.

LINDEGREN, C. C.: The Yeast Cell, Its Genetics and Cytology, Educational Publishers, St. Louis, 1949.

MCELROY, W. D., J. E. CUSHING, and H. MILLER: The induction of biochemical mutations in *Neurospora crassa* by nitrogen mustard, *Jour. Cellular Comp. Physiol.* **30**: 331–346, 1947.

MACRAE, R.: Interfertility studies and inheritance of luminosity in *Panus stypticus*, *Can. Jour. Research*, Sec. C, **20**: 411–434, 1942.

MARTIN, W. J.: Strains of *Phytophthora infestans* capable of surviving high temperatures, *Phytopathology* **39**: 14, 1949.

NOBLES, M. K.: Conidial formation, mutation and hybridization in *Peniophora allescheri*, *Mycologia* **27**: 286–301, 1935.

ROBBINS, W. J., and R. MA: Biotin and the growth of *Fusarium avenaceum*, *Bull. Torrey Botan. Club* **68**: 446–462, 1941.

ROBBINS, W. J., and I. MCVEIGH: The dual phenomenon and *Trichophyton mentagrophytes*, *Mycologia* **41**: 128–140, 1949.

RYAN, F. J.: Back mutation and adaptation of nutritional mutants, *Cold Spring Harbor Symposia Quant. Biol.* **11**: 215–227, 1946.

SKOOG, F. K., and C. C. LINDEGREN: Adaptation in a yeast unable to ferment glucose, *Jour. Bact.* **53**: 729–742, 1947.

SPIEGELMAN, S.: *in* J. B. Sumner and K. Myrbäck (Editors), The Enzymes, Vol. I, Academic Press, Inc., New York, 1950.

SPIEGELMAN, S., C. C. LINDEGREN, and G. LINDEGREN: Maintenance and increase of a genetic character by a substrate-cytoplasmic interaction in the absence of the specific gene, *Proc. Natl. Acad. Sci. U.S.* **31**: 95–102, 1945.

STEINBERG, R. A., and C. THOM: Mutations and reversions in reproductivity of Aspergilli with nitrite, colchicine and d-lysine, *Proc. Natl. Acad. Sci. U.S.* **26**: 363–366, 1940.

STEINBERG, R. A., and C. THOM: Reversions in morphology of nitrite-induced "Mutants" of Aspergilli grown upon amino acids, *Jour. Agr. Research* **64**: 645–652, 1942.

THOM, C., and K. B. RAPER: A Manual of the Aspergilli, The Williams & Wilkins Company, Baltimore, 1945.

THOM, C., and R. A. STEINBERG: The chemical induction of genetic changes in fungi, *Proc. Natl. Acad. Sci. U.S.* **25**: 329–335, 1939.

THREN, R.: Zur Entwicklungsphysiologie der Dikaryophase von *Ustilago nuda* (Jensen) Kellerm. et Sw., *Arch. Mikrobiol.* **12**: 192–228, 1941.

WOLF, F. A.: A squash disease caused by *Choanephora cucurbitarum*, *Jour. Agr. Research* **8**: 319–328, 1917.

WOLF, F. A., and F. T. WOLF: The Fungi, Vol. II, John Wiley & Sons, Inc., New York, 1947.

SUGGESTED LABORATORY EXERCISES

Each of the laboratory exercises is designed to illustrate one or more important principles regarding the physiological activities of the fungi. Each represents a more or less complete unit or phase, but there is necessarily some overlapping and duplication of the techniques. Laboratory exercises requiring greenhouse space and living plants have been omitted. This does not mean that an understanding of the cultural activities should be the only aim of the laboratory work. Rather, it is believed that such information regarding the nutritional and environmental requirements of fungi in pure culture will aid in the better understanding of the behavior of both parasitic and nonparasitic fungi in nature.

The laboratory exercises are planned so as to allow a high degree of flexibility. The instructor may wish to omit, change, or add to some of the suggested exercises to suit the equipment and other facilities available. The exercises are outlined to require a minimum of laboratory equipment. Any or all of the exercises may be expanded or shortened as desired for a large or small number of students.

Each student should select one or more laboratory exercises in which he has a particular interest. He may then be designated as the *leader* of that exercise or exercises. His duties would include (1) the general planning of the exercise in consultation with the instructor, (2) the directing of the execution of the exercise, (3) collecting and organization of the data from other students, and (4) the writing of a comprehensive report on the subject. References should be read and discussed in comparison with the results of the experiments in the laboratory.

The supply of test fungi is almost unlimited. In most exercises each student will use two or more species of fungi. The total number for the class should be sufficiently large to emphasize the differences as well as the similarities that exist among various fungi. In many instances our knowledge is limited, and little or nothing has been published regarding the physiology of some of the species used. In this sense, much of the work done in the laboratory will be experimental and should be carried out with all the care of a research problem. Fungi should be selected so that both growth and sporulation may be studied in the same experiment. In each exercise are listed a few fungi, some of which have been used in our laboratory and have given quite satisfactory results. Other species may be added or substituted.

Notes should be recorded in a full-sized permanent notebook reserved

only for the laboratory work in this course. The student should take notes and record data so that anyone familiar with the subject could organize the data and write an intelligent summary. Data taken during the experiments are important and in themselves may be quite convincing at that time, but data alone without organization and discussion of their meaning are often sterile and soon forgotten.

The paper should be written as soon as possible after the termination of the laboratory exercise. It is suggested that all students read the excellent discussion of Riker (1946) on the preparation of manuscripts.

The student should become familiar with the microscopic characteristics of the fungi used in the experiments. He should make frequent microscopic observations during the experiment and look for microscopic changes, such as abundance and maturity of spores. Contaminants are often more easily recognized under the microscope. Drawings are often desirable as records of differences in microscopic characteristics.

EXERCISE 1

General Laboratory Procedure

Exercise 1 is suitably carried out by the instructor as a *demonstration* of general laboratory techniques. This affords the instructor an opportunity to discuss the details of various procedures and to acquaint the students with the laboratory facilities. It is convenient to prepare sufficient stock culture medium, *e.g.*, medium 1 (Ex. 2), for growing the inoculum required by the class for the next exercise.

