NUTRITIONAL REQUIREMENTS OF *CEPHALOSPORIUM SALMOSYNNEMATUM* 3590A AS DETERMINED IN A CHEMICALLY DEFINED MEDIUM

By

Michael A. Pisano

A Thesis
Submitted to the School of Graduate Studies
of Michigan State College of Agriculture
and Applied Science in partial
fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

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Approved Charles L. San Clemente

ABSTRACT OF THESIS

The object of this work was to develop a chemically-defined medium to supply the nutritional requirements of Cephalosporium salmosynnematum 3590A. A review of the literature has revealed that a study of this nature has not been reported for fungi of the genus Cephalosporium.

The essential components of a synthetic medium are: carbon and nitrogen sources in utilizable forms and minerals in gross and trace amounts. Certain microorganisms also require accessory growth substances. In the development of the synthetic medium for the growth of Cephalosporium salmosynnematum 3590A, the use of appropriate analytical procedures made it possible to determine the exact requirements of the fungus.

No special treatment of the medium was necessary for the study of the gross mineral requirements, but the medium used in the trace metal studies required special purification. Dithizone and carbon tetrachloride were used to remove the trace metals, zinc and copper, from the medium and 8-hydroxyquinoline and chloroform were employed to remove iron.

Each essential component other than those which served as carbon or nitrogen sources was included in the final fermentation medium at twice the concentration needed for maximum yield. The medium finally developed is shown in Table I.

TABLE I

COMPOSITION OF THE FINAL FERMENTATION MEDIUM

Component	Concentration per liter
Glucose	10 g
L-glutamic acid	5 g
Potassium (as KCl)	0.2 g
Magnesium (as $MgCl_2.6H_2O$)	0.04 g
Sulfur (as Na_2SO_4)	0.1 g
Phosphorus (as $NaH_2PO_4.H_2O$)	0.2 g
Zinc (as $ZnSO_4.7H_2O$)	4.5 mg
Iron (as $FeSO_4.7H_2O$)	5 mg
Copper (as $CuSO_4.5H_2O$)	0.08 mg
Distilled water to 1000 ml	
pH adjusted to 7 with NH_4OH	

This medium gave yields in terms of dry weight of mycelium of 45 percent based on the weight of the glucose and L-glutamic acid added. This is comparable to the best yields obtained by various workers in studies dealing with fungi of other genera.

Glucose served as the major carbon source in the final fermentation medium. Cephalosporium salmosynnematum 3590A was found to require organic nitrogen to attain maximum yield. A variety of amino acids was found to be able to serve as suitable nitrogen as well as part of

the carbon sources. Best results were obtained with L-proline and L-glutamic acid.

The following minerals were required in gross amounts: potassium magnesium, sulfur, and phosphorus. Zinc, iron, and copper were found to be essential in trace amounts.

The addition of the common vitamins to the final fermentation medium did not affect cell yields.

In the final developed medium an initial pH of 6.3 proved to be optimum for growth of the fungus.

Analytical data obtained during the submerged cultivation of the fungus in this medium showed that practically no glucose remained at sixty hours, by which time the greatest yield of mycelium had been produced. It was further demonstrated that although organic nitrogen was preferred by the fungus, some utilization of ammonia nitrogen took place.

Maximum yields were obtained only under conditions of adequate aeration using a shaking apparatus, and at a temperature of 30°C.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The nutrition of fungi is best studied through the use of synthetic media. A synthetic medium is one of known concentration and composition. It employs none of the chemically undefined substances found in natural media. The chemicals used are of the highest possible purity. In devising a synthetic medium, attention must be paid to carbon and nitrogen sources in utilizable forms, phosphate and sulfate ions, the metallic ions potassium, magnesium, iron, zinc, manganese and others. In addition to these it is sometimes necessary to supply accessory growth substances such as vitamins in order to obtain satisfactory growth. Since a chemically defined medium employs no complex natural substances, it is essential that specific metabolites be supplied to the organism. In supplying specific substances to a given organism it is possible to gain valuable information concerning its nutritional requirements.

In this study an attempt has been made to develop a synthetic medium for the growth of the mold Cephalosporium salmosynnematum. The organism was originally isolated as a laboratory contaminent at the Michigan Department of Health, Lansing, Michigan. The fungus produces an antibiotic, "synnematin," which has been shown to be active against a variety of microorganisms.[28] It seemed desirable to study the nutritional requirements of this organism for optimal growth in a chemically defined medium. It was decided to investigate the following points:

1. Carbon and nitrogen sources.
2. Gross inorganic mineral requirements.

3. Vitamin and other growth factor requirements.

4. Amino acid requirements.

5. Trace metal requirements.

In order to follow the utilization of the various components of the medium, the glucose, ammonia nitrogen, and total nitrogen levels were determined by appropriate analytical procedures. While the primary goal of this study is to develop a synthetic medium able to support good growth of the organism and to provide information as to its nutritional requirements, it is possible that this project will eventually be instrumental in obtaining greater antibiotic activity from the fungus. Finally, a study of this nature may make a contribution to the field of fungal physiology.

HISTORICAL

Raulin[77] is usually given credit for having devised the first synthetic medium for cultivating fungi. It contained sucrose as the carbon source and ammonium nitrate as the major nitrogen source as well as essential minerals. In 1881 Brefeld[7] advised adding cigar ashes dissolved in nitric or citric acid to a solution containing a soluble carbohydrate such as glucose, and an ammonium salt. Much of the early work in the field of fungal nutrition was conducted on solid media. Media which contain agar or semisolid substrates, such as corn meal, offer advantages in that the culture vessels can be freely handled without disturbing the fungus. This is a desirable feature when it is necessary to follow the development of a fungus. Microscopic examination is facilitated, and contaminants are more easily detected. Single spore isolations can be made more easily from solid media. Agar media are also used to maintain stock cultures. Frau Hesse[36] introduced the use of agar into microbiological procedures in 1881. It has been shown that agar introduces physiologically active elements into media such as zinc,[51] as well as other essential micro-essential elements. Agar may contain growth factors such as thiamine[15] and biotin.[79] It is obvious that for precise investigations where it is desired to control as many variables as possible, liquid media should be used. The composition of the medium may be controlled and the amounts used measured accurately. Liquid media may be stationary or may be aerated by shaking or by blowing

sterile air through the media. The determination of the dry weight of mycelium is facilitated in a synthetic medium. It is easier to study metabolic by-products and isolation of by-products is less complicated when liquid media are used. The references reported in this paper present data obtained on both liquid and solid media. In order to facilitate the presentation of the data compiled from these references, the arrangement of the material discussed in this paper is made from the viewpoint of chemical requirements. The utilization of amino acids is discussed under the sections labeled carbon and nitrogen requirements.

