PHYSIOLOGICAL STUDIES ON *CALVATIA* SPECIES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Magdalena Sedlmayr
nee Buzna
1960

This is to certify that the

thesis entitled

Physiological Studies on Calvatia Species

presented by

Magdalena Sedlmayr nee Puzna

has been accepted towards fulfillment of the requirements for

PhD **degree in** Mycology

(Department of Botany & Plant Pathology)

Major professor
E. S. Beneke

Date Feb. 18, 1960

O-169

PHYSIOLOGICAL STUDIES ON *CALVATIA* SPECIES

By

Magdalena Sedlmayr
nee Buzna

A THESIS

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1960

ACKNOWLEDGMENTS

The author wishes to express her sincere thanks to Dr. Everett S. Beneke, under whose constant interest, helpful criticism and guidance this investigation was undertaken. The writer is also greatly indebted to Dr. G. B. Wilson for his valuable suggestions and kind help in the preparation of this thesis. Sincere thanks are also due to Dr. Hans A. Lillevik for his aid and criticisms in the chemical evaluations in this thesis.

The author especially wishes to express her thanks and gratitude to the late Dr. E.H. Lucas for his encouragement and assistance towards her acceptance into Graduate School.

This work was made possible by a grant from the National Institute of Health for which the author expresses grateful appreciation.

PHYSIOLOGICAL STUDIES ON *CALVATIA* SPECIES

By

Magdelena Sedlmayr
nee Buzna

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1960

Approved William

ABSTRACT

The genus *Calvatia* has assumed a greater importance in recent years following the discovery of tumor-retarding properties in some of its species. A review of the literature revealed no detailed reports on the nutritional-physiological requirements of these fungi. Consequently, this was undertaken as the general purpose of this work.

The specific purpose of this investigation was to determine the vitamin and carbon requirements of four strains of *Calvatia*. The utilization of the nutrients were determined by the growth response of the organisms and measured on the basis of the average mycelial dry weights of two or four replicates after four weeks of incubation.

The superiority of the submerged to the surface culture method, as a technic for the study of the nutrition of the fungi, was demonstrated. A synthetic medium was found available for culturing *Calvatia* species. The optimum pH and temperature range for the mycelial growth of the organism was determined.

The investigations of the vitamin requirements indicated that *Calvatia* strains have in common a total deficiency for thiamine. The capacity to synthesize the other vitamins studied, however, (biotin, pyridoxine and inositol) appeared to be adequate. The action of thiamine seemed not to be quantitative within certain limits because an increase in the dosage caused no significant increases in the growth.

Different carbon sources and their utilization by the four strains were investigated. The ability to utilize these carbon sources by the *Calvatia* strains tested depended both upon the configuration of the compounds and the particular abilities of the organisms.

TABLE OF CONTENTS

INTRODUCTION

Physiological studies of the higher Basidiomycetes have proved in recent times to be an important field of investigation. These organisms have not only been used for routine biological studies, but also for bioassays, for determination of steps involved in the synthesis and degradation of essential metabolic substances, as well as for investigations which followed the discovery of several antibiotics produced by them.

In the last 10 to 15 years several Basidiomycetes have been found to produce antibiotics. Wilkins (1945) investigated the production of bacteriostatic substances by certain Basidiomycetes. Hervey (1947) surveyed 500 Basidiomycetes for antibacterial activity. Bose (1947) found an antibiotic in *Polyporus* species; Kavanagh *et al* (1949) in *Marasmius conigenus*; Doery *et al* (1951) in *Coprinus quadrifidus*; Anchel *et al* (1952) in *Fomes juniperus*. Atkinson (1954) named and described Psalliotin, the antibiotic produced by *Psalliota xanthoderma*.

In the Basidiomycetes the nutritional-physiological experiments are limited mostly to studies on mycelial growth. The production of basidiocarps by *Collybia velutipes* on synthetic medium was studied by Plunkett (1953) and with *Coprinus* species by Bille-Hansen (1953). Ashan (1954) reported on the influence of different culture conditions on the growth of *Collybia velutipes*, and Scheler-Correns (1957) studied the effect of various sources of nitrogen on fruiting body production by *Coprinus lagopus*. Recently Koch (1958) reported his investigations on the mycelial growth and fruiting body formation of *Polystictus versicolor*, *Polysporus annosus*, *Pleurotus ostreatus* and *Psalliota bispora*. Lucas *et al* (1958) found,

that Calvatia species have certain tumor-retarding properties. Consequently there has arisen a requirement for physiological studies with these organisms to provide a basis for further investigations of the circumstances under which Calvatia species will be able to produce agents active against tumors.

As far as Calvatia species are concerned, just a few reports dealing primarily with the taxonomy and morphology of this fungus have been mentioned in the literature. One of the first comparative descriptions on the Gasteromycetes has been reported by Hollos (1904). Since this publication, a number of reports have been published about the development of Gasteromycetes in different geographical zones, by Fries (1919) for South America, and by Garner (1956) for central America. Coker and Couch (1928) described the Gasteromycetes of the eastern United States and Canada; Cunningham (1944) the Gasteromycetes of Australia and New Zealand; Dennis (1953) the West Indian Gasteromycetes.

Schwartz (1933) discussed the morphological and taxonomical characters of several Lycoperdaceae. Ritchie (1948) investigated the development of the fruit body of Lycoperdon oblongisporum.

Various synthetic or semi-synthetic media have been developed for growing Basidiomycetes, but the nutritional aspects of Lycoperdaceae have not been studied extensively, and a chemically defined medium for their growth has not been developed.

An excellent growth of Calvatia gigantea has been observed by Stevens (1957) in modified Czapek-Dox medium. Successful attempts to germinate the spores of Calvatia species have not yet been reported. This fact eliminates the possible genetical approach to solve physiological problems

in the organism. Results of nutritional experiments can best be expressed by the mycelial growth of the fungus studied. Therefore, it seems to be necessary to determine the meaning of growth. Growth is an increase in mass or in number of cells, which requires time for its various manifestation. Cochrane (1957) claims that the precise definition of growth in fungi depends on the method of measurement used.

The Calvetia species used in this investigation were supplied and originally identified by Dr. J.A. Stevens of Michigan State University, East Lansing, Michigan. The identification was confirmed by Dr. A. H. Smith of the University of Michigan, Ann Arbor, Michigan. The following strains were employed in this study:

1. Calvatia gigantea (1018F) was obtained from an area five miles west of Lansing. The spores average 3.7 u, are globose or sphaerical with smooth walls and under high power minute echinulations can be observed.

2. Calvatia gigantea (1019B) was obtained from a woods on the Michigan State campus. The spores average 4.0 u, are globose or sphaerical with smooth walls and under high power minute echinulations can be observed.

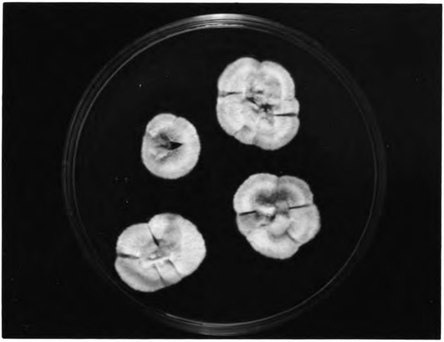
3. Calvatia gigantea (766) was obtained in Bronson, Michigan. It is thought to be close to Calvatia gigantea because of the following characteristics. The spores measure between 3.5-4.2 u, are nearly smooth and globose or sphaerical. The capillitium is long, thin, 6 u in diameter, and unbroken. It differs from the hundreds of other Calvatia gigantea species in that the peridium measured minimally 3-4 mm thick, which is at least twice the size of the normal Calvatia gigantea peridia. Culturally, the growth of this organism is typically flat, sparse and

relatively fast growing. This may be a new *Calvatia* species. It appeared to have characteristics midway between *Calvatia gigantea* and *Calvatia Bovista*. The capillitium of the latter is thick, 17 u in diameter and breaks up easily at maturity. In addition, the peridium is suggestive of *Calvatia pachyderma*, however, the spores of the species are ellipsoid and the capillitium curved like a boomerang.

4. *Calvatia fragilis* (1020) was obtained northwest of East Lansing. This organism is considered to be *Calvatia fragilis* based on strictly its morphological characteristics. The culture was isolated from an immature sporophore, which had the typical "top" shape of the species. Since no spores were available, its tentative identification was made on the basis of information obtained from the owner on whose property it was found; namely that in previous years sporophores arose in the same location and never grew larger than 4 cm high.

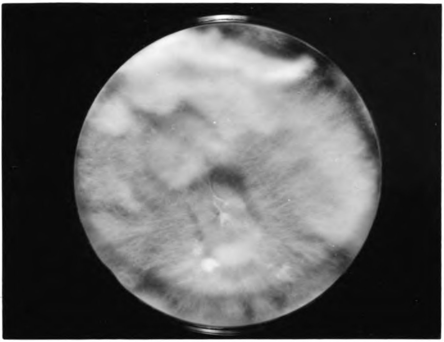
These four strains were chosen for this investigation because of their different types of growth and their responses to the Sarcoma 180 test as performed by the Sloan-Kettering Institute for Cancer Research. The photographs of the four strains of *Calvatia* illustrate the types of growth and the different growth rates. The cultural characteristics of these four strains are described below:

Strain 1018F. The mycelium-growth in the colony is a dense tan mat which shows submerged growth with furrows and cracks in the medium. The mycelial mat exhibits a colored zonation. The spores are smooth and spherical, with a diameter of 3.7 u. The mycelial growth at 24°C is 8 mm after 7 days; 21 mm after 14 days; 28 mm after 21 days and 44 mm after 28 days. It is a relatively slow growing strain. The sporophore extract

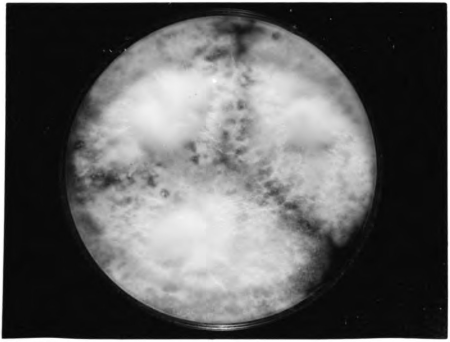


Three weeks old culture of <u>Calvatia gigantea</u> (1018 F)

Three weeks old culture of <u>Calvatia gigantea</u> (1019 B)



Three weeks old culture of Calvatia gigantea (766)



Three weeks old culture of Calvatia fragilis (1020) for this strain did not inhibit Sarcoma 180 in mice at a dilution of 1:25.

Strain 1019B produces a dense, tan matted colony which does not have any cracks in the agar. The diameter of the spores are 3.7 u, smooth and spherical. The mycelial growth at 24°C is 10 mm after 7 days; 22 mm after 14 days; 31 mm after 21 days; and 48 mm in 28 days. This strain is relatively slow growing. The sporophore extract for this strain inhibited ($\underline{+}^{-}$) Sarcoma 180 in mice at a dilution of 1:100.

Strain 766 has a thin, flat type of growth during the first 10 days, later developing aerial hyphae which are never so dense as the other strains. The mycelial growth at 24°C is 23 mm after 7 days, 51 mm after 14 days, 80 mm after 21 days and 90 mm after 28 days. It is considered a relatively fast grower. The sporophore extract from this strain has shown a strong inhibition ($\underline{+}^{+}$) of Sarcoma 180 in mice at a dilution of 1:120.

Strain 1020 exhibits a thin flat type of colony growth in 10 days. Toward the end of 18 days, the colony becomes cottony and granular. In addition, a water soluble brown pigment is produced. Mycelial growth at 24°C is 20 mm after 7 days; 49 mm after 14 days; 77 mm after 21 days; and 90 mm after 28 days. The rate of growth for this strain can be considered as relatively fast. The sporophore extract for this strain inhibited ($\underline{+}^{-}$) Sarcoma 180 in mice at a dilution of 1:10.

This work is limited to the physiology of *Calvatia* species. It deals with the effect of various growth promoting substances and carbon sources on the metabolism of the four strains.

PRELIMINARY EXPERIMENTS

In order to obtain certain necessary information to perform the investigation on the effects of the different growth factors and carbon sources, some preliminary experiments were made.

It is well known that internal and external environmental factors may affect the mycelial growth of a fungus. In the case of Calvatia species there is not much information about the internal factors as far as genetical constitution is concerned; but the age, source and kind of inoculum can be controlled. Among the external environmental factors, temperature, pH, and the type of nutrient in the medium have the most influence on growth of Calvatia. Therefore, the first two external factors were investigated at this time.

Temperature

Temperature affects every function of the organism, and has a very important effect upon enzyme systems. It is known, that the rate of enzyme activity, enzyme production and enzyme destruction are all increased by a rise in temperature up to a point. Slight changes in temperature may markedly alter the rate at which a certain nutrient is utilized.

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. The two extremes in temperatures indicate the temperature range for the organism, which varies to some extent with the various species. Most fungi do not grow or grow very slowly at 0°C, and they are usually unable to grow at temperatures above 30°-35°C. The optimum temperature for growth is

usually between 20°C and 30°C. There are, however, certain very striking exceptions to this generalization, in both directions.

It has to be considered that the reported optimum temperatures for the fungi are valid only under specified conditions of time, medium and method of measurement. That there is no single temperature optimum for growth has been stated by Cochrane (1958). A given metabolic process, respiration, antibiotic production or vitamin synthesis does not necessarily respond to temperature in the same way as does the process of growth. Fries (1953) mentioned an example of the dependence of temperature characteristics on other factors. He reported that Coprinus fimetarius grows poorly at 44°C because of the failure of methionine biosynthesis; however, if exogenuous methionine is supplied, growth at this temperature is normal.

Physiological experiments with Basidiomycetes are most often performed at 25°C. Optimum growth of some of the mycorrhiza forming Boleti has been found by Melin (1925) to occur at 25°C. Mikola (1948) found the same true for Cenoccocum species. Norkrans (1950) reported similar results with Tricholoma species. Certain other species preferred a slightly lower temperature. Several Mycena species showed the best growth at 20°C, according to Fries (1949). The coprophilic Psalliota bispora showed a good growth in a temperature range between 20°C and 27°C, with the optimum at 24°C as observed by Treschow (1944). The influence of temperature on the growth of seventeen different Coprinus species has been investigated by Fries (1956). About half the tested species showed optimum growth at 25°C. Two species preferred temperature as low as 15-25°C. All of the species which grew at higher temperatures

were coprophilic.

To determine the optimum temperature of Calvatia species studied, a simple agar plate method was used. To get comparable replicates and to avoid the drying out of the agar, equal amounts of 40 ml of modified Czapek-Dox agar (medium A), (see experimental), was poured into sterilized flat bottom Petri dishes. Mycelial plugs measuring 3.5mm were used as the inoculum. These were obtained from the growing edge of a 14-day old culture, using a sterile cork-borer. The excess agar was removed and the plugs were then placed in the center of a Petri dish, containing medium A agar. Quadruplicate plates were incubated at 8, 12, 16, 20, 22, 24, 26, 28, 30 and 37°C. The recording of the diameter of each growing colony at each temperature was made after 7, 14, 21 and 28 days.

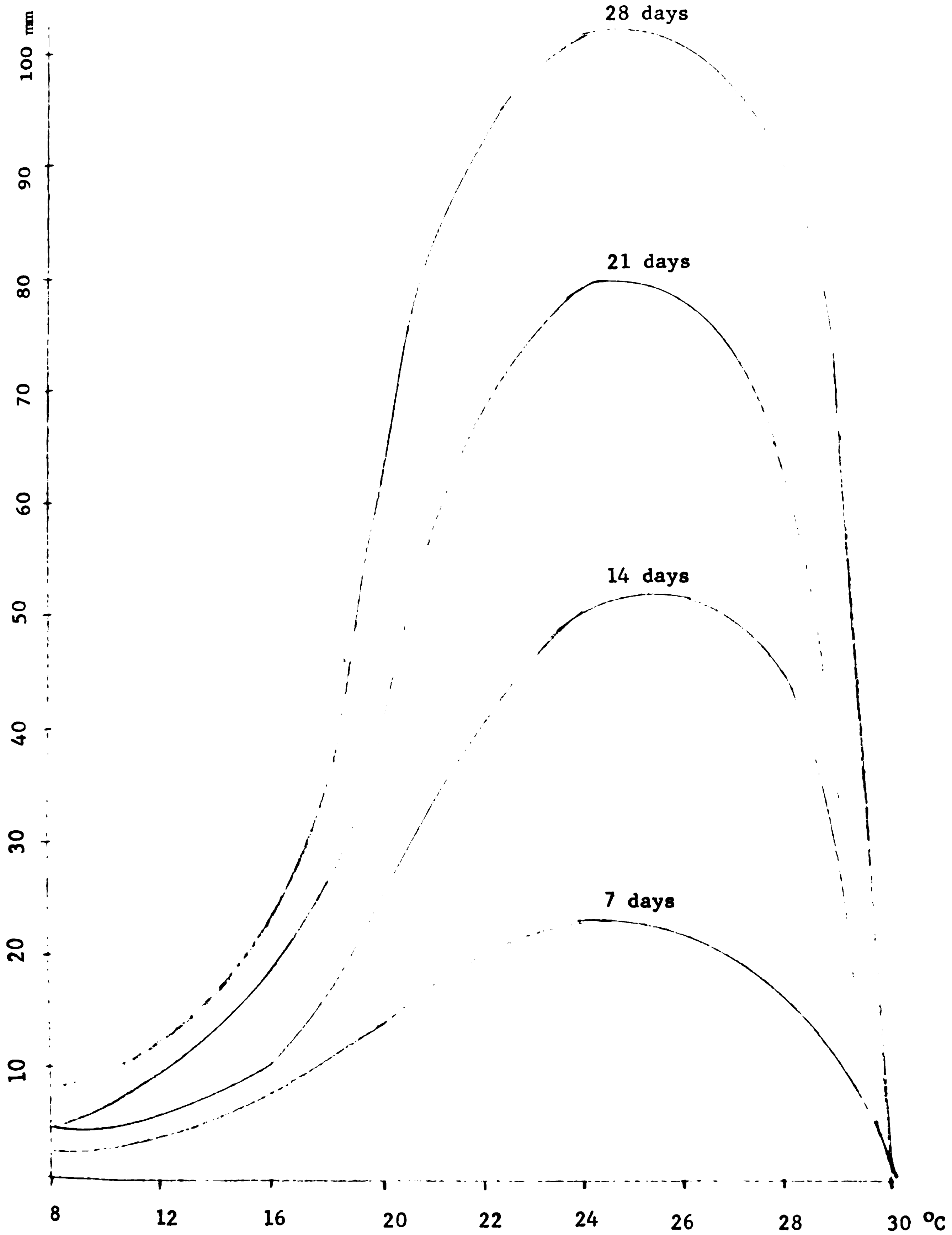
The average resultant values of a typical fast growing and a typical slow growing strain are shown diagrammatically. (Figure 1, 2). It was established from the results of the temperature investigations, that Calvatia species grew at temperatures ranging from 8°C to 28° C, with the optimum temperature range between 20°C and 24°C.