General directions for preparing media. The culture medium should be selected with the purpose of the experiment in mind. The precautions to be observed may be elemental or elaborate, depending upon the purpose for which the medium is to be used. The accuracy of one measurement should be consistent with the accuracy of the others. The weights of each constituent of a given lot of medium should be written in a notebook. As each constituent is measured, make a check mark against this constituent.

Never weigh chemicals directly on the balance pans. Use a clean piece of paper or watch glass. The weights are placed on the right-hand pan as you face the balance. Be sure the spatula is cleaned between weighings of different chemicals. If you remove more of a chemical than necessary, discard the excess. (Material still on the spatula may be returned to the stock bottle.) Keep the stock bottles closed. This prevents the entrance of dust and atmospheric moisture. Malt extract, yeast extract, and peptone quickly absorb water from the air, and when these substances have done so, an intractable mass results.

For work of ordinary accuracy use a graduated cylinder for measuring liquids. Volumetric glassware should be used for precise work. The

chemicals should be dissolved in less than the desired volume of water, and after they are in solution, the medium should be made up to volume by the addition of distilled water.

Constituents of media. The salts used in making media should be of c.p. grade. These will generally contain enough of the micro essential elements to satisfy the needs of most fungi. Iron, zinc, and manganese should be added routinely to all synthetic media. It is convenient to make up a solution containing the above micro essential elements in such strength that 1 ml. of the master solution will contain 0.1 mg. Fe^{++}, 0.1 mg. Zn^{++}, and 0.05 mg. Mn^{++}. The following amounts of the given salts have been found convenient to use: $Fe(NO_3)_3 \cdot 9H_2O$, 723.5 mg.; $ZnSO_4 \cdot 7H_2O$, 439.8 mg.; $MnSO_4 \cdot 4H_2O$, 203.0 mg. Dissolve these three salts in 600 ml. of distilled water, add sufficient c.p. sulfuric acid to yield a clear solution, and make up the volume to 1 liter with distilled water. Use 2 ml. of this solution per liter of medium. Add the source of carbon at the rate of 10 to 25 g. per liter, depending on the use of the medium. Add the nitrogen source at the rate of 2 g. per liter, or the amount which will give an amount of nitrogen equivalent to that furnished by 2g . of anhydrous asparagine (0.425 g. of N).

The activated carbon used in Ex. 9 to absorb residual traces of vitamins is a commercial preparation, Norit A (pharmaceutical grade). Use at the rate of 5 g. per liter, or more if necessary. The vitamins used routinely are thiamine and biotin. Inositol and pyridoxine are also used in Ex. 9. These vitamins may be purchased from Merck and Co., Rahway, N.J., and many other pharmaceutical houses. It is convenient to make up master solutions of these four vitamins. Make the master solutions in 20 per cent alcohol, store in 100-ml. volumetric flasks, and keep in a refrigerator when not in use. It is convenient to make the master solutions of thiamine and pyridoxine to contain 100 μg per ml. (10 mg. per 100 ml.). The master solution of inositol contains 5 mg. per ml. Biotin is used at the rate of 5 μg per liter. Either the contents of an ampule may be made up, or a microbalance used to weigh this vitamin. A master solution containing either 1 or 5 μg per ml. is convenient.

For a semisolid medium, agar is commonly used at the rate of 20 g. per liter. Agar is not a pure compound, and its use introduces various unsuspected constituents into media. No medium may be said to be of known composition if agar is used, although such media are very useful. Some of the impurities in agar may be removed by leaching with aqueous pyridine solution. Place 1 lb. of agar in a 6-liter flask, add 5 liters of distilled water and 500 ml. of pyridine. Allow to stand 24 hr. Insert a piece of 6-mm. glass tubing of sufficient length to admit air to the flask, tie a piece of cheesecloth over the neck of the flask, invert the flask, and allow the pyridine solution to drain. Wash the agar three

times with distilled water. Wash the agar twice with 95 per cent alcohol, allowing the alcohol to stand on agar overnight before draining. Dry the agar in thin layers between cheesecloth. This procedure takes about 10 days (see Robbins, 1939). In some instances the agar and other constituents of the medium may be autoclaved separately and the two solutions mixed, using antiseptic precautions. This should be done when it is required to have a very acidic agar medium. A known amount of sterile acid may be added to the agar medium after sterilization.

pH or reaction of the medium. See Chap. 8 for a discussion of pH. An approximate method of determining pH is sufficiently accurate for many purposes. On a white porcelain spot plate place one drop of Hellige (or other) wide-range indicator in each depression. Have the drops of indicator of equal size. Then add one drop of the medium to a drop of indicator in one of the depressions of the spot plate. The color of the mixed drops indicates the pH of the medium. Thus, red indicates a pH in the neighborhood of 4, light green 7, purple 10. Standard buffers (solutions of known pH) may be provided so that the student may have standards with which to make comparisons.

The buffers used in testing pH are most conveniently made from buffer tablets (Coleman). Dissolve one tablet in 100 ml. of distilled water. Add a crystal of thymol as a preservative. Thymol aids in preventing contamination and does not appreciably affect the pH of the buffer. It is convenient to store the buffers in brown-glass dropping bottles fitted with pipettes.

Unless otherwise specified, media used in the laboratory should be adjusted to pH 6 before autoclaving. This may be done by the use of the spot plate, adding a drop of pH 6 buffer to a drop of indicator. This is the standard color to which the media should be adjusted. Add either 6*N* NaOH or 6*N* HCl to the medium until the color produced by one drop of medium matches the color produced by the standard buffer. Always agitate the medium after each addition of acid or base and then test the pH. The use of concentrated acid and alkali is recommended so that dilution of the medium may be avoided. More precise methods of measuring pH may be used if desired.

Autoclaving usually lowers the pH of a medium. In general, this change will not be great, but the student should never assume that the pH will remain unchanged in autoclaving.