Carbon Requirements

Carbon makes up almost half of the dry weight of fungus cells. Protoplasm, enzymes, the cell wall, and reserve nutrients stored within the cells are compounds of carbon. Carbon compounds also play an important role in the nutrition of fungi. Fungi secure energy by oxidizing organic compounds. Carbon serves as both a structural and functional element. The number of carbon compounds known far exceeds the total of known compounds of all other elements because of the property of carbon-forming compounds in which carbon is linked to carbon in the form of chains and rings. Other elements such as nitrogen, oxygen, and sulfur may serve as linking elements. While many carbon compounds are stable at ordinary temperatures, others are extraordinarily sensitive to a wide range of chemical reagents and to slight changes in the physical environment.

In attempting to culture fungi of unknown nutritional requirements on synthetic or semi-synthetic media, glucose seems to be the customary

sugar to use first. Some fungi, however, are unable to utilize glucose. Leptomitus lacteus[89,90] was unable to utilize glucose, fructose, galactose, or sucrose. Weimer and Harter[128] reported that Sphaeronema fimbriatum was incapable of utilizing glucose. Skoog and Lindegren[97] reported the behavior of twelve strains of Saccharomyces cerevisiae which did not utilize glucose when first isolated. These strains became adapted to glucose on sufficiently long exposure to this sugar. The yeasts probably were able to form adaptive enzymes.

No carbon source will be utilized if the medium is lacking in any essential element or compound. Kinsel[43] reported that Diplodia macrospora would grow only on disaccharides and not on media containing glucose or other monosaccharides. Several investigators, among them, Wilson,[132] found that the organism was deficient for biotin. It is not unlikely that other vitamin-deficient fungi have been reported in the past as unable to utilize certain sugars owing to the absence of specific growth factors.

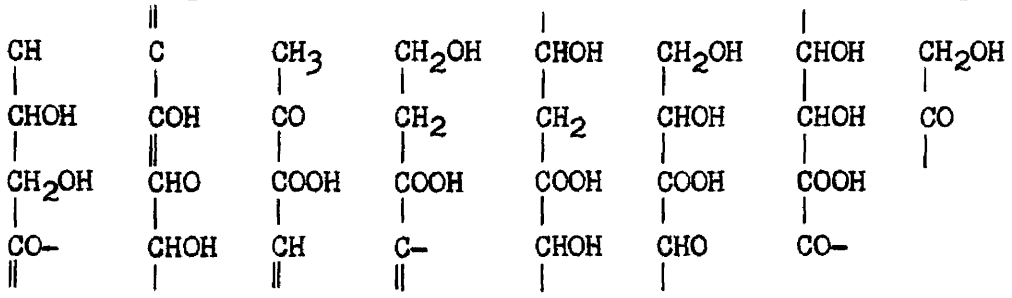
Taking other sugars and related compounds into consideration, Ezekiel, Taubenhaus and Fudge[19] found that the root rot fungus, Phymatotrichum omnivorum, grew best in media containing dextrose, maltose and xylose and obtained less growth with mannose, mannitol, lactose and sucrose. Each sugar was used singly. Mosher, Saunders, Kingery and Williams[62] reported that Trichophyton interdigitale was able to metabolize all sugars except lactose. Steinberg[107] found D-glucose, D-fructose, D-mannose, L-sorbose and sucrose to be equally effective in the nutrition of Aspergillus niger whereas D-galactose, lactose,

glycerol, and mannitol were poor sources of carbon for this fungus. Herrick[35] reported that two isolates of Stereum gausapatum grew on glucose, fructose, mannose, and galactose. One isolate grew best on fructose; the other grew equally well on all four sugars. This is an indication that not all isolates of a species are alike in their ability to utilize a given sugar. Pieters[73] stated that Saprolegnia ferax, S. monoica, Achlya racemosa, and A. prolifera were unable to utilize sucrose. Schopfer and Blumer[91] found that Ustilago violacea grew well on D-sorbitol. Herrick[35] stated that Stereum gausapatum utilized xylose better than arabinose whereas Tamiya[120] reported that Aspergillus oryzae utilized arabinose better than xylose. Growth has been reported on several methylpentoses. Steinberg[113] found that Aspergillus niger utilized L-rhamnose to some extent, but L-fucose was not utilized. Tamiya[120] stated that Aspergillus oryzae made much poorer growth on L-rhamnose than on D-xylose or L-arabinose.

Various investigators have employed sugar alcohols and sugar acids as carbon sources. Steinberg[113] obtained growth of Aspergillus niger on 2-keto-D-gluconic, 5-keto-D-gluconic, D-gluconic, D-glucuronic, and mucic acids whereas Tamiya[120] found that Aspergillus oryzae utilized D-gluconic acid. Frequently a mixed carbon source results in better growth of a given fungus. Horr[39] found that combinations of galactose and glucose gave better growth of Aspergillus niger than when each sugar was used alone.

Tamiya's paper[120] presents an elaborate study on the relationship between structure and assimilability. He used Aspergillus oryzae as

the test organism. Percentage of maximum yield of mycelium (sucrose = 100 percent) was less than 120 with inulin, dextrin, gluconic acid, mannose, starch, and glycogen; 90 to 100 with maltose, raffinose, fructose, and sucrose; 60 to 90 with galactose, trehalose, dihydroxyacetone, adonitol, dulcitol, glucose, sorbitol, and quinic acid; 30 to 60 with protocatechuic acid, arabinose, saccharic acid, monoacetin, and inositol; and 10 to 30 with malic, tartaric, citric, succinic, lactic, and salicylic acids, mannitol, ethyl alcohol, glycerol, etc. Other compounds were also tested. He observed that some compounds can serve for respiration but not for growth and that no constant relation exists between respiration and growth, since respiration was found to vary with the carbon source. Tamiya[120] further noted that practically every compound suitable for growth contained one or more of the following groups:



The differences in assimilability of the same hexose by two different strains of the same fungus may, and often do, account for the variations encountered. In the absence of any experimental evidence to the contrary, however, the differences may be explained equally well on the basis of salt proportions or trace elements, and organic impurities in different samples of carbon compounds.