Stevens (1957) investigated the elaboration of the tumor retarding material of Calvatia maxima (gigantea) #642 by using shake cultures at five temperature levels: 16, 19, 22, 25 and 28°C. The result was that the active principle reached a maximum concentration on the 24th day of submerged culture at 19°C.

pH of the Medium

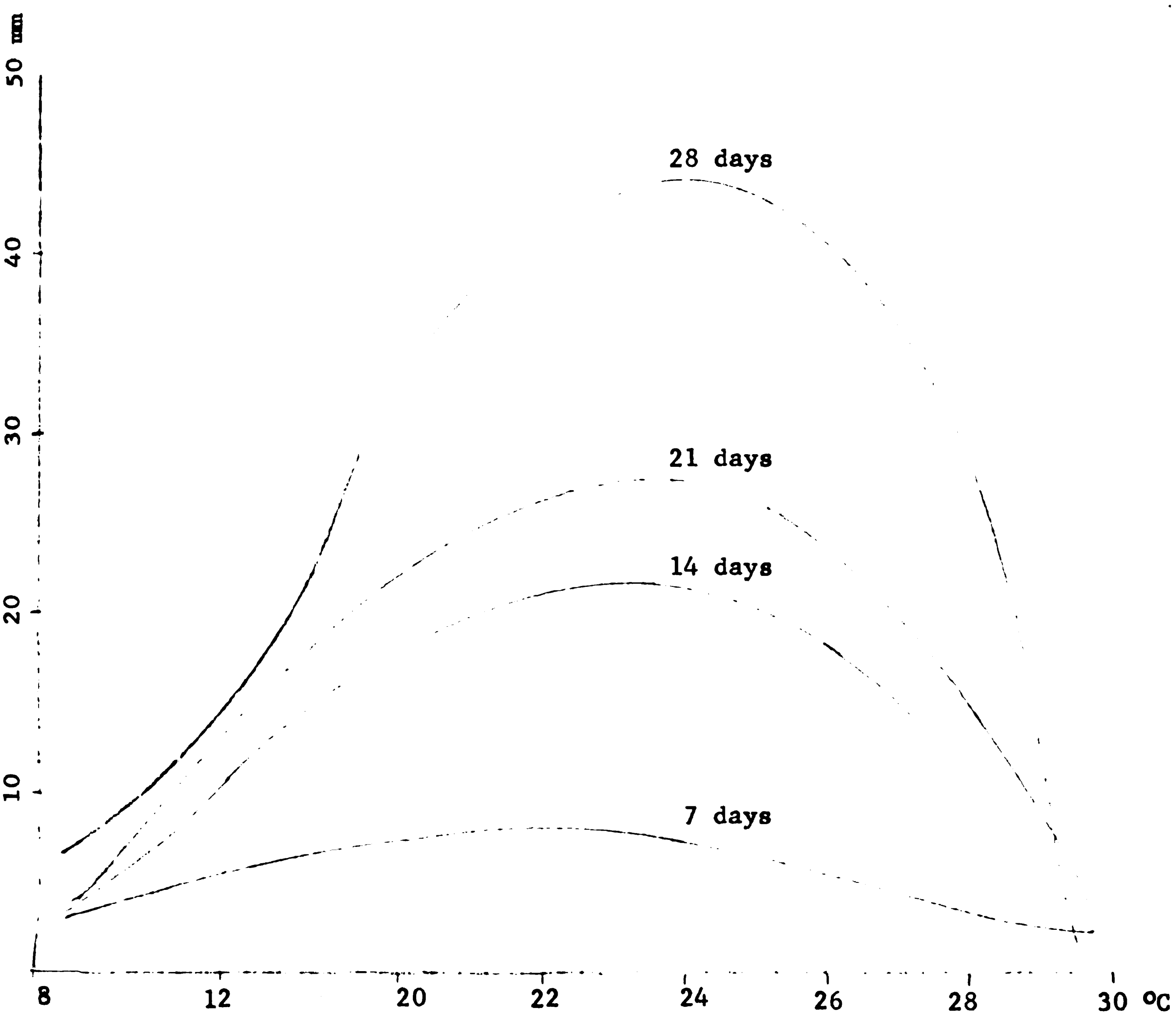
Most fungi will tolerate a wide range of hydrogen-ion concentration of the medium. Inhibition of growth is usually rather sharply defined at the limits of this range. Neutral or slightly acid reaction of the

FIGURE 1



The effect of temperature on the growth of <u>**Calvatia gigantea**</u> (766)

FIGURE 2



The effect of temperature on the growth of <u>Calvatia gigantea</u> (1018F) medium has been found the best pH for growth of most of the fungi. Growth is usually stopped on the acid side at pH 3 and on the alkaline side at pH 8-9. There are, however, exceptions to these generalizations.

Fungi, as a result of their metabolic activities, ordinarily change the pH of the media in which they grow. The pH is raised by absorption of anions or production of ammonia from nitrogenous compounds, and lowered by formation of organic acids or absorption of cations. These metabolic products of growth complicate pH experimental results, particularly in the poorly buffered media commonly employed. Since fungi differ in their metabolic activities and their rates of growth, the pH changes produced in the culture medium will differ too. The pattern of pH changes for the same fungus will depend upon the composition and concentration of the media used.

The changes in environmental factors, which affect the rate of growth of the organism such as temperature, time of harvest, gross changes in medium, growth factor supply, etc., may also affect the changes of pH of the culture medium.

As far as the Basidiomycetes are concerned, earlier investigations have shown that most species prefer an acid or neutral reaction of the medium as summarized by Wolpert (1924).

Modess (1941) surmised that the acidity of the habitat might be a guide to finding a most suitable pH range for cultivation of <u>Coprinus</u> species and, therefore, some samples of substrate from the habitats were collected to determine the required acidity of the fungi. His findings seemed to prove his hypothesis. In culture many of the larger Basidiomycetes are often unable to grow at an initial pH above 7.0.

Melin (1925) found that pH 5.0 was the optimal hydrogen-ion concentration for *Boletus variegatus*. Norkrans (1950) determined the optimum pH for *Tricholoma nudum* to be between 5.0-6.0. The optimal pH range was even narrower in case of *Fomes annosus*; it was between 4.6 and 4.9, as was observed by Etheridge (1955). Lindeberg (1944) reported the pH range of 5.7-6.4 as the optimal for most of the *Marasmius* species. However, he also reported that *Marasmius rotula* grew in culture and has been found in nature on substrates of a widely different pH. Several other fleshy Basidiomycetes require alkaline conditions for best growth. In nature *Coprinus* species grow on manure or soil rich in humus, which may indicate special demands for a less pronounced acid reaction of the environment, as was noted by Johnson and Jones (1941). They also found that *Coprinus cubensis* could grow at pH 5.9-9.2 on potato agar.

Fries (1956) investigated the pH requirements for different *Coprinus* species. She frequently found two pH optima for certain organisms, and between these two optima a depression at pH 7.5-8.0. This minimum merely reflects pH dependent on unavailability of one or more inorganic elements. The provision of iron, zinc and calcium in available forms eliminates this double optimum and replaces it with a broad single optimum zone. Fries concluded, from her results, that the coprophilic *Coprinus* species are extremely basiphilic and even other species of this genus prefer a more alkaline pH range than most other fungi.

The initial pH resulting in optimum growth of *Calvatia* species was determined by agar plate method. The initial pH was adjusted to varying values from pH 3.5-pH 7.0. The technic employed was the same as with the temperature experiments, but only one temperature, 24°C, was used.

The mycelial growth was measured after 7, 14, 21 and 28 days.

Figure 3 shows diagrammatically that the best mycelial growth was attained at initial pH 5.5, which was the unadjusted (natural) pH of both medium employed in this work on the physiology of Calvatia species. In most physiological studies it is necessary to have the pH controlled. In the case of Lindeberg's medium, M/25 phosphate buffer (KH_2PO_4) was employed. Lindeberg (1944) observed that a concentration up to 0.04 M phosphate had a sufficient buffering capacity and just a slight inhibitory effect for the higher Basidiomycetes. The use of calcium was found to reduce this slight toxic effect of phosphate.

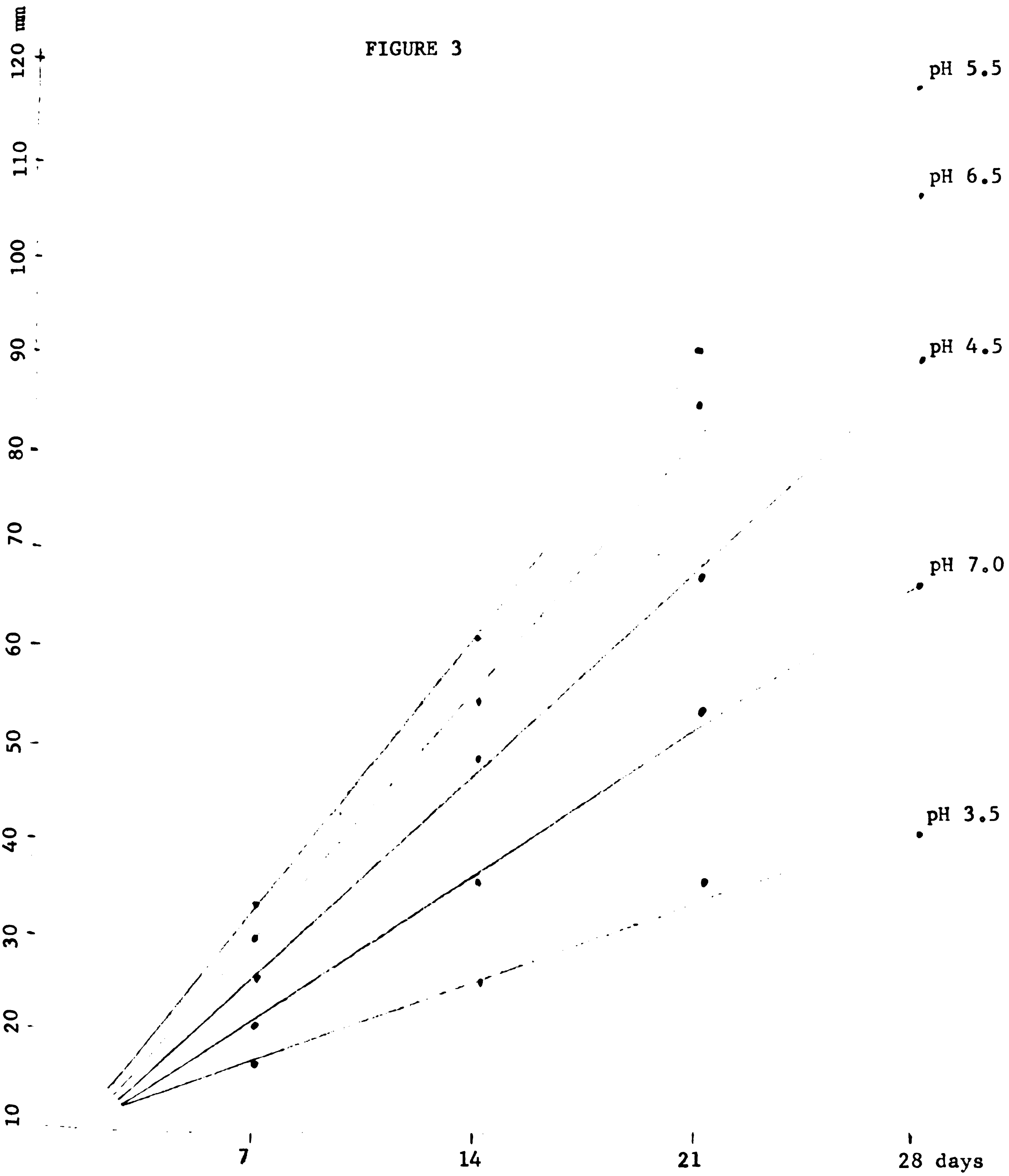
Nitrogen Requirement

In this physiological study of Calvatia species, the nitrogen requirement of these fungi was not investigated extensively. Therefore, Lindeberg's conclusion that ammonium-tartarate and asparagine are the best nitrogen sources to use in chemically defined medium for studying the higher Basidiomycetes has been assumed.

Later publications have confirmed this assumption. For example, Fries (1955) reported that ammonium-tartarate and asparagine gave the widest range of growth and the smallest change of pH in case of Coprinus species.

Lamprecht (1957) investigated the physiological influence of pH, concentration of constituents of the medium and the isoelectric point in the growth of Marasmius species. He considered ammonium-tartarate the best nitrogen source for these fungi. However, NH_4, NO_3 and KCN uptake was not dependent on the isoelectric point. He also found that the NO_3-uptake was better in lower pH and the pH close to neutral was more suitable for the NH_4-uptake.

FIGURE 3



The effect of various pH levels on the growth of <u>Calvatia gigantea</u> (766) at 24°C

To find out how the Calvatia species were able to utilize ammonium, nitrate and organic nitrogen, the growth response on four nitrogen sources were tested: ammonium-tartarate, asparagine, NH_4Cl, and KNO_3. In this experiment a quantity of nitrogen identical to that employed by Lindeberg (1944), 0.28 gram per liter was used for each source of nitrogen investigated. Ammonium-tartarate turned out to be the best of the four nitrogen sources tested as observed by growth studies on Calvatia species.

VITAMIN UTILIZATION BY CALVATIA SPECIES

Review of Literature

All living organisms, including fungi, require minute amounts of specific organic compounds for normal growth, reproduction and other vital processes, in addition to those which yield energy or are used for structural purposes. The cell may synthesize its own supply of one of these growth factors, i.e. vitamins, or it may be dependent in whole or in part on an exogeneous supply. So, organisms differ widely in their synthetic capacities for the various growth factors, i.e., vitamins.

Some fungi are self-sufficient with respect to growth factors. They are able to synthesize their vitamins from pure inorganic chemicals of a synthetic medium, autotrophic fungi. Others lack the ability to synthesize sufficient quantities of one or more growth factors and are called vitamin deficient fungi, heterotrophic fungi.

Both terms, growth factors and vitamins, will be applied to the same compounds, although the terms are not synonymous. The term growth factor has a broader meaning than vitamin. It includes the components and derivatives of some vitamins as well as other compounds which can not be classified otherwise at present. Many of the known vitamins have a catalytic function in the cell as coenzymes or constituent parts of coenzymes.

Schöpfer (1934) was one of the first investigators who studied extensively the vitamin requirements of the filamentous fungi. He recognized the thiamin deficiency of Phycomyces blakesleeanus. There are circumstances, when the cell depends absolutely on an external supply and at least over a certain range, growth will be proportional to the

supply of the required factor or factors. In this case, the deficiency is total, as it was reported for thiamine in *Phycomyces blakesleeanus* by Schöpfer (1934).

There are certain other fungi able to synthesize vitamins, but so slowly that under the usual condition of culture, the rate of all other processes is potentially faster than vitamin synthesis. In this case the organism grows slowly in the absence of exogeneous vitamin, but responds to an external supply by a faster rate of growth. Such partial deficiencies are also common, perhaps even more so than complete deficiencies, according to Lindeberg (1944) who observed partial deficiencies for thiamine in species of *Marasmius*. The degree of partial deficiency may vary widely from slight to nearly total and it is more pronounced during the early stages of growth.

Multiple requirements are especially common in the yeast, however, it can be found among the filamentous fungi also. The multiple deficiencies may be total or partial. Lindeberg (1946) found that *Collybia dryophila* was not able to assimilate the nutrient solution in the presence of either thiamine or biotin. But if thiamine and biotin were both added, a satisfactory growth was obtained. Absolute deficiencies are not known to be influenced by environment, while conditioned deficiencies may be affected by nutritional factors or by factors of the physical environment. According to Cochrane (1958) the known types of conditioned deficiency in fungi may be classified as follows:

1. The deficiency is apparent or more severe at particular temperatures, pH levels or salt concentrations.

2. The requirement for a vitamin is reduced, or more rarely,

eliminated by provision in the medium of a precursor or of a metabolite for the synthesis of which the vitamin is essential.

3. The deficiency is limited to, or more acute, at a particular stage of development.

Since temperature, vitamin supply and pH are closely associated with activities of enzyme systems, it seems logical that these factors might be closely associated in their effects upon growth. It is a possibility that fungi change in their synthetic capacity and in their needs for certain vitamins during development. It is quite possible that spore germination may require factors which mature mycelium can synthesize for itself.