Sterilization of media and glassware. Except in special instances, the autoclave may be used to sterilize both media and glassware. Fifteen minutes at 15 lb. steam pressure is adequate for test tubes and flasks which do not contain over 150 ml. of medium. Larger lots of media should be autoclaved 20 min. at 15 lb. steam pressure. Petri dishes may be sterilized 20 min. in the autoclave. It is convenient to wrap two Petri

dishes in a paper towel or several in a paper bag before placing in the autoclave. This wrapping should remain on the Petri dishes until they are used, to prevent contamination. Do not remove wrapped glassware from the autoclave until several minutes after the pressure is down. Pipettes should be wrapped and placed in pipette cases. Sterilize in the same way as Petri dishes.

Use either a water bath or the autoclave for melting agar. Never melt agar in or sterilize flasks which are more than half full. Test tubes should not be more than one-fourth full. The reason for this lies in the fact that the autoclave cools quicker than the medium. This leaves the medium superheated, and under this condition it is likely to boil violently. Never remove a flask of melted agar from the autoclave as soon as the pressure is down. Agitation may cause violent boiling. Your instructor will give you full instructions for operating the autoclave. Follow these instructions carefully.

On handling cultures. It will be necessary for each student to maintain the identity of his cultures. This may be done by name or by stock-culture number. Each medium used in the course will receive a number. If a medium is used more than once, it will be given another number. The composition of each medium should be entered in the laboratory notebook. The name of the fungus (or stock-culture number) and the number of the medium should be also written on each culture vessel. The date of inoculation and the kind of inoculum used should be entered in the notebook. It is convenient to fasten together duplicate or triplicate cultures in test tubes with a rubber band.

Preservation of stock cultures. The maintenance of a stock culture collection of filamentous fungi for class use is highly desirable. Such a collection need not be extensive but should include a sufficient number of selected species of known physiological reaction and any others which may be desired for general use. The method of preserving cultures in our laboratory has proved quite satisfactory when frequent transfers are needed for research or class use. Test tubes with constricted tops and plastic screw caps are used. Malt extract or any other suitable agar may be used. After inoculation the tubes are allowed to remain at room temperature for a few days until the inoculum starts to grow. Then the caps are screwed down tightly and the cultures stored at 5 to 10°C. Most species continue to grow slowly, and under these conditions the tubes remain free from contamination and the agar dries out very slowly. This method also excludes mites. Some vegetative cultures have remained viable for a period of more than 2 years without being transferred. However, it is suggested that all cultures be transferred every year, and the entire stock should be looked over carefully every few months, as some species may require more frequent transfers. The first transfer from

stock culture should be to another stock-culture tube, and the old tube should be kept until the new culture begins to grow free from contamination. Other methods of storing stock cultures of fungi are described by Greene and Fred (1934), Thom and Raper (1945), Fennell *et al.* (1950), and Buell and Weston (1947).

Methods of inoculation. It is customary to use a bit of mycelium from a growing culture to inoculate fresh media. For ordinary uses this is satisfactory, if only a few cultures are to inoculated at a time and no special precautions need be taken. Some fungi produce a tough mat of mycelium difficult to cut with a needle. Often this can be overcome by growing the mycelium for inoculum on an agar medium quite low in sugar. A small cork borer may be used for cutting out uniform disks of mycelium and agar from Petri dishes.

Spores alone may be transferred by a dry needle, or they may be suspended in water and inoculated by use of a loop or a sterile pipette with a cotton plug at the upper end. The use of a pipette fitted with a small rubber bulb greatly decreases the inoculation time when many cultures of the same fungus are made. It is preferable to use spores as inoculum in studies of vitamins or micro elements, where none of the previous medium should be added.

Nonsporulating mycelium may be fragmented by placing it with about 50 ml. water in a sterile Waring Blendor jar for about 30 sec. Either agar or liquid medium may be used if the addition of the medium is of no consequence. In vitamin studies the mycelium may be grown in liquid medium and, when ready for use, washed in sterile distilled water and fragmented in the Blendor. Either a loop or a pipette may be used to dispense the mycelial suspension.

Methods of obtaining single-spore cultures. In certain physiological studies it is desirable to use single-spore cultures. These may be obtained by a number of different methods. A review of the literature on these techniques has been given by Hildebrand (1938). Other modified techniques are described by Georg (1947) and Thom and Raper (1945). Still another modification may be worthy of brief mention. In this laboratory we have used a specially prepared small sewing needle as a tool for picking out single germinated spores. The eye of the needle is rounded and the thick metal portion filed down, making a rather thin edge for cutting agar. The pointed end is fastened in a convenient holder, and the needle bent in such a way that, when held over a Petri dish, the eye portion will be parallel with the surface of the medium so that it can be pushed straight down into the agar. An isolated, germinated spore is located on a dilution plate by use of a microscope. The needle is then held in place under the objective so that the spore is visible through the eye of the needle. The eye is pressed down around the spore

and is lifted up with a bit of agar and the germinated spore. Another needle may then be used to transfer the bit of agar and spore to a tube or plate. This method requires a steady hand but has the advantage of being rapid and simple. It may be employed with high magnification of the stereoscopic microscope or the low-power objective of the compound microscope.

EXERCISE 2

The Influence of Temperature on Growth and Sporulation

This exercise is outlined to illustrate three main points: (1) the general effects of different temperatures upon growth; (2) the approximate optimum temperature for growth of a few fungi; (3) that the temperature limits for sporulation are narrower than those for vegetative growth. Inasmuch as the composition of the medium may influence temperature limits, only one medium should be used in this experiment.

Medium

1. Malt extract, 20 g.; yeast extract, 2 g.; agar, 20 g.; distilled water, 1,000 ml.

Adjust the pH, if necessary, to approximately 6, and autoclave. Pour into sterile Petri dishes, about 20 ml. per plate, and inoculate them at the center. Inoculate plates in duplicate or in triplicate for each condition. Incubate the plates at a range of temperatures with convenient intervals. The range and the exact temperatures used will depend upon the facilities available. Suggested temperatures are 10, 15, 20, 25, 30, 35, and 40°C. Since light affects some of the species listed below, it is desirable to have the cultures illuminated for a part of each day.