Other classes of compounds may serve as carbon compounds. Among these are the amino acids. Steinberg[112] investigated the utilization of amino acids as carbon sources by Aspergillus niger. He found that

certain combinations of "primary" amino acids (amino acids synthesized first by the organism) were utilized about three-fourths as efficiently as sucrose. Gottlieb[27] studied the utilization of individual amino acids by Penicillium roqueforti and Fusarium oxysporum var. lycopersici. Not all the naturally occurring amino acids were utilized as carbon sources by these fungi. The six-carbon straight-chain amino acids, norleucine and lysine, and the sulfur-containing amino acids, cysteine and methionine, were not utilized as carbon sources. Although glycine and valine were poor carbon sources for P. roqueforti, F. oxysporum var. lycopersici grew well on these amino acids. Alternaria solani, Helminthosporium sativum, Rhizoctonia solani, Fusarium moniliforme, Chaetomium globosum, and Aspergillus niger were unable to utilize the naturally occurring sulfur-containing amino acids as a source of carbon. Shultz, McManus and Pomper[93] tested the ability of yeasts to utilize different amino acids as the sole source of carbon. Glutamic acid and proline were available to more species than any other amino acid. Fungi which utilize amino acids as the sole source of carbon characteristically cause the medium to become alkaline. This may be due to an accumulation of ammonia which results from deamination. In general, the amino acids appear to be poor sources of carbon.

Nitrogen Requirements

Robbins[78] has set forth a classification of the fungi based upon their ability to assimilate various forms of nitrogen. The classification is as follows: Group I - fungi able to utilize atmospheric nitrogen, nitrate nitrogen, ammonia nitrogen, and organic nitrogen;

Group II - fungi capable of assimilating nitrogen as nitrates, ammonia nitrogen, and organic nitrogen, but not able to utilize atmospheric nitrogen. Group III - fungi able to utilize ammonia nitrogen and organic nitrogen, but unable to utilize atmospheric or nitrate nitrogen. Group IV - fungi capable of assimilating organic nitrogen, but unable to utilize atmospheric, nitrate, or ammonia nitrogen.

Disagreement exists concerning the ability of fungi to fix atmospheric nitrogen. Allison, Hoover, and Morris[1] obtained negative results on nitrogen fixation with eight strains of Aspergillus niger, A. fuscus, A. cinnamoneus, A. niger var. altipes, and five species of actinomycetes. Duggar and Davis[17] obtained fixation only with Phoma betae out of a large number of fungi investigated. Much work remains to be done in this field.

Some fungi are able to utilize nitrite (NO_2^-) nitrogen. According to Leonian and Lilly,[49] Blakeslea trispora made some growth on nitrite nitrogen. Because of the instability of nitrites in acid solution and the destructive effect of nitrous acid on proteins and amino acids, nitrite nitrogen is little used in making media. The nitrates on the other hand are more commonly used in preparing media. Dimond and Peltier[16] found that Penicillium notatum was able to utilize $NaNO_3$. The pH of the medium containing $NaNO_3$ rose slowly from a value as low as four to the range favorable for penicillin production. The trend in pH was apparently determined by the utilization of the nitrate ion at a more rapid rate than the sodium ion. Ezekiel, Taubenhaus and Fudge[19] reported that Phymatotrichum omnivorum was able to utilize nitrates as a source of nitrogen. Some fungi apparently use nitrate

nitrogen in preference to ammonia nitrogen when both are supplied in the medium. Fusarium lini appears to be such a fungus.[133]

Most fungi appear to utilize ammonia nitrogen before nitrate nitrogen when both are supplied in the medium. Thornberry and Anderson[121] reported that Streptomyces griseus utilized ammonia nitrogen as the nitrogen source whereas nitrate nitrogen was not. Lockwood, Ward and May[57] found that a wide range of NH_4NO_3 concentration of from 1.5 to 6 grams per liter was favorable for D-lactic acid production by Rhizopus oryzae. Ezekiel, Taubenhaus and Fudge[19] reported that Phymatotrichum omnivorum grew well on NH_4NO_3. Steinberg[108] pointed out that Hagem investigating a large number of mucors found that Mucor griseocyamus, M. racemosus, M. christiaienis, M. spinosus, and M. sphaerosporus were able to employ either amonia or nitrate nitrogen with glucose, but required nitrate nitrogen with glycerol. The last two fungi named also were found to require nitrate with mannitol. Steinberg felt that these findings demonstrated that the nitrogen requirements of an organism are not fixed but vary with the source of carbon supply. Several investigators have found that the utilization of ammonia and some forms of organic nitrogen may be modified by the presence of other compounds in the medium. Leonian and Lilly[51] found that the growth of Phycomyces blakesleeanus, in a medium with ammonia nitrogen as the nitrogen source, was within certain limits directly proportional to the amount of succinic acid in the medium. Brian, Curtis and Hemming[8] suggested, on the basis of studies of Myrothecium verrucaria that a definite antagonism exists between the metabolic pathways involved in nitrate and ammonia utilization, and in the presence of ammonia

nitrogen the nitrate pathway is blocked. It was further suggested that ammonia nitrogen was poorly utilized unless certain organic acids were present in the medium. They suggested that different pathways of carbohydrate utilization may be followed, depending on whether nitrate or ammonia nitrogen is present.

Among the numerous possible organic nitrogen sources perhaps the most important for the nutrition of fungi in synthetic media are the amino acids. Whereas such compounds as proteins and peptones introduce chemically undefined substances into a medium, the use of amino acids makes it possible to supply a chemically defined organic source of nitrogen. As nitrogen sources the amino acids are not of equal value in the nutrition of fungi. Leonian and Lilly[49] tested the relative value of twenty-four amino acids for fourteen fungi and found that no one amino acid was best for all these species. Steinberg[112] found alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, orinthine and proline to be excellent sources of nitrogen for Aspergillus niger. He felt that these amino acids were those which were synthesized first (primary amino acids) by this fungus and from which the other amino acids (secondary amino acids) were normally formed. It is assumed that the "primary" amino acids enter directly into the metabolic pathways, whereas the "secondary" amino acids must undergo preliminary deamination before use. The primary amino acids are probably not the same for all fungi. Reporting on the amino acid metabolism of Penicillium chrysogenum Q176, Wolf[134] found that alanine, glutamic acid and proline were rapidly oxidized by this organism. This author pointed out that proline and glutamic acid are said to be the