In fungi the relative effect of the presence of vitamins in the medium usually is measured by the resultant vegetative growth, although vitamins are known to affect reproduction and other processes also. To find out the vitamin deficiency of a fungus, we must consider two important features as noted by Lilly and Barnett (1951):

1. The effect of different amounts of vitamin in the medium.

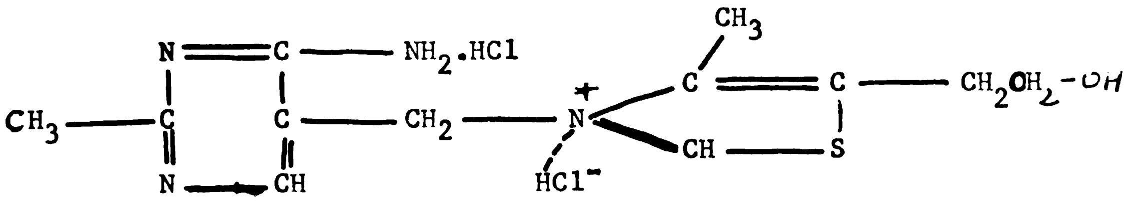
2. The response of the fungus over a period of sufficient duration to allow maximum growth.

Vitamin deficiencies among the fungi have been detected only for certain members of the water soluble B complex group. The most common vitamins are: thiamine, biotin, pyridoxine and inositol.

Thiamine

Thiamine was the first vitamin to have been studied as a known entity in the nutrition of fungi. Most of the filamentous fungi are deficient for this vitamin. Their requirement for thiamine varies widely depending on time of harvest, temperature, stage of development, composition of the

medium, etc., but for most fungi 100 γ of thiamine per liter of medium is near to optimum for growth and sporulation. The structural formula of thiamine is:

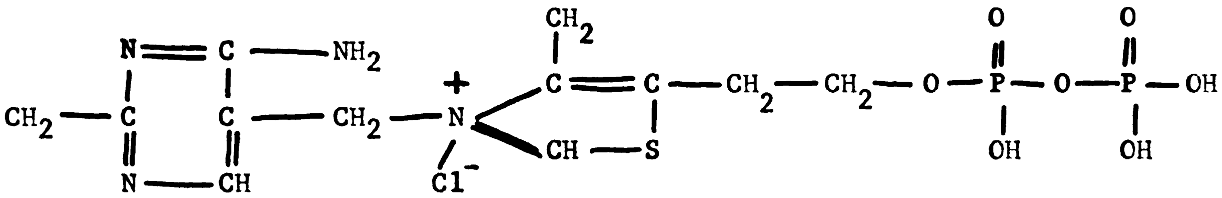


$C_{12}H_{17}N_4OSCl{\cdot}HCl$

Thiamine Chloride Hydrochloride

The thiamine molecule contains two rings, a substituted pyrimidine and a substituted thiazole. Thiamine has an important role in the regulation of carbohydrate metabolism of fungi and is undoubtedly involved in other metabolic functions as well.

The metabolic active form of thiamine is the pyrophosphate, long known as cocarboxylase because of its coenzyme function in the decarboxylation of pyruvic acid. Cocarboxylase or thiamine pyrophosphate is the pyrophosphoric ester of thiamine.



Thiamine Pyrophosphate

This molecule is the coenzyme or prosthetic group of the enzyme decarboxylase. It also participates in a variety of enzymatic reactions on α-keto acids, and it is also a coenzyme for transketolase (Jansen, 1954).

Pyruvic acid is one of the key intermediate products of carbohydrate metabolism; it is transformed into CO_2 and acetaldehyde by enzyme carboxylase.

$$\underset{\text{pyruvic acid}}{CH_3-CO-COOH} \xrightarrow[\uparrow\ \text{carboxylase (TTP)}]{} \underset{\text{acetaldehyde}}{CH_3-CHO} + CO_2$$

Lilly and Barnett (1951) reported that pyruvic acid accumulates in the culture medium of many thiamine deficient fungi when insufficient thiamine is present in the medium and at the same time the pH of the medium decreases. They found the accumulation of pyruvic acid in the culture medium in case of *Sordaria fimicola* and *lenzites trabea*, especially during the early period of growth. Haag and Dalphin (1940) mentioned that the maximum accumulation in *Phycomyces blakesleeanus* cultures occurred when about 1/20th of the optimum amount of thiamine was added. Nyman and Melin (1940) noted that all the examined species of Basidiomycetes were heterotrophic with respect to thiamine but autotrophic or self-sufficient with respect to biotin when grown in nutrient solution.

Robbins and Hervey (1955) reported *Stereum murraii* to be deficient for thiamine. Fries (1955) found in her physiological studies of *Coprinus* species that in these thiamine deficient fungi several degrees of thiamine heterotrophy can be observed. Hawker (1939) stated that thiamin also influences the reproduction of many fungi. Some grow fairly well, but remain sterile (i.e. no reproductive structures) in the absence of thiamine. Thus the requirements for sporulation are higher than those for growth. Fungi with moderate powers of synthesis can produce enough for vegetative growth, but not enough for sporulation. The role of

thiamine in the metabolism of pyruvic acid is clear from its acceleration of ethanol formation in Rhisopus cohnii, as mentioned by Schöpfer and Guillaud (1945). Fraser and Fugikova (1958) observed that the presence of thiamine was necessary for an appreciable growth response to the amino acids in the case of Agaricus bisporus.

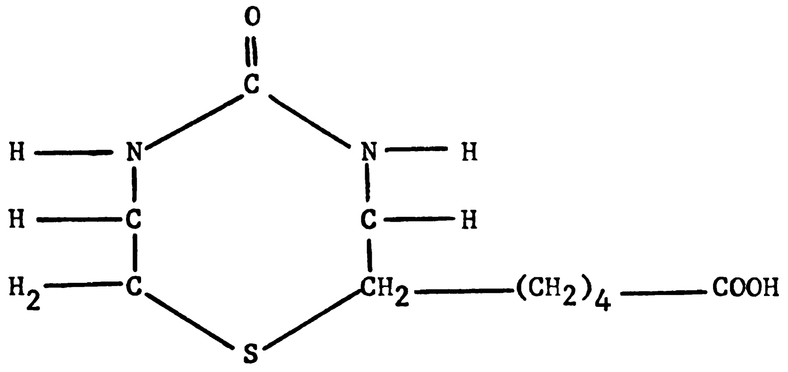
Shunt reactions are often found in mold metabolism in culture media. Wirth and Nord (1942) reported that the metabolic shunt in Fusarium lini cultures resulted in the accumulation of pyruvic acid in the medium. In these strains an induced cocarboxylase deficiency results in a retarded rate of pyruvate decarboxylation, as compared to the rate of the formation of this acid from carbohydrate. Addition of thiamine to the cultures restores the cocarboxylase level essential for maximum efficiency of carboxylase activity. So the bottleneck is eliminated and the pyruvic acid no longer accumulates in the culture medium. Grimm and Allen (1954) reported the effect of thiamine in promoting cytochrome synthesis in the case of Ustilago sphaerogena.

A great deal of work has been done with the thiamine deficient fungi which differ in their ability to utilize or synthesize the moieties of thiamine. The most common requirement is for the pyrimidine moiety according to Leonian and Lilly (1940). Most of the thiamine deficient fungi can synthesize thiazole and couple the two moieties to make the complete molecule.

Biotin

Biotin appears to be the most important growth factor for yeast, but numerous filamentous fungi have been reported to be deficient for this vitamin according to Burkholder and Moyer (1943). The structure of

biotin is:



Biotin $C_{10}H_{16}O_3N_2S$

Biotin is active at greater dilution than thiamine. The absolute requirement is usually less than 5 γ /liter of medium. Some species of Marasmius commonly occurring on the litter under forest trees require an external supply of thiamine, while others also require biotin. Some have a partial need of biotin so that growth in the presence of thiamine is further increased by the addition of biotin as has been concluded by Lindeberg (1941).

A number of enzymatic reactions have been discovered and analyzed in which biotin appears to participate in a direct or indirect manner. Gyorgy (1954) suggested that enzymatic action of biotin was related to the synthesis of asparatic acid. Later he also found that the oxidation of pyruvic acid was probably the result of faulty carbon dioxide transfer in the absence of biotin. Biotin is also involved in the deamination of certain amino acids and in the biosynthesis of oleic acid. It probably plays a role in the succinic acid dehidrogenase and amino acid oxidase.

Evidence from studies of Memnoniella echinata by Perlman (1948) and of Eremothecium ashbii by McNutt (1954) implicates biotin in the synthesis of asparatic acid, since asparate partially replaces biotin

in nutrition. Lardy et al (1947) reported that the inability of biotin-deficient Lactobacillus arabinosus to synthesize asparatic acid results from the failure to condense pyruvic acid and CO_2 to form oxalacetic acid which could then be transaminated to form asparatic acid.

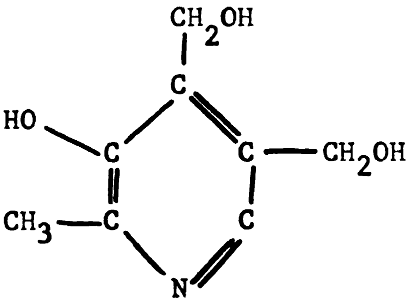
Another function of biotin is as a coenzyme in the fixation of CO_2 in living systems as has been mentioned by Foster (1949):

$$\underset{\text{pyruvic acid}}{CO_2 + CH_2\text{-}CO\text{-}COOH} \xrightleftharpoons[\text{oxaloacetic decarboxylase}]{\text{biotin}} \underset{\text{oxalacetic acid}}{HOOC\text{-}CH_2\text{-}CO\text{-}COOH}$$

A considerable portion of the oxalacetic acid so produced is aminated to yield asparatic acid which is utilized for protein synthesis. Another probable function of biotin in fungi is in the synthesis of essential fatty acids. According to Hodson (1949) oleic acid partially replaces biotin for a Neurospora crassa nutant. The same is true for Ophiostoma pini described by Mathiesen (1950). Mandels (1955) reported that growth of Myrothecium verrucaria on glucose agar is interrupted shortly after germination. Biotin at 10^{-3} - 10^{-4} γ /liter induces continued growth after germination. It has been shown that biotin is released from the spores after germination and that growth then stops due to a deficiency within the cells. Growth is resumed when biotin accumulates in the environment of the sporling to a sufficient concentration.

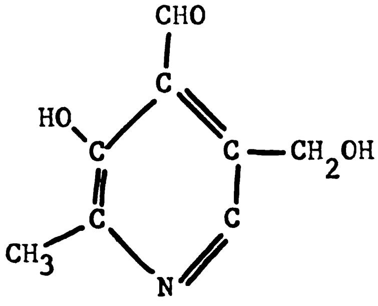
Pyridoxine or Vitamin B_6

Certain fungi are able to synthesize pyridoxine while others require it for growth, but there are not so many pyridoxine-less fungi as there are fungi deficient for thiamine or biotin.



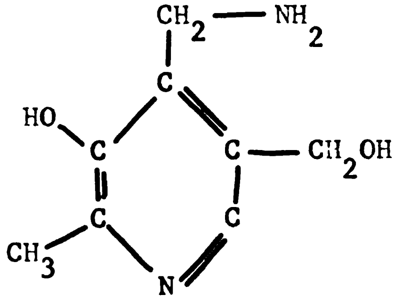
Pyridoxine

$C_8H_{11}O_3N$



Pyridoxal

$C_8H_9O_3N$



Pyridoxamine

$C_8H_{12}O_2N_2$

The structure of the compound is shown along with two other derivatives, an aldehyde and an amine which are found naturally and are active vitamins. That pyridoxine requirement is highly specific in Ophiostoma has been determined by Robbins and Ma (1942). Fries (1943) and (1950) investigated the role of pyridoxine in promoting the growth of certain Ascomycetes. Pyridoxine was the only vitamin that was required by all species investigated.

Pyridoxine requirement for Basidiomycetes has not been mentioned in the literature. The deficiency for this growth factor is very characteristic for many Ascomycetes. The fungi can be partially or totally deficient for this vitamin. Some species reported to be deficient for pyridoxine but this organism was also heterotrophic with respect to other vitamins. The presence of one vitamin for which a fungus is partially deficient may enable the fungus to synthesize other vitamins with greater ease. Stokes et al (1943) found a relationship between pyridoxine and thiamine metabolism in Neurospora sitophila. That is evident from the fact that at any given level of pyridoxine nutrition the mutant - deficient for pyridoxine and thiamine shows a growth response proportional to the amount of thiamine added,

although some growth is made in the absence of thiamine. The effect is appreciably greater at low levels of pyridoxine nutrition. Stokes suggested that apart from its own coenzyme functions, pyridoxine participates in the synthesis of thiamine. The same relationship between pyridoxine and thiamine has been noted by Tatum and Bell (1946) in *Neurospora crassa*. Harris (1956) explained this phenomenon: pyridoxine inhibits the biosynthesis of thiamine by preventing the incorporation of the pyrimidine moiety; but thiamine competitively inhibits the endogenous destruction of pyridoxine.

The action of pyridoxine appears to be connected with either amino acid synthesis or amino acid utilization or both. Like the other vitamins, pyridoxine also acts in the cell as a part of a coenzyme. The coenzyme form of pyridoxine is pyridoxal-5-phosphate; it constitutes prosthetic groups of metabolic enzymes: decarboxylases, transaminases and racemases.

Pyrodoxine is also involved in the synthesis and metabolism of tryptophane, described by Umbreit *et al* (1947). Silver and McElroy (1954) reported that pyridoxine-less mutants of *Neurospora crassa* accumulate nitrite from nitrate, indicating that pyridoxal-5-phosphate may have something to do with nitrate reduction ($NO_3 \rightleftarrows NO_2$).

According to Snell (1945) pyridoxine, pyridoxamine and pyridoxal are equally used by the filamentous fungi. The two derivatives can easily be converted in the cell to pyridoxine. Snell also discovered that autoclaving pyridoxine with the basal medium for 20 minutes increased the activity of pyridoxine forty-one times and that this change in activityfor certain organisms was correlated with oxidation and

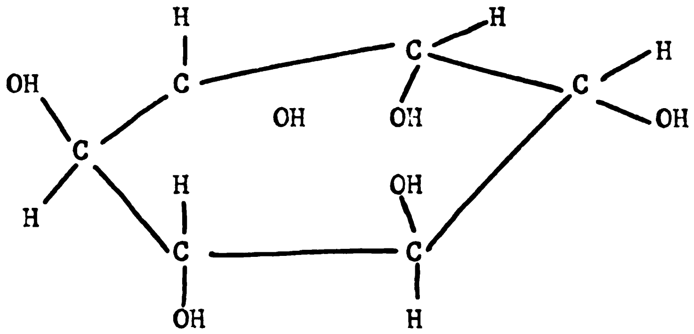
heating with certain amino acids.

Myo-Inositol

Seven optically inactive forms and a pair of active isomers of hexa-hydroxy-cyclo-hexane can exist, but only one of the inactive forms has biological activity.

Meso (i) - inositol

(hexahydroxy - cyclohexane)



Deficiency for inositol is not so common in filamentous fungi as are the requirements for other vitamins. Kögl and Fries (1937) reported that there are more fungi partially deficient for inositol than completely deficient. Partial deficiency was determined for inositol in Sclerotinia camellia by Lilly and Barnett (1948) but the response to inositol was conditioned by temperature. Deficiencies for inositol are usually accompanied by deficiencies for thiamine and biotin. Shirakova (1955) concluded that Diplocarpon rosae is totally deficient for thiamine and partially for inositol. The same was found earlier in certain Marasmius species by Lindeberg (1939). Melin (1946) mentioned partial need for inositol in certain mycorrhizal fungi.

Inositol is active only in high concentration, therefore, the amount of the inositol requirement is much greater than that of other vitamins (5 mg/liter). It has been suggested by Lane and Williams (1948) that inositol is an active part of pancreatic amylase. But later on it has been found that the function of inositol as a coenzyme in this system is very doubtful. Fuller and Tatum (1956) reported that in Neurospora crassa most of the inositol present is bound in the form

of phospholipids. The inositol-less mutant strain, which results from an inositol deficiency, has a characteristically low level of inositol-phospholipid compared to the wild type. Therefore, it was suggested that inositol is a structural component of the cell.

No general function of inositol is known in fungi. However, a favorable effect of inositol on the growth of certain fungi which are inhibited by the presence of excess thiamine has been observed. Schöpfer (1945) mentioned that in Rhizopus sinuis inositol overcame the inhibition of growth due to excess thiamine. The high specificity of inositol requirement was described and discussed by Lardy (1954). Snell (1954) concluded that even though inositol is required for growth of certain microorganisms, nothing is known of the essential metabolic role played by it; no distinctive metabolic aberrations due to its lack have been reported. Presumably in those organisms inositol may be required for the formation of essential lipid components of the cell.

Experimental Procedure

In this study, three strains of *Calvatia gigantea* and one strain of *Calvatia fragilis* were utilized: *Calvatia gigantea* 1018F; *Calvatia gigantea* 1019B; *Calvatia gigantea* 766 and *Calvatia fragilis* 1020.

Pyrex Erlenmeyer flasks of 125 ml capacity were used as culture vessels. The glassware was treated with sulfuric acid-dichromate cleansing solution, rinsed with top water and finally with distilled water. Each flask received 25 or 40 mls of medium according to the method used. The flasks were stoppered with non absorbent cotton and sterilized by autoclaving 15 minutes at 15 pounds steam pressure.

The completely synthetic basal medium which was used is Lindeberg's medium:

Glucose	20.0 gram
NH_4-tartarate	5.0 gram
KH_2PO_4	1.0 gram
$MgSO_4.7H_2O$	0.5 gram
$FeCl_3$(sol 1/500)	0.5 ml
$ZnSO_4$(sol 1/500)	0.5 ml
$MnCl_2$(sol 0.1 M)	0.5 ml
$CaCl_2$(sol 0.1 M)	5.0 ml
H_2O distilled	955.0 ml

The pH was determined electrometrically and adjusted to 5.5 before autoclaving.

The vitamins were added to the basal medium in the following quantities:

Thiamine hydrochloride	100 γ/liter

Pyridoxine hydrochloride	100 γ/liter
Biotin crystalline	5 γ/liter
i-inositol	5000 γ/liter

A stock solution was made for each vitamin, and the amounts required for each experiment were obtained from this source. This stock solution was kept refrigerated.