Records. Observations of growth should be made daily or at least every 2 days. For the purpose of this exercise the radial extension of the mycelium may be used as a measurement of growth. This usually can be easily measured by placing a rule (preferable one calibrated in millimeters) on the bottom of the Petri dish and looking through it toward a light. In order to have an accurate measure of the effect of different temperatures, the cultures must be compared before the mycelium reaches the edge of the Petri dish. A more accurate measure of growth may be obtained by determining the dry weight of mycelium grown in liquid medium. Records may be made in table form for each fungus, giving the days of incubation and the diameter of the colony. The average daily radial extension of the mycelium may then be calculated for each temperature. The amount of sporulation should likewise be recorded each time.

List of test fungi: *Alternaria* sp., *Aspergillus rugulosus*, *Botrytis cinerea*, *Cephalotheeium roseum*, *Ceratostomella fimbriata*, *Choanephora cucurbitarum*, *Glomerella cingulata*, *Guignardia bidwellii*, *Monilinia fructicola*, *Penicillium expansum*, *Phytophthora infestans*, *Sclerotinia sclerotiorum*, *Septoria nodorum*.

EXERCISE 3

The Influence of Light on Growth and Sporulation

This experiment is designed to illustrate the variable effects of visible and ultraviolet light, particularly upon reproduction of some common fungi. Exposure to light is essential to spore formation in some fungi, while other fungi may sporulate abundantly in total darkness. For a more complete discussion, see Chaps. 3 and 15.

Media

2. Same as medium 1
3. Any semisynthetic medium, such as the basal medium in Ex. 4

Adjust the pH of the media to approximately 6.0, autoclave, and pour into sterile Petri dishes. Inoculate plates in triplicate for each condition. Place the inoculated plates under the following conditions:

A. Continuous total darkness at 25°C.
B. Continuous artificial light at 25°C.
C. Alternating light and darkness at 25°C.
D. Same as *C*, but expose to ultraviolet three times for 2 min each time, at intervals of 2 days

The exposure to ultraviolet light should be made in an inoculating chamber, with the lids of the dishes removed, at a distance of approximately 10 to 12 in. from the source (the G.E. germicidal lamp is quite satisfactory). Wearing of spectacles or sunglasses will protect the eyes from the ultraviolet rays. The first exposure should be made when the fungus colony is approximately 1 in. in diameter. Subsequent exposures should be timed so that the last one is made before the mycelium reaches the edge of the plate. With rapidly growing species, the interval between exposures may be shortened. At the time of each exposure, use a wax pencil to outline the extent of the mycelium by marking the bottom of the Petri dish. The plates kept in total darkness should be wrapped in paper or stored in a lighttight cardboard box. Examination of most fungi should be made after about 7 days. The rapidly growing species should be placed in a separate box which can be opened earlier.

List of test fungi: *Botrytis cinerea, Cephalothecium roseum, Ceratostomella fimbriata, Choanephora cucurbitarum, Dendrophoma obscurans, Endothia parasitica, Monilinia fructicola, Neocosmopara vasinfecta, Penicillium expansum, Septoria nodorum, Trichoderma lignorum.*

EXERCISE 4

The Effect of the Carbon Source on Growth and Sporulation

This exercise is designed to show that fungi differ in their ability to utilize certain compounds as a source of carbon. For discussion of this

topic see Chaps. 7 and 14. In this study, the nitrogen source in the media should be simple and available to as many fungi as possible. For this purpose, asparagine is quite satisfactory.

Malt extract-yeast extract agar may be used as a standard control medium, since this is an excellent natural medium for most fungi. If desired, any other standard natural medium may be used as a control medium. All other media used in the exercise will have the same basal composition, with the carbon source as the only variable. Adjust the pH of all media to approximately 6.0 before autoclaving.

Basal Semisynthetic Medium

Carbon source	10 g.
Asparagine	2 g.
KH_2PO_4	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Fe^{+++}	0.2 mg.
Zn^{++}	0.2 mg.
Mn^{++}	0.1 mg.
Biotin	5 μg
Thiamine	100 μg
Distilled water to make	1 liter
Agar (for solid media)	20 g.

For the most accurate measure of growth, liquid media should be used, so that the mycelium may be filtered off, washed, dried, and weighed. However, agar media are often more satisfactory for reproduction. It is suggested that this exercise be carried out on agar slants in test tubes.

Media

4. Same as medium 1
5. Glucose. Media 5 to 13 will all contain the basal medium above.
6. Sucrose
7. Sorbose
8. Lactose
9. Maltose
10. Galactose
11. Starch
12. Cellulose[1]
13. No sugar

Each student should select two or more species of fungi and inoculate with each fungus three tubes of each of the 10 media listed above. Incubate at 25°C., or at room temperature. Use for inoculum spores or small bits of mycelium with as little agar as possible.

Records. The student will be responsible for taking notes or data on the growth and sporulation of the fungi he selects, but he should follow the form suggested by the leader of the exercise. He should also observe the results of students who use other species. Records on growth may be made at the end of 3 to 7 days, depending upon the fungus, while a greater time should be allowed before making final records on sporulation. For most purposes a record of the relative amount of growth or sporulation,

[1] Use good grade of filter paper, add water, and cut to a pulp in a Waring Blendor.

when compared with that on a control medium, is quite satisfactory. Thus, if the growth and sporulation (if any) of each fungus on medium 4 are arbitrarily given the values of 4+, the estimated abundance on other media may be designated as greater or less than 4, as the case may be. Such a rough method has been found satisfactory for illustrating principles and determining the availability of carbon sources utilized by the fungi. If a more accurate measurement is desired, liquid media should be used and dry weights of the mycelia obtained. Observations and records should be made on any other characteristics which are affected by changes in carbon source.

List of test fungi: *Aspergillus rugulosus, Ceratostomella fimbriata, Dendrophoma obscurans, Endothia parasitica, Glomerella cingulata, Guignardia bidwellii, Melanospora* sp., *Monilinia fructicola, Phycomyces blakesleeanus, Pleurage curvicolla, Sordaria fimicola, Sphaeropsis malorum, Ustilago striiformis.*

EXERCISE 5

The Effect of the Nitrogen Source on Growth and Sporulation

The purpose of this exercise is to illustrate the utilization of different sources of nitrogen by different fungi. This exercise should follow soon after Ex. 4, and the procedure should be the same. The use of some of the same test fungi in this exercise should emphasize the importance of a suitable semisynthetic medium for growth and reproduction. In this case the carbon source (glucose) shall be kept constant and the nitrogen source varied with each medium. The other constituents of the basal medium will be the same as listed under Ex. 4. Medium 1 may again be used as a control, but if a different lot is made, it must carry a new number.