amino acids present in the highest concentrations in corn proteins. This is significant in that corn products are frequently employed in the cultivation of fungi. A mixture of amino acids may or may not be utilized better than a single amino acid. The effect of one amino acid on the utilization of another varies with the amino acids involved and the specific fungus used. Leonian and Lilly[51] tested the growth of Phycomyces blakesleeanus upon five single amino acids and upon a mixture of five amino acids with the following results: Mixture of five amino acids, 214; asparagine, 209; DL-alanine, 151; arginine, 50; aspartic acid, 203; glycine, 201; and glutamic acid, 189 mg, respectively. Although arginine was a poor source of nitrogen for P. blakesleeanus, the presence of arginine in the amino acid mixture did not depress growth. Halpern et al.[30] found that when proline and glutamic acid were added to Czapek-Dox medium containing four percent lactose, the yield of mycelium was greatly increased. These authors also reported that a straight line relationship existed between the concentration of proline and the yield of mycelium in a basal medium containing Czapek-Dox minerals and four percent lactose. Nielsen and Hartelius[67] found that beta-alanine increased the growth of Saccharomyces cerevisiae only if aneurine and asparagine (or glutamic acid) were added. If those compounds were not added beta-alanine had a poisonous effect on the yeast. Addition of asparagine and glutamic acid increased the growth of yeast even if beta-alanine was not added.

Organic acids, especially the four-carbon dicarboxylic acids, affect the utilization of some amino acids. Phycomyces blakesleeanus on a medium containing arginine produced 43 mg of mycelium per flask.

Addition of 0.1 percent succinic acid to the medium increased the yield to 192 mg.[51]

Mineral Requirements

Several methods have been employed in order to determine which elements are essential for the growth of fungi. One of these methods is considered from the standpoint of ultimate analysis of mycelium and spores. Ultimate analyses of mycelium and spores have revealed the presence of a variety of elements including phosphorus, potassium, magnesium, sulfur, iron, zinc, copper, lead, tellurium, silver, gold, and others. However, the mere presence of an element in fungus cells does not necessarily mean that it is essential. Another way of approaching the problem of essentiality is by the omission of the element in question from the medium. Omission of an essential element prevents or limits the growth of the organism. In general, the experimental work in which specific elements have been omitted from the medium is more convincing than the method of ultimate analysis.

Gross Inorganic Mineral Requirements

The essential gross inorganic minerals are needed in larger amounts than the so-called essential "trace metals." They may be either metallic or non-metallic. Of the non-metallic elements in this category, hydrogen, oxygen, sulfur and phosphorus have been found to be essential. Carbon and nitrogen have already been discussed under separate headings.

Essential Non-metallic Elements

Hydrogen. This element enters into the composition of nearly all organic compounds of interest to physiology except carbon dioxide.

Elemental hydrogen is not used by fungi. All the hydrogen used by fungi is in chemical combination. Certain bacteria, however, are able to obtain energy by oxidizing hydrogen. Hydrogen is a component of the water molecule. The chemistry of life processes is largely confined to reactions which take place in the presence of water or in solution. Water ionizes to form hydronium (H_3O^+) and hydroxyl (OH^-) ions. The effects of these ions on biological processes are well known.

Oxygen. Many fungi are strictly aerobic and apparently none are obligate anaerobes. Some fungi are facultative anaerobes. Thus, whereas an aerobic organism requires uncombined oxygen, a facultative anaerobe may use combined oxygen in addition to free oxygen. The amount of oxygen required for optimum growth varies with the species. The ability of certain soil fungi to exist under conditions of low oxygen supply is important for survival. Hollis[38] found Fusarium oxysporum to survive under essentially anaerobic conditions for thirteen weeks, whereas F. eumartii perished within three weeks when exposed to the same conditions. The mycelium of F. oxysporum grown under reduced oxygen tension was abnormal in its morphology.

Sulfur. This element is present in many types of compounds, both inorganic and organic. Sulfate sulfur ($SO_4^=$) is the most common source of sulfur used in media. Some fungi, however, require specific organic sources os sulfur. Steinberg[106,111] has made an exhaustive study of sulfur sources for Aspergillus niger and reached the general conclusions that inorganic sulfur compounds containing oxidized sulfur were utilized, whereas sulfide and disulfide sulfur were not utilized. Many fungi utilize organic sulfur contained in natural metabolites to better advantage

or require these compounds as a source of sulfur. Leonian and Lilly[49] found that the addition of cystine to a synthetic medium was necessary for the growth of Saprolegnia mixta, achlya conspicua, Isoachlya monilifera, and Aphanomyces camptostylus. Volkonsky[126,127] observed that certain of the aquatic Phycomycetes failed to utilize sulfate sulfur. These species were Saprolegnia parasitica, Isoachlya monilifera, Achlya prolifera, A. polyandra, A. oblongata, A. conspicua, Dichtyuchus monosporus, and Aphanomyces sp. A total of twenty-six isolates failed to utilize sulfate sulfur.

Phosphorus. Raulin[77] found phosphorus to be an essential element for Aspergillus niger. Phosphorus seems to be utilized only when in the form of phosphate. Jarvis and Johnson[41] found that the growth of Penicillium chrysogenum Q176 appeared to be a linear function of the phosphorus level between 0 and 65 mcg per ml. Smith[99] studied the phosphorus metabolism of Merulius lacrymans and Marasmius chordalis in connection with the utilization of different carbon sources. In glucose medium M. lacrymans grew better when supplied with inorganic phosphate, whereas M. chordalis grew more rapidly when supplied with organic phosphorus (adenylic acid). On cellulose medium M. lacrymans grew faster when supplied with organic phosphorus. Phosphorus compounds play an important role in the functions of chemical transformations and energy transfers.

Other non-metallic elements. It has not been established that fungi require non-metallic elements other than hydrogen, oxygen, sulfur, phosphorus, and nitrogen. Evidence is lacking as to whether or not boron and iodine are essential. Sodium and chloride ions are often added to media, but neither has been proved necessary for the growth of fungi.

Essential Metallic Elements

The essential metallic elements are generally classified as functional elements although they may have structural functions. The metallic elements known to be essential to fungi include potassium, magnesium, iron, zinc, copper, calcium, gallium, manganese, molybdenum, vandadium, and scandium. It must be realized that the above elements are only essential for some fungi under certain conditions. While it may be assumed that all fungi require many of the same elements, experimental evidence is lacking for most species.