A modified Czapek's-Dox's formula, which Stevens (1957) called "Medium A", was used as the control for each experiment. It has the following composition:

Glucose	15.0 gram
Sucrose	15.0 gram
Bactopeptone	5.0 gram
Bacto-yeast	5.0 gram
$Mg\ SO_4.7H_2O$	0.5 gram
KH_2PO_4	1.0 gram
KCl	0.5 gram
$Fe\ SO_4.7H_2O$	0.01 gram
H_2O (Distilled)	1000 ml
Final pH (not adjusted)	5.5

Henceforth in this thesis the author shall also refer to this medium by the title "Medium A". A solid version of this medium was made by adding 1.5% Bacto-Agar.

Two methods of inoculation and incubation were employed and compared in this investigation.

1. Floating method (Lindeberg 1944)

The inoculum for the experiment consisted of 3.5 mm discs cut from the growing edge of a 14-day old culture, grown on "Medium A" agar in Petri dishes. The discs of inoculum were floated on the surface of 25 ml liquid medium in 125 ml Erlenmeyer flasks. The inoculated culture vessels were incubated stationary at room temperature.

2. Shake method (Derrick 1952)

In order to obtain reproducible quantitative results among replicate cultures, Kluyver's (1933) method was used to prepare a standard inoculum. To produce the inoculum for an experiment, mycelium from stock cultures was transferred into 40 ml of "Medium A" solution and grown for 14 days on a reciprocating shaker. The shaking machine had a stroke of 0.5 inches, and gave 100 one-inch excursions per minute.

The mycelial pellets of the 14-day old shake cultures were fragmented for 30 seconds in a sterile Monel metal semi-micro Waring blendor. The material in the resulting homogenous suspension was washed with sterile distilled water and centrifuged at 4000 rpm for 10 minutes. This procedure was repeated twice to give a total of three washings. The last resuspension was standardized by suspending 1 part mycelium fragments with 30 parts of distilled water.

This first step inoculum was then used to prepare the second step inoculum which was to be used to inoculate the experimental flasks. This was accomplished by putting the first step inoculum into Lindeberg's medium and allowing the mycelium to grow for 14 days on shaker. The mycelium thus produced was then fragmented, washed, centrifuged and resuspended (as described previously) and this constituted the second step inoculum used for the vitamin studies. It was felt that by employing this technique, carry over of the nutrients could be avoided.

The density of the inoculum was determined with a Klett-Summerson photoelectric colorimeter using the green filter (500-570 mμ). Each flask was inoculated with 1.0 ml of the final blended suspension which gave a reading of 12-15% transmission under the above described condition.

The cultures were incubated at approximately 25°C on one reciprocating shaker, described previously, with no additional aeration other than that caused by the continuous agitation.

The length of incubation necessary for optimum growth for each method was determined previously by running a series of growth curves.

The contents of each flask were filtered on tared Whatmann No. 1 filter using a Buchner funnel. The mycelial pellets were washed with distilled water to remove any excess medium and dried at 96°C in an oven for 24 hours.

In every experiment, four, or sometimes even more, replicate cultures were set up for each treatment. All quantitative data are based on the average dry weight of mycelium produced in the test medium in quadruplicate flasks. In all the experiments, the pH was checked at the conclusion.

DISCUSSION AND RESULTS

The experiments with the different vitamins show that all four tested strains of *Calvatia* are totally deficient for thiamine (Table 1 and 2), and that *Calvatia* species do not grow on a purely synthetic medium. Upon the addition of crystalline thiamine (100 γ /liter of medium) a good growth of mycelium was produced. The addition of biotin, pyridoxine or inositol singly to the basal Lindeberg medium did not add greatly to the growth, and if it did, just a very small amount of sparse mycelium developed. *Calvatia* species are heterotrophic with respect to thiamine, i.e., they are unable to synthesize this vitamin. Luxurious growth depends on an external supply of this growth factor.

There are no known environmental conditions for *Calvatia* species which allow the synthesis of thiamine as far as different temperatures, different pH and composition of the medium are concerned. According to these results, it can be assumed that the deficiency of *Calvatia* species for thiamine is absolute.

The combinations of different growth factors show that *Calvatia* species have only this single deficiency. (Table 3). When thiamine and biotin both were present in the basal medium, all four strains of *Calvatia* species were able to assimilate the nutrient solutions the same way as when thiamine alone was present, as evidenced by the mycelial yields. The yields were even a little less in most instances following the addition of biotin and thiamine. Biotin alone produced very little mycelial growth compared to the yield in the control (synthetic media).

It is well known that some vitamins have a depressing effect on growth of certain fungi not deficient for these particular vitamins.

TABLE 1 - The utilization of vitamins in surface culture by *Calvatia* sp. (The average mycelial dry weight of four replicates.)

Incubation time: 28 days

	BIOTIN		THIAMINE		PYRIDOXINE		INOSITOL		CONTROL	
Strains	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	22	5.3	50	4.7	11	5.2	10	5.3	11	5.3
1019 B	18	5.3	42	4.8	15	5.5	18	5.4	11	5.5
766	7	5.5	22	5.2	3	5.5	2	5.5	3	5.5
1020	29	5.3	60	4.9	23	5.4	20	5.4	16	5.5

TABLE 2 - The utilization of vitamins in submerged culture by *Calvatia* sp. (The average mycelial dry weight of four replicates.)

Incubation time: 28 days

	BIOTIN		THIAMINE		PYRIDOXINE		INOSITOL		CONTROL	
Strains	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	7	5.0	40	4.5	4	5.0	2	5.0	6	5.0
1019 B	8	5.2	27	4.5	4	5.5	3	5.5	4	5.5
766	4	5.5	10	5.0	0	5.5	0	5.5	2	5.5
1020	7	5.5	58	5.0	6	5.5	4	5.5	3	5.5

TABLE 3 -- The utilization of vitamin combinations by Calvatia sp. (The average mycelial dry weight of four replicates.)

Incubation time: 28 days

Strains	THIAMINE		THIAMINE & BIOTIN		THIAMINE & PYRIDOXINE		CONTROL	
	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	40	4.5	37	4.5	30	4.8	6	5.0
1019 B	27	4.5	25	4.5	23	4.8	4	5.5
766	10	5.0	9	5.0	8	5.3	2	5.5
1020	58	5.0	44	5.0	39	5.0	3	5.5

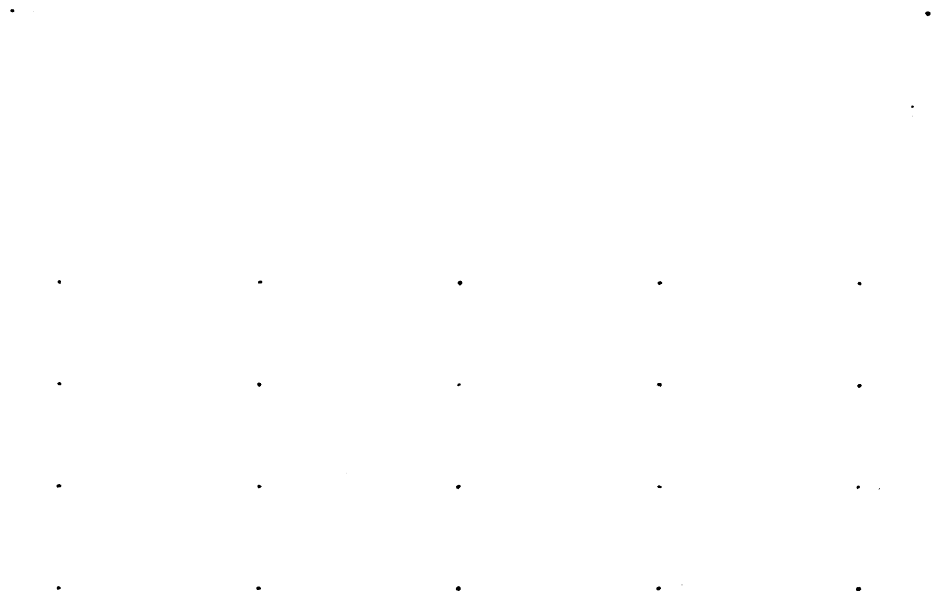
TABLE 4 - Variations in the basal medium. (The average mycelial dry weight of three replicates.)

Incubation time: 15 days (in Medium A)

Strains	Medium A		Medium A —PEPTONE		Medium A -YEAST EXTR.		Lindeberg's + THIAMINE*		Lindeberg's CONTROL	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	300	5.5	48	4.2	250	5.3	40	4.5	6	5.0
1019 B	280	6.0	55	4.5	140	5.5	27	4.5	4	5.5
766	66	5.5	37	5.3	47	5.5	10	5.0	2	5.5
1020	407	5.5	204	6.5	492	5.5	58	5.0	3	5.5

---

* inoculation time: 28 days



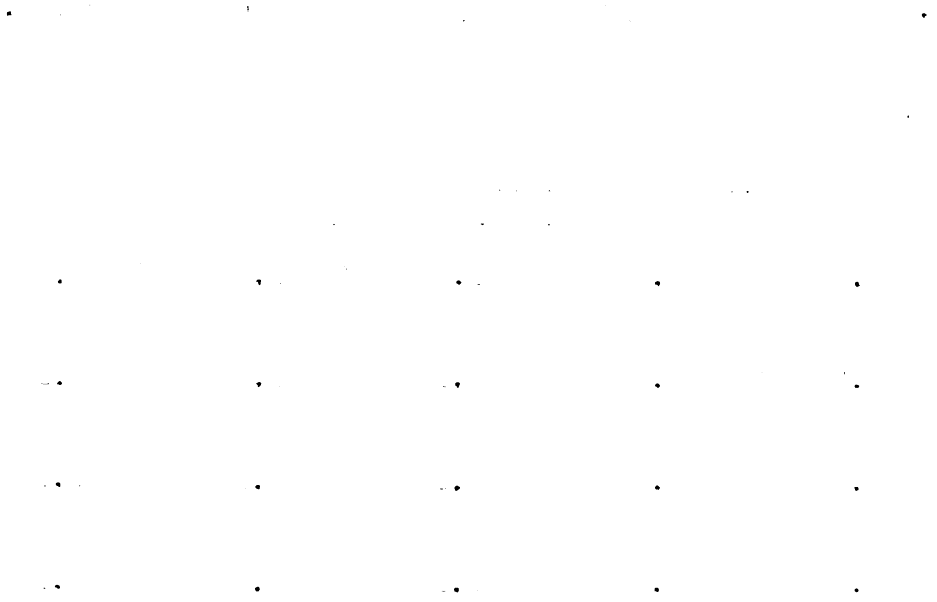


TABLE 3 -- The utilization of vitamin combinations by Calvatia sp. (The average mycelial dry weight of four replicates.)

Incubation time: 28 days

Strains	THIAMINE		THIAMINE & BIOTIN		THIAMINE & PYRIDOXINE		CONTROL	
	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	40	4.5	37	4.5	30	4.8	6	5.0
1019 B	27	4.5	25	4.5	23	4.8	4	5.5
766	10	5.0	9	5.0	8	5.3	2	5.5
1020	58	5.0	44	5.0	39	5.0	3	5.5

TABLE 4 - Variations in the basal medium. (The average mycelial dry weight of three replicates.)

Incubation time: 15 days (in Medium A)

Strains	Medium A		Medium A —PEPTONE		Medium A -YEAST EXTR.		Lindeberg's + THIAMINE*		Lindeberg's CONTROL	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	300	5.5	48	4.2	250	5.3	40	4.5	6	5.0
1019 B	280	6.0	55	4.5	140	5.5	27	4.5	4	5.5
766	66	5.5	37	5.3	47	5.5	10	5.0	2	5.5
1020	407	5.5	204	6.5	492	5.5	58	5.0	3	5.5

---

* inoculation time: 28 days

Shirakova (1955) found that Diplocarpon rosae produced more dry weight in the absence of biotin than when this vitamin was added. Biotin may reduce growth or enzyme formation in the fungus, Calvatia species must be self-sufficient with respect to biotin. They apparently are able to synthesize this vitamin from the chemical constituents in the basal medium. An external excess supply of biotin in the presence of thiamine may even depress the growth of the organism.

The addition of thiamine and pyridoxine to the Lindeberg medium produced an even more noticeable loss of mycelial weight, when this was compared to the growth resulting when thiamine alone was present. Pyridoxine alone resulted in very poor mycelial growth. Calvatia strain 1020 grew somewhat better than the control; Calvatia strains 1018 F and 1019 B were about the same as the control, and Calvatia strain 766 did not grow at all in shaker culture. It can be concluded that Calvatia species either do not require pyridoxine during metabolism or they are capable of synthesizing this vitamin from the constituents of the medium. Similar cases have been reported in the literature. Fries (1943) mentioned that in certain species of Ophiostoma, both the rate of growth and the maximum amount of mycelium were greater in pyridoxine-free medium than when this vitamin was added.

The use of inositol as a growth factor in the Lindeberg medium resulted in very poor mycelial growth. In most experiments, the presence of inositol apparently caused a smaller amount of mycelial development than produced in the control. It can be presumed that inositol may have a certain inhibitory effect on the metabolism of Calvatia species. The mechanism of this inhibition is not known and

has not been studied yet. Because of the results discussed above, it did not seem necessary to combine inositol with any of the other vitamins.

There are no reports in the literature concerning any organism requiring inositol as a coenzyme function. It is doubtful whether the compound should be considered as a vitamin. Fuller and Tatum (1956) suggested, as was mentioned before, that inositol is involved in a structural component of the cell.

Judging from the results which appear in Table 4, there is an indication that in addition to the thiamine requirement, Calvatia species have a partial deficiency for an unidentified growth factor(s) present in peptone and yeast extract. In medium A, the four strains of Calvatia had 7 - 10 times more mycelium on a dry weight basis than in Lindeberg's medium plus thiamine.

After comparing the chemical components of the two media, one concludes that the slightly greater amount of sucrose in medium A could not be responsible for the differences in yields. The presence of an unknown growth factor in peptone and yeast extract seems to be evident because of the favorable effect on growth following the addition of 5 grams of Bacto-peptone and 5 grams of Bacto-yeast extract per liter to a medium containing the adequate amount of available carbon, nitrogen and trace elements. The hypothetical substance is apparently water soluble and thermostable since it is resistant to autoclaving. It may be a single compound but more likely several. This (these) could be in addition to those growth factors already observed in a typical analysis of Bacto-peptone and Bacto-yeast extract which appears in the appendix.

The presence of unidentified growth factors in natural media is not an unknown phenomenon as evidenced by the various reports. Yusef (1953) found that the addition of small amounts of malt extract to the basal medium containing vitamins and casein hydrolysate, markedly increased the growth of different *Polyporus* species. He suggested that the presence of unidentified growth-stimulating factors in malt extract ("malt factor") appeared to play a role in the nutrition of this fungus. Melin and Das (1954) observed that roots of pine and other plants exert a strong growth-promoting effect on tree-mycorrhizal Basidiomycetes. They concluded that the roots produce one or more metabolites designed as "factor M", which are essential to the growth of these fungi. This growth factor could not be replaced by either the vitamins of the B complex group or the amino acids in casein hydrolysate, or by the components of hydrolysed yeast nucleic acid. Melin (1959) reported that a mixture of nucleic acid components, however, seemed to lower the growth-promoting effect of the "M factor".

Comparing the results reported in Table 4, it is noticeable that with one exception (strain 1020), the strains of *Calvatia* gave the best growth when Bacto-peptone and Bacto-yeast extract were both present in medium A. However, in all four cases the yield was much lower when Bacto-yeast extract was the sole additive with peptone lacking. The Bacto-yeast extract results reported above can be due to pH changes of the media during incubation in the absence of the buffering effect of peptone, or it may be an actual inhibitory effect by the yeast extract which can be overcome by the addition of peptone. Judging from the results obtained with strain 1020, the latter supposition seems to be more likely because, in spite of the buffering effect of peptone, the organism gave a better growth in the absence of yeast extract.

MacLead (1959) discovered while determining the optimum yeast-extract-dextrose concentration for *Hirsutella gigantea* that concentrations higher than 1.5% yeast extract appeared to have an inhibitory effect, and at 2.5% very little growth was produced by the fungus during the first 12 days of incubation. Growth began shortly thereafter and at the end of 16 days there was a substantial yield. Thus it would appear that yeast extract has an inhibitory effect in the early stages of growth. At the same time, in view of the fact that some growth eventually takes place, *Hirsutella gigantea* must adapt itself to the high yeast-extract concentration. In the case of *Calvatia* species, the adaptation of strain 1020 to yeast extract was not an important problem and was not investigated.

Fries (1950) mentioned the superiority of yeast extract to thiamine alone for the growth of some Basidiomycetes, but he emphasized the fact that yeast extract contains inhibitory compounds as well as unidentified beneficial substances. The effect of different concentrations of thiamine has been investigated (Table 5). Increasing the thiamine concentration from 25 γ /liter of medium to 150 γ /liter of medium did not show significant differences in the amount of mycelial yield in the four tested strains of *Calvatia* species.

A review of the literature disclosed that there are contradictions about the value of surface (floating) culture and submerged (shaker) culture methods for growing fungi. Several investigators have attributed various effects to the use of a disc, floating on the surface of the

TABLE 5 - Different thiamine concentrations on the growth of Calvatia species. (The average mycelial weight of four replicates.)

Incubation time: 30 days

	150 /1		100 /1		50 /1		25 /1	
Strains	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	49	4.5	48	4.5	48	4.5	45	4.5
1019 B	30	4.5	27	4.5	28	4.5	23	4.6
766	11	5.2	11	5.2	11	5.2	10	5.2
1020	74	4.9	69	4.9	70	4.9	67	5.0

medium. Also several authors have claimed that nutrients could be carried over in this fashion and, therefore, the submerged culture method has been accepted as superior to the surface method.

Leonian and Lilly (1939) found that the food material and growth factors present in the agar-disc inoculum do not exert an appreciable influence upon the growth of the colony. They also noted that the size of the inoculum had no effect on the growth of the culture of several organisms.