Media

14. Malt extract–yeast extract
15. Potassium nitrate
16. Ammonium sulfate
17. Ammonium tartrate
18. Asparagine
19. Glutamic acid
20. Glycine
21. Urea
22. Casein hydrolysate
23. No nitrogen

The amount of nitrogen should be kept constant. The weights of the compounds used should be calculated to contain a weight of nitrogen equivalent to that in 2 g. of asparagine.

EXERCISE 6

Special Nutritional Conditions Which Influence Growth and Sporulation

The two previous exercises have dealt mainly with the effect of the constituents of the medium on a qualitative basis (see Chaps. 3 and 14). This exercise is outlined to emphasize some of the effects of quantitative

differences in media. This may be illustrated by altering the concentration of one or more components of the medium. It is suggested that this exercise be carried out in Petri dishes, or in flasks if liquid media are used.

Media: This exercise may be divided into four parts based upon the variations in media.

A. Dilutions of the entire medium. Either liquid or agar media may be used.

24. Basal medium, containing asparagine and 20 g. glucose per liter
25. Medium 24 diluted to one-half strength
26. Medium 24 diluted to one-fourth strength
27. Medium 24 diluted to one-sixteenth strength
28. Medium 24 diluted to one sixty-fourth strength

B. Varying concentrations of sugar. Use either liquid or agar media. The same controls as in *A* may be used, if the same fungi are tested.

29. Basal medium, with 40 g. glucose
30. Basal medium, with 10 g. glucose
31. Basal medium, with 5 g. glucose
32. Basal medium, with 2 g. glucose

C. Change in medium during incubation. Use liquid media in flasks (15 ml. in 125-ml. Erlenmeyer flasks, or 25 ml. in 250-ml. flasks).

33. Basal medium, same as medium 24 above. Inoculate 10 flasks; after growth is near maximum (see instructor), separate the flasks into three groups.
    a. Replace old medium with fresh medium
    b. Replace medium with sterile distilled water
    c. Leave as control

D. Different natural products as media. Use as agar media.

34. V-8 juice (diluted to one-half strength)
35. Potato extract (200 g. potatoes per liter)
36. Malt extract (20 g. per liter)
37. Stems of bean, pea, etc., in water agar

Each student should select one fungus for use in this exercise. It is suggested that the fungi used should ordinarily produce considerable mycelium before fruiting.

Records. Careful notes must be taken regarding time of appearance of fruit bodies and spores. The amount of growth and sporulation may be compared to that on medium 24. Consult the leader or your instructor for further details on recording data.

List of test fungi: *Aspergillus rugulosus, Ceratostomella fimbriata, Choanephora cucurbitarum, Endothia parasitica, Glomerella cingulata, Guignardia bidwellii, Helminthosporium victoriae, Melanospora* sp., *Monilinia fructicola, Neocosmopara vasinfecta, Phoma betae, Sordaria fimicola,* and *Sphaeropsis malorum.*

EXERCISE 7

The Influence of Hydrogen-ion Concentration on Growth and Sporulation

This experiment is outlined to demonstrate (1) that the pH requirements for optimum growth and reproduction vary with the different species of fungi, (2) that the pH of the culture medium changes during the growth of the fungus, (3) the techniques by which pH changes may be followed during growth, (4) that the pH at the time of sporulation may be considerably different from that during most rapid growth, (5) that the pH changes are also influenced by the composition of the medium. For a discussion of pH, see Chap. 8. The pH of liquid media is more easily tested than that of solid media by colorimetric methods; hence liquid media should be used in this exercise. Twenty-five milliliters of medium in a 250-ml. Erlenmeyer flask gives rapid, even growth of many fungi and has been found to be quite satisfactory. Two different media are given below, each set at four different pH values. For convenience each is given a separate number.

Media

38. Glucose-asparagine (otherwise, basal medium as given in Ex. 4), pH 3.0
39. As above, pH 4.0
40. As above, pH 6.0
41. As above, pH 8.0
42. Sucrose-nitrate (otherwise, basal medium as given in Ex. 4), pH 3.0
43. As above, pH 4.0
44. As above, pH 6.0
45. As above, pH 8.0

Prepare these media, adjust the pH of each, and distribute to flasks before autoclaving. Then use one flask of each medium to determine the pH after autoclaving. This value should be considered the "initial pH." Each student should select one fungus and inoculate eight flasks of each medium listed above. Incubate the flasks at 25°C. or at room temperature. The pH of the culture filtrate should be determined at three different times during the period of active vegetative growth (about the fourth and eighth days) and at about the time or shortly after maximum growth is reached (sporulation of some fungi will occur at this time). Duplicate cultures should be used for each determination. See your instructor regarding the method of determining pH of culture medium. The sucrose-nitrate media may not be favorable for the growth of some of the fungi selected for use in this exercise.

Records. The relative amounts of growth should be recorded at the time of each pH determination. If more accurate growth measurements are desired, the mycelium can be dried and weighed. Also record the time of the earliest sporulation and the amount at subsequent intervals.

Consult the leader of the exercise or the instructor as to when the experiment should be terminated. A portion of these data may be presented in the form of a graph, plotting changes in pH against time for each fungus and each medium.

List of test fungi: *Aspergillus rugulosus, Cephalothecium roseum, Ceratostomella fimbriata, Glomerella cingulata, Monilinia fructicola, Neocosmopara vasinfecta, Penicillium spiculosporum, Penicillium expansum, Sordaria fimicola, Sphaeropsis malorum, Phycomyces blakesleeanus* (plus and minus).

EXERCISE 8

Methods of Inoculating Agar Media and Their Effect upon Growth and Sporulation

This is a brief and simple exercise, but it is outlined to demonstrate a principle which seems to be fundamental, at least for certain fungi. The most common way of inoculating agar media is to place a bit of actively growing mycelium or a few spores at the center of the medium surface. For most purposes this is entirely satisfactory, but in special cases other methods may be used. A drop of spore suspension or of finely cut mycelium may be placed at the center of the agar plate, or the entire surface may be flooded with heavy spore suspension or suspension of cut mycelium. The mycelium may be fragmented by cutting in a Waring Blendor in 50 ml. of sterile water for 30 sec. to 1 min.