The essential metallic elements may be divided into two groups, macro- and micro-metallic elements. This grouping is made only for convenience and on the basis of amounts ordinarily employed in culturing fungi under laboratory conditions.

Essential Macro-metallic Elements

Potassium. As far as is known potassium is essential for all organisms. Steinberg[115] found that the optimum amount of potassium required by Aspergillus niger was 150 mg per liter. The relative amounts of mycelium formed increased as the potassium content of the medium decreased. The fungus produced almost three times as much mycelium per milligram when fifteen instead of 150 mg per liter were used. Jarvis and Johnson[41] reported that Penicillium chrysogenum Q176 required 40 mg of potassium per liter of medium for optimum growth. Steinberg[115] studied the problem of biological substitution using Aspergillus niger as the test fungus. Biological substitution means that one element can replace another, in whole or in part. It was found that sodium gave increased yield of mycelium in a medium containing suboptimal

amounts of potassium. Beryllium was found to give increased yields in a medium containing suboptimal amounts of magnesium. Studies of biological substitution require great care and knowledge of the composition of the media employed and of the behavior of the fungus under the experimental conditions used.

Magnesium. Steinberg[115] found that within certain limits of concentration, the amount of growth of Aspergillus niger was proportional to the concentration of magnesium in the medium. Jarvis and Johnson[41] studying the nutrition of Penicillium chrysogenum Q176 reported that the fungus required 8 mg of magnesium per liter of medium for optimum growth. Rabinovitz-Sereni[76] found that Penicillium glaucum, Botrytis cinerea, and Alternaria tenius failed to grow in the absence of magnesium. These fungi were able to grow in a medium with a concentration of magnesium sulfate of 40 percent. These three species were able to grow in the presence of traces of magnesium but sporulated only when the concentration of magnesium was increased. Respiration also increased as the magnesium content of the medium increased. Failure to sporulate unless sufficient magnesium is available is probably to be expected with many fungi. Magnesium is involved in many of the enzymatic reactions involved in fermentation.

Calcium. The calcium requirements of the fungi are not uniform. Young and Bennett[139] reported that Rhizoctonia solani did not grow in the absence of this element. The pathogenic mold Trichophyton interdigitale was found to require calcium.[62] Steinberg[116] found that Aspergillus niger and Fusarium oxysporum needed no more than spectroscopic traces of calcium, if they required the element at all.

Several other fungi were found to require calcium by this investigator. The concentrations of calcium required for maximum growth varied from two to six mg per liter of medium.

Essential Micro-metallic Elements

These metallic elements are just as important nutritionally for fungi as the carbon source, nitrogen source, and the traditional minerals like magnesium, potassium, phosphorus, sulfur, etc. In general, however, the amounts required are so small relative to the others that the designation "trace elements" is applied. Raulin[77] showed that in addition to the usual mineral nutrients, certain other elements, such as zinc and iron, must be furnished in the medium in order to obtain the maximum dry weight yield of the fungus. Besides zinc and iron, copper, manganese, molybdenum and gallium, and possibly a few other metals, are now considered essential for the growth of the fungi.

Iron. So far as is known, iron is essential for all fungi. Because of the almost universal occurrence of a group of iron-containing enzymes (catalase, the cytochromes, cytochrome oxidase, etc.), the essential role of iron is taken for granted. Steinberg[102,107] showed that iron is essential for the growth of Aspergillus niger. Rogers[85] found that iron was stimulatory at certain concentrations for Phymatotrichum omnivorum. It is interesting to note that Koffler et al.[47] were able to reproduce the ability of corn steep ash to increase penicillin production in a basal synthetic medium by the addition of iron and soluble phosphates. Yoshimura[138] showed that the amount of catalase produced by Aspergillus oryzae increased as the amount of iron in the medium increased. Jarvis and Johnson[41] reported that iron

was essential for Penicillium chrysogenum Q176. Lilly and Leonian[56] showed that a relation existed between the amount of iron supplied in the medium and the ability of Rhizobium trifolii to synthesize certain vitamins. In the presence of suboptimal concentrations of iron, the addition of certain vitamins replaced iron to a certain degree. A quantitative study of the vitamins synthesized by Torulopsis utilis showed that the iron concentration was important.[53] Increased amounts of thiamine, riboflavin, nicotinic acid, and pyridoxine were synthesized on media, low in iron, whereas the amounts of biotin, inositol and p-aminobenzoic acid were decreased. The iron concentration of the medium was shown to affect the amount of pigmentation of Torulopsis pulcherrima.[82]

Zinc. Both Raulin[77] and Steinberg[101] showed this element to be essential for Aspergillus niger. Mosher, Saunders, Kingery and Williams[62] found that the pathogenic mold Trichophyton interdigitale required zinc for growth. Foster and Waksman[24] found zinc indispensible for the growth of Rhizopus nigricans. Rogers[85] reported that the growth of Phymatotrichum omnivorum was stimulated by zinc in concentrations up to and including 200 ppm. Kauffman-Cosla et al.[42] found that the growth of Aspergillus niger in Raulin medium was affected by the presence of zinc in that the presence of the latter caused increased assimilation of the glucose and the formation of cellulose. Zinc was found to act in dilutions from 1/150,000 to 1/50,000,000, the action being proportional to the concentration.

Zinc ions activate various enzymes such as enolase and dipeptidase. Zinc is contained in carbonic anhydrase, an enzyme which

catalyzes the decomposition of carbonic acid to carbon dioxide and water. Foster and Waksman[24] demonstrated that a zinc deficiency brought about incomplete oxidation of glucose by Aspergillus niger with an increase in fumaric acid production. Fumaric acid was produced most efficiently when the concentration of zinc was 1.2 mg per liter. Higher concentrations of zinc resulted in increased growth and decreased production of fumaric acid. From these results it appears that zinc plays a role in the utilization of glucose, the completeness of oxidation and assimilation being favored by relatively high concentrations of zinc. Foster[22] pointed out that although zinc-containing cultures attained maximum growth rapidly, they autolyzed with considerable loss in weight. This fact should be kept in mind where mycelial weight is being determined.

Copper. This element has been shown to be essential for animals, green plants, and fungi. Marked responses by fungi to copper are difficult to achieve because of the minute amounts which are sufficient for growth. Steinberg[103] found that 0.04 mg of added copper per liter of purified medium was sufficient for the maximum growth of Aspergillus niger. Under these conditions omission of copper decreased the yield only from 984.8 to 774.3 mg. McHargue and Calfee[60] showed that copper was essential for Aspergillus flavus and Rhizopus nigricans. The full effect of copper was dependent upon the presence of other essential elements. In his review on the heavy metal nutrition of fungi, Foster[22] described the work of Wolf and Emmerie which showed the essential nature of copper for the growth of Aspergillus niger.