Margolin (1942) reported that after calculating the amount of auxithals in a disc of agar transferred into 20 mls of nutrient solution, the required minimum for growth was found to be infinitely larger than any amount that can be transferred in this fashion. He based this on the assumption that the growing fungus did not destroy any of the auxithals in the medium.

Later on, the use of fragmented mycelium in the culture of fungi seemed to be considered as a superior method of preparing inoculum. Savage and Vander Brook (1946) emphasized the importance of the fragmentation of the mycelium by a high speed blender and evaluated the blended inoculum as opposed to floating discs. Dorell and Page (1947) observed that a closer check of replicate cultures and a shorter lag period in initiation of growth are attained if fungal mycelium is fragmented rather than employed as a floating disc. Kitay and Snell (1948) concluded that time of blending, washing and suspending of fragments and amount of suspension used for inoculum are important influencing factors.

Foster (1949) compared the physiology of surface with submerged

growth. He indicated that submerged growth cultures theoretically and practically afford the closest approach to the ideal method of studying mold metabolism, although even these experimental conditions differ considerably from those of the natural habitat of the fungus. According to Foster (1949), surface culture is entirely inadequate because it represents the overall result of the metabolic processes of a heterogeneous mixture of physiological systems. Submerged culture, however, provides physiologically homogeneous fungal material, since all cells are uniformly exposed to the environmental factors, both physical and chemical, during the growth period.

Derrick (1949) reported that the organisms may store or absorb several times their requirements of vitamins when grown on a vitamin or a vitamin-rich medium. The amount of inoculum transferred to a vitamin deficient medium, therefore, is critical and repeated transfers are necessary to eliminate any carry over of vitamins. Wiken _et al_ (1951) compared the two methods of inoculation and incubation and proved the superiority of the submerged growth statistically.

The morphological alteration of several fungi in submerged shaken cultures have been reported by Burkholder (1945) - as a morphogenetic variation. Foster (1949), however, questioned whether this phenomenon should be considered as morphogenesis, since the curious growth pattern is not a fundamental link in the biological development of the organism but rather a manifestation resulting from these peculiar physical conditions.

From the results shown in Table 6, it is evident that in spite of the differences in the amount of the mycelial yield given by the floating

TABLE 6 - The statistical comparison of the surface and submerged method.

	CONTROL				BIOTIN				THIAMINE				BIOTIN + THIAMINE				PYRIDOXINE				INOSITOL			
	I		II		I		II		I		II		I		II		I		II		I		II	
Strains	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$
1018 F	6	± 0.9	11	± 1.9	7	± 1.2	22	± 4.3	40	± 4.5	50	± 6.0	37	± 3.7	49	± 7.8	4	± 0.6	11	± 1.8	2	± 1.1	10	± 2.9
1019 B	4	± 0.6	11	± 2.4	8	± 0.9	18	± 6.2	27	± 0.7	42	± 5.2	25	± 1.2	36	± 2.8	4	± 1.1	15	± 3.3	3	± 1.1	18	± 3.4
766	2	± 0.4	3	± 0.6	4	± 0.3	7	± 1.3	10	± 0.9	22	± 5.4	9	± 0.7	19	± 2.5	0	-	3	± 1.1	0	-	2	± 0.6
1020	3	± 0.6	16	± 1.6	7	± 1.3	29	± 7.5	58	± 1.0	60	± 6.1	44	± 5.4	56	± 9.3	6	± 0.5	23	± 4.3	4	± 0.6	20	± 2.8

I submerged culture (i.e. method)

II surface culture (i.e. method)

$\bar{x}$ arithmetic mean (average of replicates)

$S_{\bar{x}}$ standard deviation of the mean, calculated by the following formula: $S_{\bar{x}} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n\,(n - 1)}}$

Σ = sign of

n = number of replicates

and shaker system, both methods show the requirement of the tested strains of *Calvatia* for thiamine. It seems that mycelial growth in the presence of different growth factors was proportionally similar in both methods. It is noticeable that the amounts of growth are greater in the floating method than in the shaker method. According to the previous review of the literature, this must be due to the "carry-over" of nutrients by the inoculum, which is unavoidable in the use of nutrient discs. There is present a certain amount of food material and growth factors in the nutrient discs which influences the growth of the colony. If this were not so, then the amount of fungal growth produced by the shaking method could not be so much lower, since the dry weight of both forms of inocula was roughly the same, i.e., 0.2 - 0.4 mg.

It has to be considered a fact that in case of submerged growth, the inoculum was always standardized and was a physiologically homogeneous material, uniformly exposed to the environmental factors during the growth period. In the case of surface cultures, standardization of the environmental circumstances could not be guaranteed. Another disadvantage of the latter method is also evident from the data reported in Table 6. The replicates of the floating method show greater deviation from the mean value than in the case of the shaker method. The greater variability produced by the floating method is expressed in the comparatively higher standard deviation ($S_{\bar{x}}$) values in Table 6. The data obtained by the floating method of cultivation represents only the overall result of the metabolic processes of an extremely heterogeneous mixture of physiological systems because the inoculum was not provided with a uniform and reproducible physical environment.

The results shown in Table 7 indicate that there are no significant differences in the utilization of thiamine and thiamine-pyrophosphate by the four strains of Calvatia tested.

Lilly and Leonian (1940) compared the action of thiamine and thiamine-pyrophosphate in several thiamine deficient fungi. They found no difference in the maximum weights of mycelium formed in the presence of equivalent quantities of these two growth factors. Therefore, they concluded that cocarboxylase can replace thiamine, possibly entering the cell without dephosphorylation. Jensen (1954) reported cocarboxylase about 30 per cent more active than equimolecular quantities of thiamine for most bacteria. However, he did not mention that this is also valid for the fungi.

Lilly and Barnett (1951) suggested that pyruvate accumulates in the culture medium of some thiamine deficient fungi in the absence of the required amount of thiamine. The pyruvate accumulation was accompanied by a decrease of pH in the medium. That was not observed in the case of Calvatia species. In the absence of thiamine, the pH of the medium did not decrease which may suggest that pyruvic acid, instead of accumulating in the medium, could be further metabolized by any one of several possible pathways, except the Krebs cycle.

The greatest mycelial yield at the end of the 28 days of incubation occurred at the lowest pH value.

Since all living organisms are dynamic systems and each preparation of inoculum forms a different population which varies according to its previous environmental contacts, constant responses would not be expected.

TABLE 7 - The utilization of thiamine and thiamine-pyrophosphate by *Calvatia* sp. (The average mycelial dry weight of four replicates.)

Strains	THIAMINE 150 /liter				TPP 150 /liter			
	14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH
1018 F	13.5	5.1	37.1	4.5	12.7	5.2	39.3	4.5
1019 B	18.5	5.2	26.9	4.7	20.0	5.0	28.1	4.6
766	5.3	5.3	11.9	5.1	6.5	5.3	10.1	5.1
1020	10.5	5.1	55.0	4.7	11.7	5.0	52.3	4.7

CARBON UTILIZATION BY CALVATIA SPECIES

Review of literature

It is well known that carbon is one of the most essential elements in the nutrition of organisms. The carbon sources are utilized by fungi for the synthesis of structural and functional compounds. In addition to this, the oxidation of carbon compounds provides an energy source for their metabolism. Since nearly half of the dry weight of fungus cells consists of carbon, fungi are dependent on their carbon supply. It is obvious, therefore, that the study of carbon nutrition is fundamental to the understanding of the physiology of the Calvatia species.

Carbohydrates are the most common and best sources of carbon. All of them differ physiologically with the mirror-image configuration of the same structure. Whether a carbohydrate is utilized or not depends upon both the configuration of the certain saccharide and the particular abilities of the specific fungus. Usually only one enantiomorph is utilized, or one is utilized much more than the other. In general, L-isomers of the naturally occurring aldohexoses and xyloses do not support growth on fungi. It is known since 1923 (Brannon) that nearly all fungi which can be cultured are able to use glucose or fructose. Albritton's summary of the carbohydrates utilized by fungi for growth (1953) shows that fewer species utilize lactose, maltose sucrose and raffinose than utilize the monosaccharides (glucose, fructose and galactose) which these oligosaccharides yield on hydrolysis.

Glucose is biologically the most important hexose for fungi. This sugar is generally used as a carbon source among the carbohydrates.

Derrick (1949) attained the maximum growth for different wood-

destroying Basidiomycetes in media containing 16 percent glucose. Perlman (1949), using synthetic medium, found that the utilization of glucose by *Polyporus anceps* depended upon the concentration of thiamine. An increase in the amount of thiamine resulted in increased utilization of glucose.

Khudiakov and Vozniakovskaia (1951) reported the culture of four different strains of *Boletus* on a medium utilizing glucose as carbon source. Oddoux (1953) was able to grow 247 species of Basidiomycetes on glucose medium. Vorderberg (1949) claimed that *Coprinus lagopus* did not grow on glucose. However, this conclusion seems to be based on results obtained with an unhappy combination of too low an incubation temperature and an inhibitory decoction of manure added to the substrate. The author also used fruiting body production as an expression of the amount of growth, which is a debatable method according to Fries (1955). Fries (1955) compared the different carbon sources for the growth of *Coprinus* species. She found that glucose, mannose and fructose are almost equally effective for most of the investigated fungi. Fructose is, however, not as well utilized by *Coprinus narcoticus*.

The failure to utilize fructose as a carbon source in the higher fungi has been reported in several cases. Shirakova (1955) found that *Diplocarpon rosae* was unable to utilize fructose for growth, but it is possible that this was due to autoclaving fructose in the medium.

The utilization of galactose by fungi is variable. It is a poor carbon source for most of the Basidiomycetes. Treschow (1944) observed that in *Psalliota bispora*, galactose resulted in a mycelial growth up

to 73 per cent of that on glucose. The corresponding figure for Coprinus micaceus was 47 per cent. Among the Tricholoma species investigated by Norkrans (1950) the saprophytes seemed to utilize this carbon source much better than the mycorrhiza formers. He also found that the addition of a small amount of glucose ("start glucose") increased the growth on galactose.

Among the pentoses, xylose and arabinose were used most frequently in the nutrition of fungi. Xylose ("wood sugar"), as may be expected, is usually a satisfactory source of carbon for the wood-destroying fungi. Treschow (1944) found that the use of xylose resulted in a 32 per cent better mycelial growth than for glucose in the case of Psalliota bispora. Herrick (1940) reported that Stereum gausapatum used xylose better than arabinose.

In general, arabinose is distinctly inferior to glucose or mannose in fungal growth. Margolin (1942) deduced from his investigations that xylose was utilized either more completely or more rapidly than arabinose in most of the studied fungi. Lilly and Barnett (1956) demonstrated that several fungi were able to utilize L-arabinose and failed to grow or did not give satisfactory growth on D-arabinose. Lampert (1958) investigated the different carbon sources of Merulius lacrimans. Xylose and arabinose yielded a good mycelial growth along with glucose, saccharose, maltose, lactose and raffinose but the fungus was not able to utilize rhamnose.

The utilization of monosaccharides seems to be dependent on an enzyme system which is constitutive in some fungi and inducible in others. The strains of a given species may fall into different groups

in this regard.

The oligosaccharides are used by fungi only after conversion into reducing sugars by the appropriate extracellular enzyme. The ability to utilize the oligosaccharides, no matter whether the process takes place via hydrolysis to monosaccharides or via direct phosphorolysis depends on the production of suitable enzymes by the organism.

Maltose, as a carbon source, is almost universally utilized by the fungi. Most of the Basidiomycetes give a good mycelial growth using maltose in the medium; however, maltose is more easily hydrolysed by autoclaving than are sucrose or lactose. Fries (1955) noted that maltose can be utilized by all species of Coprinus, but to a different degree. Swartz (1935) reported maltose as a very favorable sugar for mycelium production in Calvatia saccata, Calvatia caelata and Calvatia gigantea.

Sucrose is generally a good source of carbon for fungal growth, but it is utilized by fewer fungi than maltose. The response to sucrose is correlated with the amount of invertase produced by the particular organism. Findlay (1944) mentioned the utilization of sucrose by some wood-rotting Basidiomycetes. Bille-Hansen (1953) found that sucrose appeared to be completely or almost completely unavailable to the investigated three coprophilous species of Coprinus.

Lilly and Barnett (1953) observed that Collybia velutipes utilized fructose, glucose, sucrose, mannose, maltose and cellobiose best in that order; Polyporus albellus utilized mannose, xylose, glucose and maltose best in that order; Polyporus versicolor utilized glucose, mannose cellobiose, fructose and maltose best in that order.

Cellobiose is almost as widely utilizable as maltose for fungi, but the data is more limited than those for maltose. Norkrans (1950) stated that Tricholoma flavobrunneum failed to utilize cellobiose, but other species of the same genus grow well with it as the carbon source. Madelin (1956) indicated that cellobiose is a good source of carbon for most of the Coprinus species.

According to the literature trehalose permits good growth for several fungi, however, this carbohydrate was not investigated in the nutrition of the Basidiomycetes. Lactose is a poor carbon source for most of the fungi. This may be due either to the general unsuitability of galactose which is one product of hydrolysis of this disaccharide or to the failure to produce the appropriate hydrolytic enzyme. Fries (1955) reported that lactose could not be used by any of the investigated Coprinus species. Norkrans (1950) noted, however, that Tricholoma nudum gave a satisfactory growth when lactose was used as a carbon source in the medium. Humfeld and Sugihara (1952) mentioned that Agaricus campestris was not able to utilize this carbohydrate.

Raffinose is the only trisaccharide which is important in the nutrition of fungi, although the utilization of this carbohydrate is inferior to the utilization of glucose or maltose. Among the Basidiomycetes, some of the Coprinus species were noted by Johnson (1941) to be able to use raffinose as well as glucose. Herrick (1940) observed that raffinose was an even better source of carbon for Stereum gausapatum than glucose.

The use of polysaccharides in the nutrition of fungi also depends

upon the ability to produce the appropriate hydrolytic enzymes. Only those organisms are able to utilize these carbon sources which form the necessary enzymes to hydrolyze or to degrade in some way these water insoluble carbohydrates to low molecular weight simple sugars. Starch is readily used by a number of fungi, however, only those which produce amylase are able to utilize this polysaccharide. Nørkrans (1950) indicated that because of this fact, starch is hydrolysed primarily to maltose and all Basidiomycetes checked gave a good mycelial growth on "soluble starch". Fries (1955) reported excellent growth for *Coprinus micaceus*, using starch as a carbon source. The fact that starch is often a better substrate for growth than glucose has been explained by Cochrane (1958): "This effect presumably results either from contamination of the starch with growth factors or from the fact that utilization of a slowly hydrolysable compound is accompanied by less accumulation of acids than utilization of glucose".

Cellulose is used by the majority of fungi according to Norman (1942). Fungi play an important part in the breaking down of the cellulose that remains in the soil. Some wood-destroying fungi break down the lignin of the wood cells, but others dissolve the original cellulose cell walls. Only those Basidiomycetes which form cellulase are able to utilize cellulose, the most common carbon source on the surface of the earth. Reese (1947) deduced that the wood rotting and litter-decomposing fungi were generally, although not universally, able to decompose cellulose. He also found acidity an influencing factor in the cellulose utilization. Nobles (1948) stated that the fungi which caused white rots (as for instance *Polyporus abietinus*, *Polyporus*

cinnabarinus, Armillaria mellea, etc.) were able to attack the non-cellulosic constituents of the wood. The fungi which attacked cellulose in preference to lignin caused the brown rots (Merulius lacrymans, Lenzites trabea, Polyporus betulinus, etc.). Norkrans (1950) suggested that the species within a given genus were not necessarily alike in their response to cellulose. In the case of Tricholoma species, the utilization of cellulose varied with the presence of other carbon sources. It has been also suggested that Tricholoma species are able to form an adaptive cellulase. Siu (1951) concluded that enzymes which attacked cellulose were liberated by microorganisms only in the presence of cellulose. The cellulose attacking enzymes are adaptive enzymes formed by fungi only as a specific response to the presence of certain substrate.

Lignin is also broken down by wood-destroying fungi, but attempts to cultivate these artifically on preparations of lignin from wood have not been very successful. Gottlieb (1950) reported that some of the wood-destroying fungi were able to utilize lignin, but others, as for instance Polyporus abietinus, used lignin in synthetic medium only after a process of adaptation. Such adaptation permitted the organisms to grow on many different kinds of lignin and lignin containing residues according to Van Vliet (1954).

Inulin is used by relatively few fungi. This is due to the failure to secrete the appropriate enzyme, inulase, rather than to any inhibitory effect of the inulin itself, as has been concluded by Hawker (1950). Fries (1955) noted that although all the investigated species of Coprinus were able to grow on fructose they could not utilize inulin,

which is a polyfructoside. Perhaps it would be possible to promote some growth by adding "start glucose", but it is also possible that they lack the necessary inulase.

Glycogens, the reserve polysaccharides of animals, are used by a number of fungi in artificial culture. Those fungi which are unable to satisfy their carbon requiremens from starch cannot be expected to utilize glycogens, since probably the same enzymes attack both of these polysaccharides. There is not much data in the literature dealing with the use of glycogen by the Basidiomycetes. Only Herrick (1940) mentioned glycogen as a good source of carbon for Stereum gausapatum.

Certain fungi grow better on a mixture of several carbon sources than when supplied with the equivalent amounts of the individual carbohydrates comprising the mixture. It is probably that such organisms are able to produce small quantities of several enzymes more easily than a large quantity of any one alone. Fries (1955) investigated the combinations of different carbohydrates for Coprinus species. She found that growth in the presence of mixed carbon sources was sometimes increased or even made possible. In nature, the fungi are usually supplied with a mixture of carbohydrates and other carbon sources rather than a larger amount of a single one. Therefore, it is possible that carbon sources that have proven unavailable in experiments can be utilized in nature.

Proteins and their amino acids are vital constituents of the organisms. They are attacked by many fungi chiefly as a source of nitrogen. In the absence of any other source of carbon, however, most fungi are able to use proteins to satisfy their carbon requirement.