For this exercise it is suggested that Petri dishes containing glucose-asparagine-sucrose agar (such as the basal medium in Ex. 4) be used. Fungi which ordinarily produce considerable mycelial growth before fruiting abundantly may give the best results and will provide spore-free inoculum when cultures are young. Some pycnidium-producing species should be included.

Each student should choose one or more fungi and inoculate plates in triplicate by the following methods:

A. A bit of mycelium placed at the center
B. A few spores transferred by a needle placed at the center
C. A drop of suspension of cut mycelium placed at the center.
D. A drop of spore suspension placed at the center.
E. Flooding the entire plate with suspension of cut mycelium
F. Flooding the entire plate with a heavy spore suspension
G. Diluting the spore suspension 1/1,000 and flooding the plate

Observe the cultures daily and note the abundance of vegetative growth and the time and abundance of sporulation in each case.

List of test fungi: *Alternaria* sp., *Ceratostomella fimbriata, Dendrophoma obscurans, Endothia parasitica, Fusarium* sp., *Glomerella cingulata, Guignardia bidwellii, Helminthosporium sativum, Monilinia fructicola, Neo-*

cosmopara vasinfecta, Phoma betae, Septoria nodorum, Sordaria fimicola, Sphaeropsis malorum.

EXERCISE 9

Vitamin Deficiencies in the Fungi

This exercise is outlined to demonstrate (*a*) vitamin deficiencies in the filamentous fungi, (*b*) the differences in the needs of the different species of fungi, (*c*) the techniques used to determine these deficiencies. For a discussion of vitamin deficiencies and lists of vitamin-deficient fungi, see Chap. 9 and Robbins and Kavanagh (1942).

In studying the vitamin requirements of the fungi, great care must be taken to use glassware and chemicals which are free from vitamins. The glucose-asparagine medium has the advantage of being a suitable source of carbon and nitrogen for most fungi. Casein hydrolysate may be used in the place of asparagine. In its preparation, the medium should be boiled with activated charcoal (Norit, 5 g. per liter), to remove any vitamins present, and filtered. Thus, the medium is "essentially free" of vitamins. Contamination may occur from dust, cotton fibers from the plug, dirty glassware, etc. Micro essential elements and vitamins must be added after this treatment.

Media. It is best to use liquid media for this exercise so the mycelium can be dried and weighed.

46. Basal glucose-asparagine (vitamin-free)
47. As above, with thiamine (100 μg per liter)
48. As above, with biotin (5 μg per liter)
49. As above, with thiamine and biotin
50. As above, with thiamine, biotin, inositol (5 mg. per liter), and pyridoxine (100 μg per liter)

Adjust the pH to 6.0 and distribute to tubes or flasks (25 ml. per 250-ml. flasks or 15 ml. per 125-ml. flasks) before autoclaving.

A. Screening test for vitamin deficiencies. Simple screening tests to determine roughly the deficiencies of fungi may be carried out in either liquid or purified-agar (see Ex. 1) media. Test tubes may be used for agar media, but Erlenmeyer flasks are suggested for liquid media. The student should select four or five species from the stock-culture collection, and inoculate tubes or flasks of each of the above media, in triplicate, with each species. Either spores or a small bit of mycelium may be used as inoculum. Daily observations and records of growth should be made. Visual estimates of relative amounts of growth are sufficiently accurate to detect most deficiencies. If liquid media are used, the mycelium may be weighed.

B. Growth curves of vitamin-deficient fungi. Inoculate 10 flasks each of media 46 to 50 with a filamentous fungus shown in part *A* to be defi-

cient. The fungus will be harvested at intervals and the amount of growth determined by obtaining the dry weight of the mycelium. If there is sufficient growth in the flasks, the first harvest should be made after 3 or 4 days.

Harvesting of the mycelium is accomplished by filtering the medium through a fine cloth and washing the mycelium with distilled water. Harvest duplicate cultures at each time. The mycelium is then transferred to small aluminum pans of known weight, dried for 12 to 24 hr. at 90°C., and weighed. The subsequent harvests should be made at intervals of 2 to 4 days, depending upon the growth rate of the fungus used. A convenient method of presenting the results is in the form of a graph, ploting time against weight of mycelium for each of the four media used.

EXERCISE 10

The Influence of Vitamin Concentration on Growth and Sporulation

This exercise is designed to illustrate a few important principles regarding the need for an adequate supply of vitamins in the medium for vitamin-deficient fungi. Some of these points are (1) that vegetative growth may be limited by an inadequate supply of the needed vitamins; (2) that higher concentrations of vitamins are needed for reproduction than for vegetative growth; (3) that the absolute amount of a vitamin necessary to induce reproduction varies with the amount of sugar in the medium; (4) that the number of perithecia (or other reproductive structures) is partially dependent upon the concentration of the vitamins in the medium; (5) that fungi may be used in bioassays for the vitamin content of various products.

The following experiments are suggested (these may be conducted as demonstrations before the whole class, if desired):

A. Thiamine concentration and growth; thiamine assay

Media

51. Basal glucose-asparagine medium, no vitamins
52. As above, but with 100 μg thiamine per liter
53. As above, 25 μg thiamine per liter
54. As above, 12.5 μg thiamine per liter
55. As above, 6.25 μg thiamine per liter
56. As above, 3.12 μg thiamine per liter
57. As above, 1.56 μg thiamine per liter
58. As above, add 0.5 g. cake flour per flask
59. As above, add 0.5 g. whole-wheat flour per flask

Other amounts of cake and whole-wheat flour may be used, or polished and brown rice may be used instead.

Adjust the pH of the above media to 6.0 and distribute 25 ml. each to

250-ml. flasks. Inoculate four flasks of each medium with one strain of *Phycomyces blakesleeanus* or *Ceratostomella fimbriata*.