These workers grew the fungus on an electrolytically purified medium, then removed the liquid and obtained no further growth upon reincubation. If 0.1 mcg per 90 cc was then added, growth commenced as usual, indicating that copper was the responsible agent. At least 0.2 mcg per 250 cc of nutrient medium was required for growth to occur and 0.3 mcg for the appearance of the first spores. Copper is an essential constituent of certain enzymes, including tyrosinase, which occurs in many fungi.

Other micro-metallic elements. McHargue and Calfee[60,61] noted that growth of Aspergillus flavus, Rhizopus nigricans, and Saccharomyces cerevisiae increased in the presence of added manganese. Steinberg[114] showed that omission of manganese from a balanced medium resulted in a decrease in yield of Aspergillus niger from 1,084.8 to 356,6 mg. No spores formed when manganese was omitted. Manganese (Mn++) has been shown to be a natural activator of yeast arginase. Other enzymes are activated by this element.

Under certain conditions Steinberg[105] was able to show that omission of gallium from the medium led to decreased yield and sporulation of Aspergillus niger. In view of the similar chemical behavior of gallium and aluminum, Steinberg considered it possible that the biologic activity sometimes attributed to aluminum may in reality be due to gallium.

Steinberg[108] found that scandium appeared to be essential when glycerol was used as a carbon source for Aspergillus niger. Growth was poor on this carbon source; omission of copper or manganese increased the yield somewhat. Omission of scandium decreased the yield

from 269.4 to 107.4 mg. Scandium appeared to have no effect on growth when sucrose was used as a source of carbon. Addition of lysine or proline (20 mg per liter) to the glycerol medium increased growth and at the same time prevented the effect of scandium. These results suggest that the need for certain elements may be shown only under certain nutritional conditions.

The effect of molybdenum on fungi was investigated by Steinberg.[103,104] This investigator found that more molybdenum was required by Aspergillus niger for maximum growth in media containing nitrate nitrogen than in media with ammonia nitrogen. He expressed the opinion that molybdenum is essential for Aspergillus niger even when ammonia nitrogen is available.

Whether or not vanadium and cobalt are required by fungi has not been definitely established.

Vitamin Requirements

Many fungi are able to grow and develop normally on a substrate containing no vitamins. Other fungi do not grow on synthetic media composed of pure chemicals. While certain fungi are able to synthesize required vitamins, others do not possess this ability. Robbins and Kavanagh[81] named the latter group "vitamin-deficient" fungi. These authors felt that Schopfer was the first to demonstrate satisfactorily the importance of a vitamin for the growth of fungus. Schopfer[92] showed that the addition of crystalline thiamine to a solution of pure sugar, minerals and asparagine made growth of Phycomyces blakesleeanus possible in such a medium. Lilly and Barnett[55] pointed out

that vitamin deficiencies among the fungi have been detected only for certain members of the water-soluble B-complex. The most common vitamins involved are thiamine, biotin, inositol, pyridoxine, nicotinic acid, and pantothenic acid.

Phycomyces blakesleeanus is an example of an organism having a single vitamin deficiency; i.e., it requires only one vitamin for growth. Robbins and Kavanagh[80] listed the following as aided by thiamine: Phytophthora capsici, P. cinnamomi, P. cryptogea, P. drechsleri, P. palmivora, P. parasitica, P. bochmeriae, P. cactorum, P. cambivora, Phycomyces nitens, Schizophyllum commune, Sclerotium delphinii, S. rolfsii, Sphaerulina trifolii, Pythium anhenomanes, and P. polycladon. Leonian and Lilly[49] stated that thiamine is necessary for Blakesleea trispora, collyhia tuberosa, Lentinus nitens, Phytophthora erythoseptica, Pythiomorpha gonapodioides, and Pythium polymastum.

Some fungi have multiple deficiencies (for two or more vitamins). An example of an organism having a multiple deficiency is furnished by Sclerotinia camelliae. Barnett and Lilly[4] reported that little or no growth of this organism occurred on a vitamin-free medium or that containing either thiamine or biotin alone. The fungus grew well only in media containing both thiamine and biotin. When inositol also was added, growth was consistently better than in the presence of the two vitamins. According to these investigators, this indicated a partial deficiency for inositol, in addition to the total, or near total, deficiencies for thiamine and biotin. Lilly and Barnett[54] stated that Chaetomium convolutum was deficient for thiamine and biotin. In the presence of an optimum amount of one of these vitamins, growth was

proportional to the amount of the other vitamin in the medium. White[129] reported that Ophiobolus graminis required biotin and thiamine for mycelial development. Multiple vitamin deficiencies are more common among the yeasts than among the filamentous fungi. The vitamin requirements of thirty-eight species and strains of yeast were reported by Burkholder,[9] and for one hundred and ten additional named species and varieties by Burkholder, McVeigh and Moyer.[10] A summary of the deficiencies reported in these two papers is as follows: biotin, 114; thiamine, 48; pantothenic acid, 44; inositol, 19; nicotinic acid, 19; pyridoxine, 19. No deficiency for riboflavin was found.

A number of vitamins, such as thiamine and biotin, have been shown to perform definite functions in fungi as well as in animals. The characteristic features of a vitamin include the following: (1) its organic nature; (2) its activity in minute quantities; (3) its catalytic action; (4) the specificity of its action. Some vitamins are known to be components of various enzyme systems.

METHODS AND MATERIALS

Fermentation Procedures

Cephalosporium salmosynnematum 3590A was used exclusively in these experiments. Roberts[83] has given information concerning the origin of this strain as well as its cultural behavior. The organism was obtained from the Division of Laboratories of the Michigan Department of Health.

All fermentations were carried out on a Gump rotary shaker in wide-mouth 500 ml Erlenmeyer flasks. The shaker operated at about 250 rpm, each flask describing a two and one-quarter inch diameter circle. Each flask containing 50 ml of the fermentation medium was plugged with cotton and sterilized at 121°C for twenty-five minutes. The inoculum was prepared by washing the growth of the organism from a slant of Czapek-Dox agar with approximately five ml of sterile distilled water. A flask containing the seed medium was inoculated with the aqueous suspension and placed on the shaker for seventy-two hours. The incubation temperature was 30°C. At the end of the seventy-two hours, approximately one ml of the growth suspension was transferred to a new flask also containing seed medium. This flask was placed on the shaker and allowed to remain for twenty-four to thirty-six hours. The content of the latter flask was then homogenized in a Waring Blendor for approximately one minute and 0.2 to 0.3 ml of the homogenized suspension was added per flask as the inoculum. Vegetative inoculum was used throughout the experiments. Two different seed

media were employed to prepare the inoculum. The compositions of these media are shown below.