According to Gottlieb (1946) the proteins have high calorific value and under certain conditions can furnish energy for growth and respiration. Best and Taylor (1945) stated that most of the straight chain amino acids could be assimilated by the organism, and under certain conditions as much as 58 per cent of the protein could be converted to glucose.

The products of glucose metabolism: citrate, fumarate, succinate and malate are the organic acids, mostly utilized by fungi. Peptone may serve as a source of carbon and nitrogen for many organisms.

As far as Basidiomycetes are concerned, only a few investigations have been conducted on the utilization of amino acids as carbon source. Treschow (1944) reported, however, that Psalliota bispora gave a good mycelial growth using oxalic acid in the medium to satisfy the carbon requirement. It is characteristic of fungi cultivated on amino-acid medium as the sole source of carbon to increase the alkalinety of the medium. This is probably due to accumulation of ammonia which results from deamination as was mentioned by Lilly and Barnett (1951).

Experimental Procedure

The same four strains of <u>Calvatia</u> (1019F, 1018B, 766 and 1020) as before, were employed in these studies for the utilization of different carbon sources. The basal synthetic medium was the same as that used previously, i.e. Lindeberg's medium except that 100 γ/liter of thiamine was added. The pH was determined as previously described.

Forty ml of the modified Lindeberg medium was placed in a 125 ml Erlenmeyer flask and plugged with non-absorbent cotton. Only Pyrex glassware was used and it was washed as described previously. Each flask was autoclaved for 15 minutes at 15 pounds steam pressure.

The various carbohydrates were added to the basal medium in quantities which supplied 8 grams of carbon per liter. In most of the experiments, the carbohydrates were added to the basal medium before autoclaving. Maltose, trehalose and raffinose were sterilized separately in the autoclave for different lengths of time at different pounds steam pressure (according to their heat-sensitivity) and added aseptically to the basal medium.

To avoid the breakdown of sugars during autoclaving, the following heat-sensitive carbohydrates, fructose, L-sorbose, xylose, melibiose and invert sugar, were sterilized by Seitz-filtration using a filter with 1 μ pore diameter.

Once again the density of the inoculum was determined with Klett-Summerson photoelectric colorimeter, using the green filter (spectral range 500 - 570 mμ). Each flask was inoculated with 1.0 ml of blended suspension at 30 - 35% transmission.

The cultures were incubated only on the reciprocating shaker with no additional aeration. All the experiments were incubated at

approximately 25^{o}C. The cultures were exposed to diffuse daylight with intervening dark periods at night. No attempt was made to standardize condition of illumination.

Sufficient flasks were inoculated to provide two replicates for each strain harvested after 14 and 28 days on the shaker. The mycelium was filtered on tared Whatmann No. 1 filter papers using a Buchner funnel, and washed twice with distilled water. It was then dried to constant weight for 24 hours at 96^{o} in oven. The dry weight of mycelium was calculated. In all experiments the pH was checked at the conclusion.

Results and Discussion

In this study, all carbon sources tested were present in an amount calculated to yield an equal supply of carbon. Therefore, it was possible to compare their utilization by the four Calvatia strains on an equitable basis.

Monosaccharides

The experimental evidence shown in Table 8 indicated that pentoses were utilized less than most of the hexoses by Calvatia species. If any of the 5 carbon sugars were used as the sole carbon source, the strains tested grew poorly. There are slight differences, however, in the utilization between the strains and also between the availability of the pentoses.

Ribose was the best carbon source among the pentoses for strains 1018 F, 1019 B and 1020. Strain 766 did not grow in the presence of ribose in the basal medium.

The results of Table 8 indicated that Calvatia species were not able to satisfy their carbon requirements with either xylose or arabinose.

The growth of Calvatia species was more rapid and greater dry weights of mycelium were produced in autoclaved than in sterile filtered xylose, although less yield would be expected on the autoclaved xylose because this pentose is known to be broken down to furfural in autoclaving.

Both isomers of arabinose were employed in this nutritional investigation of Calvatia species but neither of them promoted a satisfactory growth. According to Lilly and Barnett (1956) from the distribution of the isomers of arabinose in the organisms, it would be

TABLE 8 - The utilization of various monosaccharides by <u>Calvatia</u> species. (The mycelial dry weights of two replicates)

C-sources	1018 F				1019 B				766				1020			
	14 days		28 days		14 days		28 days		14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
PENTOSES																
L-arabinose	2.3	5.3	3.4	4.7	4.9	5.0	7.6	4.9	2.1	5.2	3.6	4.9	2.5	5.0	4.2	4.9
D-arabinose	2.7	5.1	4.9	4.9	2.0	5.0	3.5	4.9	0	5.5	2.7	5.1	2.3	5.0	4.8	4.9
d(+) xylose (autoclaved)	3.3	5.2	5.1	4.8	4.2	5.3	6.5	4.6	4.0	5.1	6.8	4.8	4.1	5.0	6.0	4.8
d(+) xylose (filtered)	1.7	5.5	3.7	5.3	2.2	5.3	3.7	5.2	2.0	5.2	3.5	5.1	1.6	5.4	5.3	5.2
d(-) ribose	3.3	5.5	7.0	5.2	4.3	5.2	9.5	5.0	0	5.5	0	5.4	2.8	5.3	7.5	5.0
rhamnose	0	5.5	1.9	5.3	0	5.5	3.1	5.2	0	5.5	2.7	5.4	0	5.5	2.4	5.3
HEXOSES																
d(+) glucose	13.0	5.3	39.1	4.5	14.4	5.3	27.2	4.5	4.4	5.3	11.1	5.0	11.7	5.3	52.1	4.8
d(+) galactose	1.4	5.5	4.2	5.0	1.3	5.3	11.8	4.6	4.6	5.2	8.1	4.9	5.1	4.9	28.8	4.5
d(+) mannose	14.5	5.0	15.5	4.8	8.8	5.0	9.3	4.8	4.4	5.0	6.6	4.9	2.2	5.3	24.1	4.7
l(-) fructose	3.2	5.4	22.6	4.9	2.6	5.4	15.8	5.0	2.3	5.4	7.9	5.2	3.3	5.2	20.2	5.0
L-sorbose	1.4	5.4	4.6	5.0	0	5.5	2.5	5.2	0	5.5	2.0	5.3	1.8	5.3	3.5	5.1
CONTROL																
C-free medium	0	5.5	1.2	5.5	0	5.5	1.1	5.5	0	5.5	0	5.5	0	5.5	1.4	5.5

expected that L-arabinose would be utilized readily by more fungi than D-arabinose. In the case of Calvatia species, two of the strains, 1019 B and 766, grew better on L-arabinose, and two strains, 1018 F and 1020, preferred D-arabinose.

The hexoses employed in this study were glucose, fructose, mannose, galactose, rhamnose and sorbose with growth results best in this order. In general, the 6 carbon sugars were utilized much better by Calvatia species than the pentoses, however, the ability of the strains to use them for growth was different.

Glucose, as may be expected, was the best carbon source for each strain among the hexoses tested, although it was not utilized as well as dextrin or cellobiose. Glucose is a biologically very significant sugar. It has an important role in the terminal respiration of organisms. Fruton and Simmonds (1959) presented a scheme for the pathway of anaerobic breakdown of glucose to ethanol and carbon dioxide in the microorganism. The overall reaction is:

$$\text{Glucose} + 2\text{ADP} + 2\ \text{phosphate} \longrightarrow 2\ \text{ethanol} + 2CO_2 + 2\text{ATP} + 2H_2O.$$

According to the results shown in Table 8, fructose was easily assimilated by the four strains of Calvatia tested, but it was inferior to glucose. Fructose is also metabolized by microorganisms, after phosphorylation, the same way as glucose. But while glucose is converted to glucose 6-phosphate by ATP in the presence of glucokinase, fructose is converted to fructose-1-phosphate and in the presence of fructokinase. Nilsson (1956) observed that fructose-1,6-diphosphate (an intermediate product of carbon metabolism) had a very striking growth-promoting effect on Boletus variegatus and Collybia velutipes. He also found that the effect was reversed above a certain concentration of the

diphosphate, at least for *Boletus variegatus*. The investigation was only preliminary and he offered no explanation of the effect.

Mannose was readily used in *Calvatia* fragilis (1020), although the fungus did not grow as well as on glucose. The rate of utilization was slow during the first 14 days of incubation, but later on it became faster. Limited growth took place with mannose in the strains of *Calvatia gigantea* (1018 F, 1019 B and 766) and most of the growth was completed within 14 days. Mannose can be involved in the metabolism of microorganisms, but it has to be phosphorylated previously. Slein (1950) indicated that yeast hexokinase catalyzed the phosphorylation of mannose by ATP to form mannose-6-phosphate, and phospho-mannose isomerase was responsible for the enzymic conversion of this sugar phosphate to fructose-6-phosphate.

Calvatia gigantea strains grew poorly on galactose, but *Calvatia fragilis* (1020) had a satisfactory mycelial growth on this 6-carbon sugar. According to Fruton and Simmonds (1959) the ability of an organism to use galactose, depends upon its ability to convert this hexose into a phosphorylated derivative of glucose able to enter the main respiratory pathways.

Rhamnose, a desoxy sugar of mannose, was not able to satisfy the carbon requirement of *Calvatia* species. Even *Calvatia fragilis* (1020) which gave an appreciable growth on mannose was not able to utilize this carbon source.

None of the *Calvatia* strains investigated was able to utilize sorbose. Sorbose is a keto-hexose also, but differs from fructose in the configuration of carbon atom 5. The toxicity of sorbose was mentioned by Lilly and Barnett (1953). They stated that sorbose inhibition

occurred at relatively high concentrations in certain fungi and it was strongly affected by the temperature. However, Cochrane (1958) suggested that sorbose may interfere with a respiratory pathway of the organism.

The differences in the utilization of the monosaccharides by the strains of *Calvatia* can be due to the structural differences between the sugars and to the particular abilities of the organisms also.

Oligosaccharides

As seen in Table 9, cellobiose was one of the best carbon sources for Calvatia species. Strain 1018 F produced with 10 per cent more mycelium, strain 1019 B, 25 per cent; strain 766, 50 per cent; and strain 1020 had 150 per cent more mycelial growth in the presence of cellobiose than with the generally accepted glucose as a carbon source.

According to Reese and Levinson (1952) most of the fungi are able to split cellobiose to its constituent glucose residues. Analyzing the data on cellobiose utilization (Table 9), Calvatia fragilis (1020) seemed to be superior to Calvatia gigantea (1018 F, 1019 B and 766) in the production of the appropriate extracellular enzyme. Strain 1018 F showed a relatively low mycelial growth after 14 days incubation, but later on the rate of cellobiose utilization increased. The result suggested that the involved enzyme was inducible. No attempt was made, however, to study adaptive enzyme formation with respect to carbohydrate utilization.

A satisfactory growth was obtained using maltose as a carbon source in the medium for Calvatia strains. The weight of the mycelial growth was lower than on cellobiose, with the exception of strain 1019B, where the yield on maltose even exceeded that on cellobiose. For Calvatia fragilis (1020) maltose seemed to be a less available carbon source than glucose, which may be due to the lack of the necessary hydrolytic enzyme in the fungus.

Le Mense et al (1947) found that maltose was utilized almost in every fungus investigated, although quantitative differences between strains were common.

TABLE 9 - The utilization of various carbon sources by Calvatia species. (The mycelial dry weights of two replicates.)

C-sources	1018 F				1019 B				766				1020			
	14 days		28 days		14 days		28 days		14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
OLIGOSACCHARIDES																
sucrose	3.3	5.2	10.9	5.0	5.6	5.3	13.7	5.1	1.8	5.2	5.0	5.1	4.9	5.5	10.1	5.3
d(+) maltose	2.7	5.3	40.8	4.8	2.9	5.4	46.1	4.0	2.0	5.5	10.3	5.0	5.5	5.3	26.9	5.0
d(+) lactose	3.0	5.5	4.9	5.5	1.8	5.5	5.5	5.3	2.2	5.5	5.7	5.5	2.7	5.5	6.9	5.3
cellobiose	7.6	5.4	53.5	4.5	20.5	5.0	43.0	4.5	9.7	5.2	21.3	4.9	52.3	4.6	146.0	4.0
melibiose	1.8	5.5	3.8	5.3	1.5	5.5	3.0	5.5	0	5.5	2.0	5.3	2.1	5.4	5.1	5.3
trehalose	4.7	5.2	13.0	4.9	8.3	5.2	23.1	4.9	6.3	5.3	9.3	4.9	7.2	5.2	20.5	5.0
invert sugar	5.1	5.2	22.1	4.7	8.6	4.9	46.2	4.5	5.2	5.2	16.5	4.8	10.9	4.9	64.3	4.4
d(+) raffinose	7.2	5.3	9.5	5.1	6.2	5.3	8.3	5.0	2.6	5.4	5.9	5.1	5.6	5.2	7.9	5.0
POLYSACCHARIDES																
cellulose	0	5.3	2.8	5.1	2.1	5.3	3.3	5.2	0	5.3	3.2	5.1	1.9	5.3	4.2	5.1
dextrin	4.6	5.0	20.3	4.7	7.9	4.9	85.6	4.4	12.0	4.8	31.6	4.5	73.8	4.3	153.1	4.0
starch	6.8	5.1	9.8	5.0	8.3	5.0	23.2	4.9	7.8	5.0	13.2	5.0	9.2	5.0	33.1	4.5

TABLE 9 - continued

C-sources	1018 F				1019 B				766				1020			
	14 days		28 days		14 days		28 days		14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
POLYSACCHARIDES - continued																
inulin	4.8	5.1	6.9	5.0	3.9	5.5	8.7	5.4	2.1	5.5	2.9	5.2	7.7	5.4	9.9	5.2
glycogen	5.9	5.1	13.7	5.0	9.1	5.3	21.0	5.1	10.3	5.5	14.9	5.0	15.1	5.3	90.2	4.8
CONTROL																
C-free medium	0	5.5	1.2	5.5	0	5.5	1.1	5.5	0	5.5	0	5.5	0	5.5	1.4	5.5

Although cellobiose and maltose by hydrolysis form two molecules of glucose, there did not seem to be any correlation between the capabilities of the strains to utilize these two disaccharides. In the former carbon source the two glucose molecules are connected by a β-glucosidic linkage and in the latter one by a α-glucosidic linkage. Consequently, they require different enzymes for cleavage. The difference between the utilization of these two carbohydrates by the Calvatia strains was probably due to the difference in abilities to produce the required enzymes.

The smaller amount of mycelial growth with maltose could be explained by the fact that maltose is more easily hydrolyzed by autoclaving than cellobiose. A special precaution was taken, however, to sterilize the medium containing maltose for a minimum of time.

According to the data of Table 9, sucrose, a β-fructofuranoside, was not a good carbon source for Calvatia species. It could be that the organisms produce the hydrolyzing enzyme very slowly or in a small quantity, as both components of this disaccharide (glucose and fructose) produced a satisfactory growth when used separately. Apparently the α - β glucose to fructose linkage was not easily broken.

Mandels (1954) indicated that sucrose is metabolized by a nonhydrolytic system in spores of the fungus Myrothecium verrucaria. Bealing (1953) observed that invertase preparations catalyzed the transfer of fructofuranosyl groups not only to water, but also to various alcohols and sugars by transglucosidation.

The fact that the small yield of the four Calvatia strains on sucrose was due to the slow production of invertase rather than to the

inability to utilize the components of this sugar could be proved by the substitution of invert sugar. Invert sugar is a mixture of glucose and fructose with the components present in 1 to 1 ratio. It differs from sucrose as its constituents are already hydrolyzed. Therefore, it would be classified as a mixture of sugars rather than a disaccharide. All the four Calvatia strains investigated produced a very good growth on this mixed carbon source. With exception of strain 1018 F, they gave even a better mycelial growth than on glucose in 28 day culture. This can be explained by the effect of combined carbon sources (induction of a new enzyme). Calvatia species did not need any specific enzymes to utilize invert sugar as they did in case of sucrose. One constituent, glucose, acted as an "inducer", permitting a rapid growth on the other constituent, fructose.

It could be assumed from the result shown in Table 9 that trehalose was utilized satisfactorily by the strains of Calvatia. However, Calvatia fragilis (1020) did not respond as well to this carbon source as the strains of Calvatia gigantea. (1018 F, 1019 B and 766.)

According to Cochrane (1958), in all probability trehalose in fungi has the role of a translocateable reserve carbohydrate. This is especially common in the Basidiomycetes. This disaccharide is formed early in the metabolism and used up later, however, an adequate picture of trehalose metabolism is not yet available. Calvatia species seemed to be able to produce trehalase, the special enzyme which splits the α-1-1 glucosidic linkage in this disaccharide. The synthesis of trehalase has not been studied in Calvatia species. However, a correlation between the rate of growth and the amount of the appropriate enzyme produced is apparent.

Lactose and melibiose appeared to be poor carbon sources for the Calvatia strains tested. All four of them produced very small mycelial growth on these disaccharides. The failure of Calvatia species to utilize this carbon source could be explained by the lack of the necessary hydrolytic enzymes, lactase or melibiase; or by the inability to utilize one of the component hexoses, galactose.

Just one trisaccharide, raffinose, was employed in this nutritional study of Calvatia species. Raffinose, which contains one linkage as in sucrose and another as in melibiose was utilized poorly by the strains tested. According to the earlier discussions, Calvatia species were not able to produce melibiase, but a certain amount of sucrose was formed by the organisms. Therefore, the small growth which was produced by the fungi investigated on raffinose could be explained by the availability of fructose.

Polysaccharides

Data shown in Table 9 indicates that good mycelial weights were obtained with soluble starch in three strains of Calvatia species, although strain 1018 F was not able to utilize this carbon source in an appreciable amount.

Starch is a "nutrient" polysaccharide and acts as a metabolite reserve of monosaccharides in organism. It is composed of a mixture of two different polysaccharides: aamylose and amylopecyin. According to West and Todd (1956), the glucose units of amylose are bound to each other in a (1-4)-glucosidic linkage: amylopectin, while containing chains of glucose units like those of amylose, also has branches of these glucose chains linked through a (1-6)-glucosidic linkage.