B. Thiamine concentration and sporulation. Use media 51 to 53, 55, and 57, but solidify with 20 g. purified, vitamin-free agar (see Ex. 1) per liter. Pour into sterile Petri dishes (about 20 ml. each). Inoculate four plates with *Ceratostomella fimbriata* (be sure to use ascospores or mycelium producing perithecia), *Phycomyces blakesleeanus* (plus and minus strains on opposite sides of plate), *Choanephora cucurbitarum*, and *Dendrophoma obscurans*. Incubate *Phycomyces* at 20 to 22°C., the others near 25°C. Cultures of *Choanephora* must be adequately aerated and must receive alternate light and darkness. Observe cultures of *Ceratostomella* for production of perithecia, *Phycomyces* for zygospores, *Choanephora* for conidial heads, and *Dendrophoma* for pycnidia.

Allow sufficient time for the above cultures to grow; then add one or two drops of sterile (autoclaved) solution of thiamine to some of the thiamine-starved, nonsporulating cultures (leave controls). Observe the effects.

C. Effects of added thiamine on thiamine-starved mycelium

Media

60. Distilled water and purified agar
61. Distilled water, 100 μg thiamine per liter, purified agar

NOTE: The addition of agar in media 60 and 61 is not essential.

From a thiamine-starved culture of *C. fimbriata* which has produced no perithecia cut quarter-inch disks with a cork borer and place them in tubes of media 60 and 61. If liquid media are used, make sure that the disks of inoculum float on the surface. Observe the results after a few days.

D. Relation of required thiamine to sugar in medium. To show that the concentration of thiamine required for the production of perithecia depends upon the amount of sugar in the medium, this short experiment may be performed.

Media. Liquid glucose-asparagine

62. Glucose 25 g., thiamine 1 μg per liter
63. Glucose 2.5 g., thiamine 1 μg per liter
64. Glucose 0.25 g., thiamine 1 μg. per liter

Distribute the media in 250-ml. Erlenmeyer flasks, inoculate with *C. fimbriata*, and incubate at 25°C. Observe the rate of growth, time of perithecium formation, and relative number of perithecia formed in each medium. Harvest and weigh the mycelium of each culture after perithecia have formed.

E. Effects of biotin starvation on a biotin-deficient fungus

Media

65. Glucose-asparagine, purified agar; no biotin
66. As above, 5 μg biotin per liter
67. As above, 1 μg. biotin per liter
68. As above, 0.5 μg biotin per liter
69. As above, 0.1 μg biotin per liter

Pour into Petri dishes and inoculate each medium in triplicate with *Sordaria fimicola*. Observe the results after about 6, 8, and 10 days. Note the amount of growth, the time of perithecium formation, and the relative numbers of perithecia. Examine microscopically the perithecia formed in the low concentrations of biotin, and look for deformed asci and ascospores. Add a drop or two of sterile biotin solution to some of the nonfruiting, biotin-starved cultures. Observe the effects in a few days.

Records. Take full notes on all observations of the above experiments. Write out a full explanation of the results with interpretations based upon physiological processes in fungi (see Chap. 14).

EXERCISE 11

Factors Affecting Spore Germination

This exercise demonstrates the effects of nutrients, humidity, pH, and temperature upon the time and percentage of germination of the spores of some common fungi. While the germination of fungus spores may be influenced by a number of factors, only a few of them can be easily studied in the laboratory. See Chap. 16 for a discussion of factors which influence spore germination.

A. Effects of nutrients upon spore germination. Place filter paper in the bottoms of Petri dishes. Cut two holes in the filter paper about ½ in. in diameter and place a glass slide over these. Add water to moisten the paper, and autoclave. Make up a spore suspension in media 70 and 71, and place drops of this suspension on the slide over the holes in the paper, which must be kept moist with sterile water. Incubate at 25°C. After incubation the slide may be examined by placing the Petri dish on the microscope and removing the lid.

Media

70. Distilled water
71. Distilled water plus 2 g. yeast extract per liter
72. Same as medium 71 but solidified with agar; adjust to pH 8.0
73. Same as medium 71, but pH 7.0
74. Same as medium 71, but pH 6.0
75. Same as medium 71, but pH 5.0
76. Same as medium 71, but pH 4.0

B. Effect of acidity. This experiment may be carried out in liquid media 72 to 76, using drops of spore suspension as described above in part *A*, or the media may be solidified with agar and Petri dishes used. Observe the results at intervals up to 48 hr. Your records should include the approximate time required for germination of the first spores and the percentage of germination at each examination. The first appearance of a germ tube may be considered as germination.

C. Effect of relative humidity. Place drops of spore suspension in distilled water on sterile glass slides to serve as controls. On three other dry glass slides, place dry spores. Place these in desiccators as follows: (1) spores in water and in desiccator which will maintain saturated atmosphere; (2) spores on dry slide in saturated atmosphere; (3) spores on dry slide in desiccator with relative humidity at approximately 98 per cent; (4) spores on dry slide in desiccator with relative humidity at approximately 92 per cent. To maintain 98 per cent humidity, use a 1.00 molal solution of sucrose; for 92 per cent humidity use a saturated solution of K_2HPO_4 (see Clayton, 1942). Open the desiccators after 24 to 30 hr. and examine the spores for germination.

D. Effect of temperature. Use four Petri dishes with medium 74 or 75. On each place three or four drops of spore suspension and mark these spots on the bottom of the dish. Incubate these as follows: (1) in an incubator at 30°C.; (2) at 25°C.; (3) in a refrigerator at 18°C.; (4) in a refrigerator at 10°C.

Examination of spores. The time required for spores of the various species to germinate under the usual conditions varies from 2 to 24 hr. or more. For some fungi all the experiments in the exercise may be examined and compared after 12, 18, or 24 hr. One examination should be made after 48 hr. For most species the experiments may be concluded at this time.

List of test fungi: *Alternaria* sp., *Cephalothecium roseum, Choanephora cucurbitarum, Glomerella cingulata, Guignardia bidwellii, Helminthosporium sativum, Monilinia fructicola, Penicillium* sp., *Phytophthora infestans.*

EXERCISE 12

The Associative Effects among Fungi

Pure cultures of a single organism seldom exist in nature. Instead, each organism is constantly exposed to a biotic as well as a physical environment. As a result, there is usually competition between different fungi and between fungi and other organisms in the same substrate, particularly the soil. On the other hand, many organisms are benefited by their association with others. Often the metabolic products of one favor the growth of another.