TABLE I

COMPOSITION OF THE SEED MEDIA

Medium A		Medium B	
Component	Concentration per liter	Component	Concentration per liter
Glucose	10 g	Glucose	10 g
$(NH_4)_2SO_4$	4 g	L-glutamic acid	5 g
KH_2PO_4	1 g	Potassium (as KCl)	0.2 g
$MgSO_4.7H_2O$	1.5 g	Magnesium (as $MgCl_2.6H_2O$)	0.04 g
L-asparagine	1* g	Sulfur (as Na_2SO_4)	0.1 g
Zinc (as $ZnSO_4.7H_2O$)	4.58 mg	Phosphorus (as $Na_2HPO_4.H_2O$)	0.2 g
Iron (as $FESO_4.7H_2O$)	2 mg	Zinc (as $ZnSO_4.7H2O$)	4.58 mg
Copper (as $CuSO_4.5H_2O$)	1.25 mg	Iron (as $FeSO_4.7H_2O$)	2 mg
Distilled water to 1,000 ml		Copper (as $CuSO_4.5H_2O$)	1.25 mg
pH adjusted to 6.5 with NaOH		Distilled water to 1,000 ml	
		pH adjusted to 7.0 with NH_4OH	

* L-asparagine was also used in a concentration of 2.5 g in some of the experiments.

Medium A was used only in the earlier phases of this study.

In the studies concerning the minerals, the element under investigation was removed from the medium and then added in graded amounts. If it proved to be essential, it was included in the medium for subsequent studies at a concentration which was in excess of the indicated level. In the case of the minerals that were required in large amounts, no special purification of the medium was attempted. The level of the element was reduced in the inoculum flasks, however. The inoculum for the mineral studies was not homogenized in the blendor in order to eliminate the possibility of introducing metals into the medium from the blendor itself.

The media used for the trace metal studies were prepared by the methods of Olson and Johnson[69] with one modification. This was that all shaking for the extraction of the trace metals was done by hand instead of on a reciprocating shaker, over a two-hour period rather than overnight. Zinc was removed from the medium through the addition of 120 ml of approximately 0.1 percent dithizone in carbon tetrachloride to 600 to 700 ml of the medium at pH 5.0. Trace metals were added after purification. The medium was extracted with dithizone in a one liter separatory funnel by shaking intermittently over a period of two hours. The excess dithizone and the dithizone-metal complex were then removed by repeated extraction with approximately 0.1 volume of redistilled carbon tetrachloride. In order to remove completely the excess dithizone, three extractions were made after all visible color had disappeared. Prior to dispensing the medium into flasks the pH was adjusted to 7.0. Iron and copper were then added in excess. Finally, zinc was added in graded amounts. The flasks were autoclaved and were ready for use.

For the removal of iron 120 ml of approximately 0.1 percent 8-hydroxyquinoline in chloroform was added to a one liter separatory funnel containing 600 to 700 ml of the medium at pH 5.0. This was shaken intermittently for two hours as was done for the removal of zinc. The metal complex and excess 8-hydroxyquinoline were removed by consecutive extractions with 0.1 volume of redistilled chloroform. Since the color of the metal complex is less intense, five extractions were made after the disappearance of visible color. The medium was adjusted to pH 7.0 and dispensed into flasks. Zinc and copper were added in excess and iron was added in known graded amounts. The flasks were autoclaved and were ready for inoculation.

Copper was removed from the medium by the same method used to remove zinc except that the dithizone treatment was conducted at pH 1.5. After the excess dithizone was removed, the medium was adjusted to pH 7.0 with metal-free ammonium hydroxide and dispensed into flasks. Zinc and iron were added in excess, whereas copper was added at the desired test levels. The flasks were autoclaved as usual.

Medium B was employed to grow the inoculum for the trace metal studies. As was done for the other mineral experiments, the inoculum was prepared in two stages. In the first stage, the organism was washed from a slant with sterile distilled water. For these trace metal studies sterile triple distilled water was employed, the last distillation being made in an all-glass still. Five ml of the suspension was placed into a flask containing medium B. This flask was allowed to remain on the shaker for seventy-two hours. After this

time 0.5 ml of the content of the latter flask was transferred to a new flask also containing medium B except that no trace metals had been added to it. This was done to minimize the possibility of carrying over appreciable amounts of trace metals into the fermentation medium. After twenty-four to thirty-six hours on the shaker, 0.2 to 0.3 ml of the growth of the second flask was used to inoculate the flasks containing the fermentation medium. The inoculum was not homogenized for these experiments. The reason for this omission was to eliminate the possibility of introducing metals into the medium from the blendor.

Earlier in this work all media were adjusted to pH 6.5 before autoclaving. It was later found that optimum growth occurred at a pH of 6.3 after autoclaving. As a consequence, all media were adjusted to this pH in subsequent experiments.

All mineral solutions added to the purified media were prepared with reagent grade chemicals and triple distilled water. The final distillation was carried out in an all-glass still. The water was stored in four liter pyrex glass bottles for three days only.

The flasks and other glassware used in these experiments were specially cleaned. They were washed with a dichromate-sulfuric acid cleaning solution. The glassware was then rinsed ten times with double distilled water and finally rinsed five times with triple distilled water. For the trace metal studies, the glassware was rinsed thirteen times with triple distilled water only. The flasks were allowed to air-dry in an inverted position without contact with any contaminated surface.

All fermentations were run in duplicate and then repeated. The data given represent an average of at least four flasks. For the analytical work only one run was made, but duplicate determinations were made on each of three flasks at each time of sampling.

The fermentation period was seventy-two hours, but for several of the amino acid experiments ninety-six and one hundred and twenty hours were used also. At the end of the fermentation period the flasks were removed from the shaker and the mycelium was collected. This was done by filtering the contents of the flask through a previously weighed filter paper (Whatman no. 1, 7 cm) on a Buchner funnel. If the filtrate was to be saved for future study it was stored at minus 20°C. The mycelium which was collected on the tared filter paper was placed in an oven (105°C) for eighteen to twenty-four hours. At the end of this time the dry weight was determined on an analytical balance and the mycelial weight was calculated.