Calvatia strains 1020 and 766 grew somewhat better on starch than they did on maltose. This may be due to the presence of some growth promoting substances in starch. Another explanation might be that starch is broken down by phosphorolysis directly to glucose compounds and not via maltose as was suggested by Bonner (1950). The fact that starch was not such a good carbon source for strain 1018 F and 1019 B as maltose could be explained by a slow or improper formation of the necessary extracellular enzyme, maltase.

The glycogen utilization in the Calvatia strains (Table 9) was proportional to the ability of using starch as a carbon source. Although the mycelial growth on glycogen was more abundant, especially in Calvatia fragilis (1020) where it even surpassed the yield on cellobiose.

Glycogen is a glucose polysaccharide similar to the amylopectin fraction of starch, in which straight-chain arrays of glucopyranose units (in (1-4)-glucosidic linkage) are cross linked by means of (1-6)-glucosidic bonds. Fructon and Simmonds (1959) reported that inorganic phosphate was an obligatory participant in the degradation of glycogen and that a phosphorylated monosaccharide, glucose-1-phosphate was formed in the reaction.

It may be possible, in the case of Calvatia fragilis, that a phosphorolysis took place to convert glycogen into glucose-1-phosphate. This phosphorylated glucose derivative entering the main respiratory pathway provided the energy for growth. Calvatia gigantea strains 1018 F and 1019 B seemed not to be able to accomplish such a phophorolysis.

According to the results in Table 9, dextrin was far the best carbon source investigated. With the exception of strain 1018 F, Calvatia species were able to satisfy their carbon requirements most effectively on this polysaccharide. Dextrin is a product of partial hydrolysis of starch. Dextrins, formed from amylose have unbranched chains, while those from amylopectin are branched.

Each Calvatia strain tested responded in the same way to dextrin, as they did to starch and glycogen. Therefore, the discussion concerning the ability to utilize starch and glycogen by this fungi are also valid for dextrin utilization. The quantitative differences between starch and dextrin as carbon sources, however, cannot be interpreted easily until the chemistry of these polysaccharides is better known, according to Cochrane (1958).

The results in Table 9 demonstrate that inulin was poorly utilized by Calvatia species. Just a small growth was observed in each strain. Inulin is a polyfructoside and a specific enzyme, inulase is necessary to break it down to its constituent fructose molecules. Palmer (1951) reported that inulin represented a linear array of about 33 fructofuranose units joined together by means of β-(2-1)-glucosidic linkages. He also found a small number of D-glucose units in inulin. Though all the investigated strains of Calvatia grow well on fructose, they could not utilize inulin. The insignificant small amount of growth on this carbon source could be explained by the few units of glucose present in inulin.

Hawker (1950) concluded that the inability of fungi to use inulin for growth was due to the failure to secrete the enzyme, rather than any inhibitory effect of the inulin itself.

The results presented in Table 9 show that Calvatia species were not able to use cellulose as a carbon source. Evidently they lacked the enzyme necessary to decompose this polysaccharide.

Cellulose is a linear polymer of D-glucose, where the glucose molecules are joined together through (1-4)- β -glucosidic linkage. Norkrans (1956) noted that cellulose was apparently attacked at several points along the chain of glucose residues.

The lack of cellulase formation by Calvatia species could be proved by the fact that cellobiose, the intermediate product of this polysaccharide, was one of the best carbon sources for the organisms. However, it should be mentioned that the technic used for testing the cellulose utilization of Calvatia strains was unsatisfactory. The ability to use this polysaccharide could be inhibited by many other factors, as for instance the water insolubility of the powdered cellulose used.

Mixture of Sugars

The results in Table 10 indicate also the effect of the combinations of carbohydrates tested on Calvatia species. Each combination of glucose resulted in a smaller amount of mycelium than when glucose alone was present in the medium. However, the yields were superior to those which were obtained using the other components separately. This may be due to the fact that glucose allowed sufficient growth so that the enzymes essential to galactose, lactose or rhamnose utilization could be synthesized.

A stimulatory effect was observed when glucose and rhamnose were

TABLE 10 - The utilization of mixed carbon sources by Calvatia species. (The mycelial dry weights of two replicates) *

C-sources	1018 F				1019 B				766				1020			
	mg	~~pH~~	~~pH~~ mg	pH	mg	~~pH~~	~~pH~~ mg	pH	mg	~~pH~~	~~pH~~ mg	pH	mg	~~pH~~	~~pH~~ mg	pH
d(+) glucose + d(+) galactose	-	-	34.2	4.5	-	-	28.1	4.8	-	-	8.6	5.0	-	-	39.4	4.8
d(+) glucose + d(+) lactose	-	-	25.5	4.9	-	-	20.9	4.7	-	-	9.7	5.0	-	-	27.5	4.7
d(+) glucose + rhamnose	-	-	37.0	4.7	-	-	36.8	4.9	-	-	8.6	5.2	-	-	38.5	5.0
d(+) lactose + rhamnose	-	-	4.5	5.5	-	-	5.0	5.3	-	-	4.8	5.4	-	-	5.1	5.1
L-arabinose + D-arabinose	-	-	4.8	5.0	-	-	7.0	5.2	-	-	3.1	5.1	-	-	4.5	5.4
CONTROL d(+) glucose	39.1			4.5	27.2			4.5	11.1			5.0	52.1			4.8

---

* only 28 days incubation was checked

both present on the growth of strain 1019 B. However, rhamnose alone was poorly utilized by the organism. It is possible that glucose acted as an "inducer" to form an "adaptive enzyme" to utilize rhamnose, although no experimental evidence is available to prove this assumption.

The mixture of the two poorest carbon sources, lactose and rhamnose, was not able to satisfy the carbon requirement of the Calvatia strains investigated.

The combination of L- and D-arabinose promoted the same mycelial growth for each strain as the more effective of these two sugars did when used separately as a carbon source.

Organic Acids

The results in Table 11 show that under these experimental conditions, Calvatia species were not able to use satisfactorily the organic acids as carbon sources. All the organic acids employed in this study supported poorly the mycelial growth in the strains investigated.

The relatively best growth was obtained in the presence of urea. That this may be due to the fact that this compound is a frequent constituent of the higher Basidiomycetes. However the yields were so small that urea cannot be considered a growth supporting carbon source. Nevertheless, strain 766 grew as well on this compound as on glucose.

Acetate, oxalate, succinate and citrate, the intermediate products of the Krebs cycle were poorly utilized by the strains of Calvatia.

No appreciable yields were obtained in the presence of asparagine or ammonium tartarate as a sole carbon source.

Alanine and arginine were also poor carbon sources for Calvatia species. Lysine was the most poorly utilized of the organic acids and

TABLE 11 - The utilization of various carbon sources by <u>Calvatia</u> species. (The mycelial dry weights of two replicates.)

C-sources	1018 F				1019 B				766				1020			
	14 days		28 days		14 days		28 days		14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
ORGANIC ACIDS																
Na-acetate (initial pH 6.4)	3.8	6.4	6.6	6.5	7.4	6.4	10.1	6.3	5.1	6.4	6.0	6.4	6.9	6.4	9.1	6.6
Na-oxalate	8.3	6.0	8.9	6.4	7.4	6.0	8.8	6.3	9.1	6.0	9.6	6.1	8.1	6.2	10.0	6.2
Na-succinate (initial pH 6.0)	5.9	6.0	9.1	6.1	6.0	6.0	7.3	6.0	5.2	6.0	6.9	6.0	10.2	6.0	12.4	6.2
Na-citrate (initial pH 6.5)	5.8	6.5	7.4	6.6	8.0	6.5	12.5	6.7	4.1	6.5	7.9	6.5	5.8	6.8	9.7	6.4
1(+) asparagine (initial pH 5.0)	4.0	5.0	5.6	5.3	5.0	5.0	7.1	5.2	4.8	5.2	8.6	5.5	6.8	5.5	11.9	6.0
dl-alanine	4.0	5.5	5.4	5.6	7.0	5.5	10.4	5.8	6.6	5.5	8.6	5.5	5.5	5.5	8.3	5.8
1(-) arginine	4.2	5.5	6.1	5.3	2.7	5.4	6.6	5.2	1.7	5.5	3.5	5.5	7.8	5.3	9.2	5.1
L-lysine	4.0	5.4	4.5	5.3	2.5	5.5	3.6	5.4	2.5	5.5	3.5	5.4	2.8	5.4	3.9	5.3
DL-leucine	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5
$(NH_4)_2$ tartarate	7.0	5.5	8.5	5.3	4.5	5.5	6.8	5.3	3.9	5.5	5.0	5.5	6.0	5.5	8.8	5.3
Urea	10.6	6.4	13.1	6.7	11.9	6.8	13.8	6.9	10.0	6.6	12.1	6.7	10.9	6.6	12.3	6.8

TABLE 11 - continued

C-sources	1018 F				1019 B				766				1020			
	14 days		28 days		14 days		28 days		14 days		28 days		14 days		28 days	
	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH	mg	pH
MIXTURE OF GLUCOSE AND																
ORGANIC ACIDS																
d(+) glucose +																
$(NH_4)_2$ tartarate	-	-	36.1	4.7	-	-	25.9	4.8	-	-	11.4	5.2	-	-	48.3	5.0
d(+) glucose +																
Na-oxalate	-	-	33.9	6.0	-	-	22.1	6.0	-	-	12.3	5.8	-	-	42.1	6.1
d(+) glucose +																
l(+) asparagine	-	-	34.2	5.0	-	-	24.5	5.0	-	-	10.5	5.5	-	-	49.6	5.6
d(+) glucose +																
dl-alanine	-	-	37.3	5.5	-	-	26.1	5.4	-	-	11.5	5.5	-	-	48.9	5.4
CONTROL																
C-free medium	0	5.5	1.2	5.5	0	5.5	1.1	5.5	0	5.5	0	5.5	-	5.5	1.4	5.5

leucine was not able to promote any growth at all.

Glucose and some of the organic acids (Table 11) were incorporated into the same medium to determine whether the lack of growth was due to the toxic effect of the organic acids. Neither compound was poisonous to the organism because when glucose was added to these compounds the fungus produced an abundant amount of mycelium.

Although there are certain chemical differences in the organic acids employed in this study, it is believed that some general conclusions can be drawn from the results obtained.

In general, the organic acids are poor carbon sources for Calvatia species. That can be due to the inability of the fungi to produce the variety of enzymes necessary to carry out the degradations and oxidations of these compounds. Another explanation might be that the cells are impermeable to the organic acids in such high concentrations used (because of the equivalent amount of carbon supply) and at physiological pH levels.

The fungi produced alkaline reaction in the organic acid medium. Increases in Ph from 5.5 to 6.8 were frequent. Higher pH values were associated with the medium on which better mycelial growth occurred. However, no relationship between the acidity of the organic acids and the final pH of the medium was apparent.

SUMMARY

Three strains of *Calvatia gigantea* (1018 F, 1019 B and 766) and one strain of *Calvatia fragilis* (1020) were studied in this nutritional investigation.

All four strains of *Calvatia* grew well on a modified Czapek-Dox medium and on Lindeberg's medium in the presence of thiamine. It was found that the optimum temperature range for mycelial growth of the organisms was between 20 and 24° C and the optimum pH at 5.5.

Each of the strains investigated were totally heterotrophic with respect to thiamine, and autotrophic with respect to biotin, pyridoxine and inositol. A partial deficiency was established for an unidentified growth factor(s), present in Bacto-Peptone and Bacto-Yeast extract.

No significant differences in mycelial growth of the strains of *Calvatia* were obtained when equivalent amounts of thiamine and thiamine-pyrophosphate were compared. The submerged culture method (using fragmented hyphae) proved to be superior to the surface culture method.

Calvatia species were able to utilize a variety of carbon sources when grown in Lindeberg's medium in the presence of thiamine. The amount of growth varied with the carbon source and was determined by the average mycelial dry weight of two replicates after 14 and 28 days of incubation.

Calvatia gigantea (strain 1018 F) grew best in the following decreasing order: cellobiose, maltose, glucose, fructose, invert sugar, mannose and dextrin. A moderately good growth was obtained using glycogen, starch, trehalose, urea, sucrose and raffinose, while the organic acids and inulin supported very little growth.

Calvatia giganetea (strain 1019 B) utilized dextrin, cellobiose, invert sugar, maltose, glucose, starch, glycogen, trehalose, and fructose, listed in the order of their decreasing effectiveness. Considerably less, but still available to the organism as carbon sources were sucrose, urea, galactose, mannose and ribose. The organism grew poorly on medium incorporating inulin, raffinose and the organic acids.

Calvatia gigantea (strain 766) produced good mycelial growth on dextrin, invert sugar, glycogen, starch, glucose, urea, cellobiose, maltose and trehalose, in that order. Somewhat lower yields were obtained in the presence of galactose, fructose, sucrose and the organic acids. Raffinose and mannose were poor carbon sources for this fungus.

Calvatia fragilis (strain 1020) satisfied its carbon requirement effectively on dextrin, cellobiose, glycogen, invert sugar, glucose and starch, listed in the order of their decreasing effectiveness. Galactose, mannose and fructose were less available for the organism. No significant growth was obtained in the presence of the other carbon sources tested.

BIBLIOGRAPHY

1. Albritton, E.C. 1952. Standard values in nutrition and metabolism. WADC. Tech. Report 52-301. Wright-Patterson Air Force Base.

2. Anchelm M., A. Hervey and W.J. Robbins. 1952. Antibiotic substances from Basidiomycetes. X. *Fomes juniperuus*, Schrenk. Proc. Nat. Acad. Sci. 38: 655.

3. Aschan, K. 1954. The production of fruit bodies in *Collybia velutipes*. I. Influence of different culture conditions. Physiol. Plantarum. 7: 571.

4. Atkinson, N. 1954. Psalliotin, the antibiotic of *Psalliota xanthoderma*. Nature 174: 598.

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

6. Bealing, F.J. 1953. Mould "Glucosaccharase": a fructosidase. Biochem J. (London) 55: 93.

7. Best, C.H. and N.B. Taylor. 1945. The physiological basis of medical practices. William and Wilkins, 2nd Ed.

8. Bille-Hansen, E. 1953a. Fructification of three coprophilous species of *Coprinus*, using glucose, sucrose and maltose as carbon source. Bot. Tidsskr. 50: 81

9. Bille-Hansen, E. 1953 b. Fructification of coprophilous *Coprinus* on synthetic medium. Physiol. Plantarum. 6: 523.

10. Bonner, J. 1950. Plant Biochemistry. Academic Press. New York.

11. Bose, S.R. 1947. Antibiotic in a *Polyporus*. Nature 158: 292.

12. Brannon, J.M. 1923. Influence of glucose and fructose on growth of fungi. Bot. Gaz. 76: 257

13. Burkholder, P.R. and D. Moyer. 1943. Vitamin deficiencies of fifty yeasts and molds. Bull. Torrey Bot. Club 70: 372.

14. Burkholder, P.R. and E.W. Sinnott. 1945. Morphogenesis of fungus colonies in submerged shaken cultures. Am. Jour. Bot. 32: 424.

15. Cochrane, V.W. 1958. Physiology of Fungi. Wiley and Sons, New York

16. Coker, W.C. and J.N. Couch. 1928. The gasteromycetes of the Eastern United States and Canada. Univ. of North Carolina Press, Chapel Hill.

17. Cunningham, G.H. 1944. The Gasteromycetes of Australia and New Zealand. 236 pp. Dunedin.

18. Dennis, R.W.G. 1953. Some West Indian Gasteromycetes. Kew. Bulletin: 307

19. Derrick, M.J. 1949. Fundamental studies of the nutrition and physiology of wood destroying Basidiomycetes. Ph.D. thesis, Syracuse University.

20. Doery, H.M., J.F. Gardner, H.S. Burton and P. Abraham. 1951. Antibiotics from a Basidiomycete, *Coprinus quadrifidus*. Antibiotics and Chemotherapy 1: 409.

21. Dorrell, W.W. and R.M. Page. 1947. The use of fragmented mycelium in the culture of fungi. J. Bact. 53: 360.

22. Etheridge, D.E. 1955. Comparative studies of North American and European cultures of the root rot fungus, *Fomes annosus* (Fr.) Cooke. Can. J. Bot. 33: 416.

23. Findlay, W.P.K. 1941. Effect of addition of sugar on rate of decay of wood. Ann. Applied Biol. 28: 19.

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

25. Fries, R.E. 1919. Über einige Gasteromyceten aus Bolivia und Argentinien. Arkiv. fur Bot. 8:1

26. Fries, N. 1943. Vitamin B_1, vitamin B_6 and biotin auf das Wachstum einiger Ascomyceten. Symb. Bot. Upsalienses 7:5

27. Fries, N. 1949. Culture studies in the genus *Mycena*. Svensk Bot. Tidsskr. 43: 316

28. Fries, N. 1950. *Ophiostoma multiannulatum* as a test object for the determination of pyridoxine and various nucleotide constituents. Arkiv Bot. 1:271.

29. Fries, N. 1950. Growth factor requirements of some higher fungi. Svensk Bot. Tidsskr. 44: 370

30. Fries, L. 1953. Factors promoting growth of *Coprinus fimetarius* (L.) under high temperature conditions. Physiol. Plantarum 6:551

31. Fries, L. 1955. Studies in the physiology of *Coprinus*. I. Growth substance, nitrogen and carbon requirements. Svensk Bot. Tidsskr. 49: 475

32. Fries, L. 1956. Studies in the physiology of *Coprinus*, II. Influence of pH, metal factors and temperature. Svensk. Bot. Tidsskr. 50:1

33. Froser, L.M. and B.S. Fugikova. 1958. The growth promoting effect of several amino acids on the common cultivated mushroom Agaricus bisporus. Mycologia 50: 538.