If one desires to demonstrate these principles in the laboratory, pure cultures must be used, and by combining two or more of these species in a culture vessel, the associative effects may be studied. This exercise is outlined to demonstrate the main types of associative reactions between species of fungi in the laboratory under controlled conditions and to show that a species may react differently in its association with different fungi.

Media

77. Malt extract–yeast extract agar
78. Glucose-asparagine purified agar (vitamin-free)
79. Glucose-asparagine liquid (vitamin-free)

A. Each student should select three pairs of the fungi and test their interaction on agar plates of media 77 and 78. Duplicate plates should be inoculated for each condition. It is suggested that the two pairs of fungi be inoculated on opposite halves of the agar plates 1 to 2 in. apart. This will allow both fungi to make some growth before they come in contact. Incubate all cultures at 25°C. Notes should be taken on about the fifth day and the tenth day, and for most cultures the final observations may be made after 2 weeks. Carefully made sketches may add greatly to the value and clarity of your notes.

The types of reactions may be grouped under (1) none, (2) stimulation, (3) symbiotic, (4) antagonistic. Each type of reaction should be explained on the basis of the present experiments.

B. Using vitamin-free liquid medium 79, inoculate flasks with *Phycomyces blakesleeanus* and *Sordaria fimicola* separately and with both species together. After a few days observe the results. This part of the exercise may be conducted as a demonstration for the entire class.

List of test fungi: *Actinomyces sp.*, *Alternaria* sp., *Aspergillus rugulosus*, *Botrytis* sp., *Cephalothecium roseum*, *Guignardia bidwellii*, *Helminthosporium sativum*, *Monilia* sp., *Monilinia fructicola*, *Penicillium chrysogenum*, *Phycomyces blakesleeanus*, *Sordaria fimicola*, *Trichoderma lignorum*.

Suggested Demonstrations

In addition to the experiments outlined in the above exercises, the following are suggested as demonstrations for the entire class. These may be expanded into complete exercises for individual student participation.

1. *Need for micro elements for growth and sporulation.* Steinberg (1919) describes the procedure for preparing a medium essentially free of micro elements to which the desired elements may be added. Use *Aspergillus niger* as a test species. See Chap. 5 for other methods of removing micro elements from media.

2. *Influence of light on spore discharge.* This experiment demonstrates the phototrophic response of sporangiophores and perithecial beaks and the discharge of the spores toward the source of light. For a discussion of this subject, see Buller (1934). Use species of *Pilobolus*, *Sordaria*, or *Pleurage*.

3. *Influence of aeration on sporulation. Choanephora cucurbitarum* is an excellent species to use in demonstrating the need for adequate aeration for the production of conidia. Grow the fungus on agar in Petri dishes. Some of the dishes may be sealed with Scotch tape, while the lids of others may be raised to permit free exchange of gases.

4. *Longevity of spores.* This may be designed as a long-time experiment to determine the longevity of spores of several fungi under different conditions of storage. It may be continued from year to year, tests for the ability to germinate being made every few months.

5. *Action of fungicides and fungistatic agents.* These experiments should be outlined to show the effectiveness of various agents in preventing spore germination. For references, see Chap. 11 and Horsfall (1945). These agents include the action of sulfur, copper, 8-hydroxyquinoline, antivitamins, and ultraviolet radiation.

6. *Action of antibiotics against fungi.* The specificity of the action of some antibiotics is easily tested against growth or spore germination of some common fungi by the use of penicylinders in agar plates flooded with spores.

7. *Inheritance of physiologic characters.* (*A*) The inheritance of vitamin or amino-acid deficiency may be demonstrated by crossing deficient mutants of *Neurospora* with a self-sufficient strain (see Beadle, 1946, and Chap. 18). (*B*) The inheritance of bioluminescence may be demonstrated by crossing the North American and European strains of *Panus stypticus* (see Macrae, 1942).

REFERENCES

Beadle, G. W.: Genes and the chemistry of the organism, *Am. Scientist* **34**: 31–53, 1946.

Buell, C., and W. H. Weston: Application of the mineral oil conservation method to maintaining collections of fungus cultures, *Am. Jour. Botany* **34**: 555–561, 1947.

Buller, A. H. R.: Researches on Fungi, Vol. VI, Longmans, Roberts and Green, London, 1934.

Clayton, C. N.: The germination of fungous spores in relation to controlled humidity, *Phytopathology* **32**: 921–943, 1942.

Fennell, D. I., K. B. Raper, and M. H. Flickinger: Further investigations on the preservation of mold cultures, *Mycologia* **42**: 135–147, 1950.

Georg, L. K.: A simple and rapid method for obtaining monospore cultures of fungi, *Mycologia* **39**: 368–371, 1947.

Greene, H. C., and E. B. Fred: Maintenance of vigorous mold stock cultures, *Ind. Eng. Chem.* **26**: 1297–1298, 1934.

HILDEBRAND, E. M.: Techniques for the isolation of single microorganisms, *Botan. Rev.* **4**: 627–664, 1938.

HORSFALL, J. G.: Fungicides and Their Action, Chronica Botanica Co., Waltham, 1945.

MACRAE, R.: Interfertility studies and inheritance of luminosity in *Panus stypticus*, *Can. Jour. Research*, Sec. C, **20**: 411–434, 1942.

RIKER, A. J.: The preparation of manuscripts for Phytopathology, *Phytopathology* **36**: 953–977, 1946.

ROBBINS, W. J.: Growth substances in agar, *Am. Jour. Botany* **26**: 772–778, 1939.

ROBBINS, W. J., and V. KAVANAGH: Vitamin deficiencies of the filamentous fungi, *Botan. Rev.* **8**: 411–471, 1942.

STEINBERG, R. A.: A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*, *Am. Jour. Botany* **6**: 330–372, 1919.

THOM, C., and K. B. RAPER: Manual of the Aspergilli, The Williams & Wilkins Company, Baltimore, 1945.

INDEX

C

I

J

K

L

M

S

T

U

X

Y

Z