34. Fruton, J.S. and S. Simmonds. 1959. General Biochemistry. Wiley, New York, 2nd Ed.

35. Fuller, R.C. and E.L. Tatum. 1956. Inositol-phospholipid in Neurospora and its relationship to morphology. Am. Jour. Bot. 43: 361

36. Garner, J.H.B. 1956. Gasteromycetes from Panama and Costa Rica. Mycologia 48: 757.

37. Gottlieb, D. 1946. The utilization of amino acids as a source of carbon by fungi. Arch. Biochem. 9: 341.

38. Gottlieb, S., W.C. Day and M.J. Pelczar, Jr. 1950. The biological degradation of lignin II. The adaptation of white-rot fungi to growth on lignin media. Phytopath. 40: 926.

39. Grimm, P.W. and P.J. Allen. 1954. Promotion by zinc of the formation of cytochromes in Ustilago sphaerogena. Plant Physiology 29: 369.

40. György, P. 1954. The Vitamins. In Sebrell and Harris (eds) (527 - 593). Academic Press Inc., New York

41. Haag, E. and C. Dalphin., Une réaction biochimique de l'aneurine d' une grande sensibilite. Compt. rend. soc. phys. hist. mat. Geneva 57: 76. 1940

42. Harris, D.L. 1956. Interaction of thiamine and pyridoxine in Neurospora. II. Competition between pyridoxine and the pyrimidine precursors of thiamine. Arch. Biochem. Biophys. 60: 35.

43. Hawker, L.E. 1939. The nature of the accessory growth factors influencing growth and fruiting of Melanospora destruens Shear, and of some other fungi. Ann. Bot. (N.S.) 3: 455

44. Hawker, L.E. 1950. Physiology of Fungi. Univ. of London Press, London.

45. Herrick, J.A. 1940. The carbon and nitrogen metabolism of Stereum gausapatum Fries. Ohio Jour. Sci. 40: 123

46. Hervey, A.H. 1947. A survey of 500 Basidiomycetes for antibacterial activity. Bull. Torrey Bot. Club 74: 476.

47. Hodson, A.Z. 1949. Oleic acid interference in the Neurospora crassa assay for biotin. J. Biol. Chem. 179:49.

48. Hollos, L. 1904. Die Gasteromyceten Ungarns. Leipzig.

49. Humfeld, H. and T.F. Sugikova. 1952. The nutrient requirements of *Agaricus campestris* grown in submerged culture. Mycologia 44: 605

50. Jansen, B.C.P. 1954. The Vitamins. In Sebrell and Harris (eds) (426 - 448), Academic Press Inc., New York

51. Johnsson, G.T. and A.C. Jones. 1941. Data on the cultural characteristics of species of *Coprinus*. Mycologia 33: 424.

52. Kavanagh, F., A. Hervey and W.J. Robbins. 1949. Antibiotic substances from Basidiomycetes. IC. *Marasmius conigenus*. Proc. Nat. Acad. Sci. 35: 343.

53. Khudiakov, J.P. and U.M. Vozniakovskaia. 1951. Pure cultures of mycorrhizal mushrooms. Mikrobiologia (USSR) 20: 13

54. Kitay, E. and E.E. Snell. 1948. Effect of size of inoculum on the apparent vitamin requirements of lactic acid bacteria. Soc. Exptl. Biol. and Med. 68: 648

55. Kluyver, A.J. and L.H.C. Perquin. 1933. Zur Methodik der Schimmel - Stoffwechseluntersuchung. Biochem Z. 266: 68

56. Koch, W. 1958. Untersuchungen über Mycelwachstum und Fruchtkörperbildung bei einigen Basidiomyceten (*Polystictus versicolor*, *Polyporus annosus*, *Pleurotus ostreatus* and *Psalliota bispora*.) Arciv für Mikrobiologie 30: 409

57. Kögl, F. and N. Fries. 1937. Über den Einfluss von Biotin, Aneurin and meso-Inosit auf das Wachstum verschiedenen Pilzarten. Zeit. physiol. Chem. 249: 93

58. Lamprecht, L. 1957. Zur Physiologie des echten Hausschwamms. Die verarbeitung anorganischer Stick stoffverbindungen in Abhängigkeit von Konzentration, pH-Wert und Isoelektrischem Punkt. Archiv fur Midrobiologie. 27: 182.

59. Lamprecht, L. 1958. Beiträge zur Physiologie des echten Hausschwamms. Über die Verwertung organisch gebundenen Stickstoffs und Kohlenstoffs. Archiv fur Mikrobiologie. 30: 190

60. Lane, R.L. and R.J. Williams. 1948. Inositol, an active constituent of pancreatic (alpha) amylase. Arch. Biochem. 19: 329

61. Lardy, H.A., R.L. Potter and C.A. Elvehjem. 1947. Involvement of biotin in the biosynthesis of oxalacetic and -ketoglutaric acids. J. Biol. Chem. 169: 451

62. Lardy, H.A. 1954. The Vitamins. In Sebrell and Harris (323 - 351) Academic Press, Inc., New York

63. Le Mense, E.H., J. Corman, J.M. Van Lanen and A.F. Langlykke. 1947. Production of mold amylases in submerged culture. J. Bacteriol. 54: 149.

64. Leonian, L.H. and V.G. Lilly. 1939. Studies in the nutrition of fungi II. Effect of inoculum on the growth of the colony. Phytopath. 29: 592

65. Leonian, L.H. and V.G. Lilly. 1940. Auxithals synthesized by some filamentous fungi. Plant Physiol. 15: 515

66. Lilly, V.G. and H.L. Barnett, 1951. Physiology of the Fungi. McGraw Hill Book Co. Inc., New York

67. Lilly, V.G. and H.L. Barnett. 1953. The utilization of sugars by fungi. W. Va. Univ. Agr. Exp. Sta. Bull. 362 T: 5

68. Lilly, V.G. and H.L. Barnett. 1956. The utilization of D and L-arabinose by fungi. Am. Jour. Bot. 43: 709

69. Lindeberg, G. 1939. Über den Einfluss der Wasserstoffionenkonzentrations auf das Wachstum verschiedener Marasmius Arten. Svensk Bot. Tidsskr. 33: 341.

70. Lindeberg, G. 1941. Über die Wirkung von Biotin auf Marasmius androsaceus (L.) Fr. Arch. fur Mikrobiologie 12:58

71. Lindeberg, G. 1944. Über die Physiologie ligninabbauender Bodenhymenomyceten. Studien on Swedischen Marasmius Arten. Symb. Bot. Upsalienses. 8(2) : 1

72. Lindeberg, G. 1946. The effect of biotin and thiamine on the growth of Collybia dryophila Fr. Svensk Bot. Tidsskr. 40: 63

73. Lucas, E.H., R.U. Byerrum, D.A. Clarke, H. Ch. Reilly, J.A. Stevens and C. Ch. Stock. 1958 - 1959. Production of oncostatic principles in vivo and in vitro by species of the genus Calvatia. Antibiotics annual

74. MacLead, D.M. 1959. Nutritional studies on the genus Hirsutella I. Growth response in an enriched liquid medium. Can. Jour. of Bot. 37: 695

75. Madelin, M.F. 1956. Studies on the nutrition of Coprinus lagopus Fr., especially as affecting fruiting. Ann. Bot. (N.S.) 20: 303

76. Mandels, G.R. 1954. Metabolism of sucrose and related oligosaccharides by spores of the fungus Myrothecium verrucaria. Plant Physiol. 29: 18

77. Mandels, G.R. 1955. Biotin and interrupted growth of *Myrothecium verrucaria*. Am. Jour. Bot. 42: 921

78. Margolin, A.S. 1942. The effect of various carbohydrates upon the growth of some fungi. (Thesis) W. Va. Univ.

79. Mathiesen, A. 1950. The nitrogen nutrition and vitamin requirement of *Ophiostoma pini*. Physiol. Plantarum 3:93

80. McNutt, W.S. Jr. 1954. The direct contribution of adenine to the biogenesis of riboflavin by *Eremothecium ashbyii*. J. Biol. Chem. 210: 511

81. Melin, E. 1925. Untersuchungen über die Bedeutungen der Baummykorrhiza. Eine okologisch physiologische Studie. Gustav Fischer. Jena.

82. Melin, E. and B. Nyman. 1940. Weitere Untersuchungen über die Wirkung von Aneurin und Biotin auf das Wachstum von Wurzelpilzen. Arch. für Mikrobiologie. 11:313.

83. Melin, E. 1946. Der Einfluss von Waldstreuextracten auf das Wachstum von Bodenpilzen mit besonderer Berücksichtigung der Wurzelpilze von Bäumen. Symb. Bot. Upsalienses. 8: 3.

84. Melin, E. and V.S.R. Das. 1954. Influence of root-metabolism on the growth of tree mycorrhizal fungi. Physiol. Plant. 7: 851

85. Melin, E. 1959. Studies on the physiology of tree mycorrhizal Basidiomycetes. I. Growth response to nucleic acid constituents. Svensk. Bot. Tidsskr. 53: 135.

86. Mikola, P. 1948. On the physiology and ecology of *Cenococcum graniforma*. Comm. Inst. Forest fennicae. 36: 3.

87. Modess, O. 1941. Zur kenntnis der Mykorrhizabildner von Kiefer and Fichte. Symb. Bot. Upsalienses. 1: 1

88. Nilsson, H. 1956. The growth-stimulating effect of fructose-1, 6-diphosphate on *Boletus variegatus* and *Collybia velutipes*. Physiol. Plantarum. 9: 74

89. Nobles, M.K. 1948. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. Can. Jour. Research Sec. C., 26: 281

90. Norkrans, B. 1950. Studies in growth and cellulolytic enzymes of *Tricholoma*. Symb. Bot. Upsalienses. 11:5

91. Norkrans, B. and B.G. Ranby. 1956. Studies of the enzymatic degradation of cellulose. Physiol. Plantarum. 9: 198

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

93. Oddoux, L. 1953. Essai de culture de 508 especes l' homobasidiomycetes. Inernat. Conf. on Sci. Aspects of mushroom growing. Proc. 2. Mush. Sci. 2: 28

94. Palmer, A.A. 1951. A specific determination and detection of glucose as a probably constituent radical of certain fructosans by means of Notatin. Biochem J. 48: 389.

95. Perlman, D. 1948. On the nutrition of Memnoniella echinata and Stachybotrys atra. Am. Jour. Bot. 35: 36

96. Perlman, D. 1949. Studies on the growth and metabolism of Polyporus anceps in submerged culture. Am. Jour. Bot. 36: 180

97. Plunkett, B.E. 1953. Nutritional and other aspects of fruit-body production in pure cultures of Collybia velutipes (Curt.) Fr. Ann. Bot. (N.S.) 17: 193.

98. Reese, E.T. 1947. On the effect of aeration and nutrition on cellulose decomposition by certain bacteria. J. Bacteriol. 53: 389.

99. Reese, E.T. and H.S. Levinson. 1952. A comparative study ofthe breakdown of cellulose by microorganisms. Physiol. Plantarum 5: 345.

100. Ritchie, D. 1948. The development of Lycoperdon oblongisporum. Am. Jour. Bot. 35: 215

101. Robbins, W.J. and R. ma. 1942. Specificity of pyridoxine for Ceratostomella ulmi. Bull. Torrey Bot. Club 69: 184

102. Robbins, W.J. and A. Hervey. Growth substance requirement of Stereum murraii. Mycologia 47: 155. 1955.

103. Savage, G.M. and Vander Brook. 1946. The fragmentation of the mycelium of Penicillium notatum and Penicillium chrysogenum by a high speed blender and the evaluation of blended seed. J. Bacteriol. 52: 385.

104. Siu, R.G.H. 1951. Microbial decomposition of Cellulose. Reinhold Publ., New York

105. Schöpfer, W.H. 1934a. Les vitamines cristallisées B comme hormones de croissance chez un microorganisme. (Phycomyces). Arch. fur Microbiologie. 5: 511

106. Schöpfer, W.H. 1934 b. Über die Wirkung von reinen kristallisierten Vitaminen auf *Phycomyces*. Ber. deut. bot. Ges. 52: 308

107. Schöpfer, W.H. and M. Guillaud. 1945. Recherches expérimentales sur les facteurs de croissance et le pouvoir de synthese de *Rhizpous cohnii* Berl. et de Toni (*Rhizopus suinus* Neilson.) Zeit. für Vitaminforsch. 16: 181.

108. Scheler-Correns E. 1957. Über die Fruchtkörperbildung von *Coprinus lagopus* bei verschiedenen Stickstoffquellen. Archiv für Mikrobiologie. 26: 52

109. Shirakawa, H.S. 1955. The nutrition of *Diplocarpon rosae*. Am. Jour. Bot. 42: 379

110. Silver, W. and W.D. McElroy. 1954. Enzyme studies on nitrate and nitrate mutants of Neurospora. Arch. Biochem. Biophys. 51: 379

111. Slein, M.W. 1950. Phosphomannose isomerase. J. Biol. Chem. 186: 753

112. 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. 1942.

113. Snell, E.E. and A.N. Rannefelt. 1945. The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. Jour. Biol. Chem. 157: 475.

114. Snell, E.E. 1954. The Vitamins. Sebrell and Harris (366 - 367) Academic Press Inc., New York

115. Stevens, J.A. 1957. Studies of environmental factors influencing in vitro growth of Basidiomycetes and their elaboration of biologically active substances. (Ph.D. thesis) Michigan State University

116. Stokes, J.L., J.W. Foster and C.R. Woodward. 1943. Synthesis of pyridoxine by a "pyridoxineless" X-ray mutant of *Neurospora* sitophila. Arch. Biochem. 2:235.

117. Swartz, D. 1933. Some developmental characters of species of Lycoperdaceae. Am. Jour. Bot. 20: 440

118. Tatum, E.E. and T.T. Bell. 1946. Neurospora. III. Biosynthesis of thiamine. Am. Jour. Bot. 33: 15.

119. Treschow, C. 1944. Nutrition of the cultivated mushroom. Dansk. Bot. Arkiv. 11:1.

120. Umbriet, W.W. 1954. The Vitamins. Sebrell and Harris (234 - 242). Academic Press Inc., New York

121. Van Vliet, W.F. 1954. The enzymic oxidation of lignin. Biochem. et Biophys. 15: 211

122. Vorderberg, K. 1948. Nutritional requirements of *Coprinus lagopus*. Z. Naturforsch. 3b: 272

123. West, E.S. and W.R. Todd. 1956. Textbook of Biochemistry. The MacMillan Co. (2nd Ed.), New York

124. Wiken, T., H.G. Keller, C.L. Schelling and A. Stockli. 1951. Uber die Verwendung von Myzelsuspensionen als Impfmaterial in Wchstumversuchen mit Pilzen. Experientia. 7: 237

125. Wilkins, W.H. 1945. Investigation into the production of bacteriostatic substances by fungi. Cultural work on Basidiomycetes. Trans. Br. Mycol. Soc. 28: 110

126. Wirth, J.C. and F.F. Nord. 1942. Essential steps in the enzymatic breakdown of hexoses and pentoses. Interaction between dehydrogenation and fermentation. Arch. Biochem. 1: 143

127. Wolpert, F.S. 1924. 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. Miss. Bot. Gard. 11: 43.

128. Yusef, H.M. 1953. The requirements of some Hymenomycetes for essential metabolites. Bull. Torrey Bot. Club 80: 43

Typical analysis of Bacto-Yeast Extract - (Furnished by the Difco Laboratories Incorporated, Detroit 1, Michigan)

PER CENT	
Ash	10.10
Total N	9.18
Chloride	0.19
Total Sulphur	1.39
PPM	
Lead	16.00
Arsenic	0.11
Manganase	7.80
Zinc	88.00
Copper	19.00
PER CENT	
Phosphorus	0.89
Iron	0.028
SiO_2	0.052
Potassium	0.042
Sodium	0.32
Magnesium	0.03
Calcium	0.0406
Arginine	0.78
Asparatic acid	5.1
Glutamic acid	6.5
Glycine	2.4
Histidine	0.94

Typical analysis of Bacto-Yeast Extract - continued

PER CENT	
Isoleucine	2.9
Leucine	3.6
Lysine	4.0
Methionine	0.79
Phenylalanine	2.2
Threonine	3.4
Tryptophane	0.88
Tyrosine	0.60
Valine	3.4
MICROGRAMS PER GRAM	
Pyridoxine	20.00
Biotin	1.40
Thiamine	3.20
Nicotinic acid	279.00
Riboflavine	19.00
Folic acid	0.30

Typical analysis of Bacto-Peptone - (Furnished by Difco Laboratories Incorporated, Detroit 1, Michigan)

Total Nitrogen	16.16%
Primary Proteose N	0.06%
Secondary Proteose N	0.68%
Peptone N	15.38%
Ammonia N	0.04%
Free amino N (Van Slyke)	3.20%
Amide N	0.49%
Mono-amino N	9.42%
Di-amino N	4.07%
Tryptophane	0.29%
Tyrosine	0.98%
Cystine (Sullivan)	0.22%
Organic Sulphur	0.33%
Inorganic Sulphur	0.29%
Phosphorus	0.22%
Chlorine	0.27%
Sodium	1.08%
Potassium	0.22%
Calcium	0.058%
Magnesium	0.056%
Manganese	Nil
Iron	0.0033%
Ash	3.53%
Lead	15.00 ppm

Typical analysis of Bacto-Peptone - continued

Arsenic	0.09 ppm
Zinc	18.00 ppm
Copper	17.00 ppm
SiO_2	0.042%
Arginine	8.00%
Asparatic acid	5.90%
Glutamic acid	11.00%
Glycine	23.00%
Histidine	0.96%
Isoleucine	2.00%
Leucine	3.50%
Lysine	4.30%
Methionine	0.83%
Phenylalanine	2.30%
Threonine	1.60%
Valine	3.20%
Pyridoxine	2.50 gamma/gm.
Biotin	0.32 gamma/gm.
Thiamine	0.50 gamma/gm.
Nicotinic acid	35.00 gamma/gm.
Riboflavine	4.00 gamma/gm.