Analytical Procedures

Glucose determinations were made with the Shaffer-Somogyi reagent no. 5 containing 5 g of potassium iodide.[94]

The method for ammonia nitrogen was adopted from Report No. 6 on Antibiotics Research from the University of Wisconsin, 1948.[3] The method is based on a steam distillation of the ammonia at alkaline pH. The ammonia is collected in acid and the excess acid is titrated with standard sodium hydroxide. The ammonia still necessary for this determination was constructed in this laboratory and was patterned after the model shown in the above-mentioned report.

Total nitrogen was determined by the semi-micro-Kjeldahl method of Ma and Zuazaga.[58]

All pH determinations were made by means of a glass electrode.

RESULTS

Composition of the Final Fermentation Medium

The object of this work was to develop a chemically-defined medium to supply the nutritional requirements of Cephalosporium salmosynnematum 3590A. The synthetic medium which fulfilled this requirement is shown in Table II.

TABLE II

COMPOSITION OF THE FINAL FERMENTATION MEDIUM

Component	Concentration per liter
Glucose	10 g
L-glutamic acid	5 g
Potassium (as KCl)	0.2 g
Magnesium (as $MgCl_2.6H_2O$)	0.04 g
Sulfur (as Na_2SO_4)	0.1 g
Phosphorus (as $NaH_2PO_4.H_2O$)	0.2 g
Zinc (as $ZnSO_4.7H_2O$)	4.5 mg
Iron (as $FeSO_4.7H_2O$)	5 mg
Copper (as $CuSO_4.5H_2O$)	0.08 mg
Distilled water to 1,000 ml	
pH adjusted to 7.0 with NH_4OH	

When grown on the final fermentation medium, Cephalosporium salmosynnematum 3590A gave yields (dry weight of mycelium) of 45 percent based on the weight of glucose and L-glutamic acid added. This figure represents the combined total of carbon sources added which is incorporated into the mycelium. It may be mathematically expressed as follows:

$$\% \text{ conversion of added carbon to mycelium} = \frac{\text{grams dry mycelium}}{\text{grams carbon sources added}} \times 100$$

The medium contains levels of minerals equal to twice those found necessary for maximum growth. The amounts necessary for maximum growth were determined on medium B (Table I), except that the concentration of the component being investigated was varied. Based on yields obtained, it is assumed that L-glutamic acid functions as a carbon source as well as a nitrogen source. Thus, the L-glutamic acid and glucose were not added in excess. The levels of sodium and chloride ion employed in the medium produced no toxic effects.

The final fermentation medium remains clear after autoclaving. The heat sterilization imparts a light tan color to the medium which is probably due to some carmelization of the glucose. The amount of glucose involved is slight and does not affect cell yields.

Carbon Requirements

Since glucose was found to be readily utilized it was added as the major carbon source. Figure 9 shows that no glucose was used during the first twenty-four hours of the fermentation. Between twenty-four and thirty-six hours approximately 19 percent of the glucose originally present was gone. The greatest utilization of glucose

occurred between thirty-six and forty-eight hours when approximately 65 percent of the original glucose concentration had been used. At sixty hours only 1.2 percent of the glucose remained in the medium. The greatest yield of mycelium was obtained at this time.

Several other carbohydrates were studied as possible sources of carbon. These included sucrose, maltose, fructose, mannose, and lactose. The results obtained with these sugars are shown in Table III. Medium A (Table I) was used for these studies except that no trace metals were added.

TABLE III

UTILIZATION OF VARIOUS CARBON SOURCES BY CEPHALOSPORIUM SALMOSYNNEMATUM 3590A

Carbon Source	Concentration per liter	Dry Weight of Mycelium per liter
Lactose	10 g	0.32 g
Sucrose	10 g	1.88 g
Fructose	10 g	2.24 g
Mannose	10 g	2.44 g
Maltose	10 g	2.74 g
Glucose	10 g	2.40 g

The yields of mycelium were much lower than those obtained on the final fermentation medium but it is apparent that fructose, mannose, and maltose were also good sources of carbon. Sucrose was utilized at a slightly slower rate. Lactose seemed to be metabolized very slowly as compared to the other sugars. The higher yields of mycelium

obtained with maltose are probably explained by the fact that the maltose used was of technical grade whereas the other sugars were of reagent grade. It is very likely that the technical maltose was "contaminated" with trace metals and that the yield of mycelium obtained represents not only a response to the maltose present but to the trace metals as well. When this experiment was repeated using reagent grade maltose, the yield was reduced.

Although glucose was selected as the major carbon source, the yields obtained based on the amounts of glucose and L-glutamic acid indicate that the latter served as a carbon source as well. It may be seen from Table VI that 6.78 g per liter of mycelium were obtained at sixty hours. Based on the sugar added only, the figure for percent conversion to dry weight of added carbon sources is 68 percent. Based on the sugar and the amino acid added, the figure is 45 percent. It has been reported that for the fungi values over 50 percent are rare.

Nitrogen and Amino Acid Requirements

Nitrogen was first supplied to the organism through the addition of $(NH_4)_2SO_4$ to the medium. Growth in media in which $(NH_4)_2SO_4$ was the sole source of nitrogen was poor. This was also found to be true for $NaNO_3$. It was noted that the addition of L-asparagine to the medium greatly increased the weight of mycelium obtained. Because of the beneficial nature of L-asparagine, it was retained as a component of the early test media. L-asparagine was added to medium A (Table I) in a concentration of 1 g per liter. In several experiments the concentration was increased to 2.5 g per liter. The medium at this

stage, therefore contained two sources of nitrogen, one organic and the other inorganic.

Other amino acids were studied for their individual effect on the growth of the fungus. Medium A (Table I) was employed for these experiments except that no trace metals were added and L-asparagine was replaced by the amino acid under investigation. Later these studies were repeated with required trace metals present. The amino acids were added in concentrations of from 0.25 to 5 g per liter. Flasks containing L-asparagine in a concentration of 1 g per liter were included as controls. Under the conditions of these experiments, it may be seen from Figures 1a and 1b, that the amino acids fall roughly into five groups based upon their ability to increase growth of the organism. It should be noted that this grouping is based upon the growth response to the highest level of amino acid used (5 g per liter). L-asparagine and L-glutamic acids gave the best yields which were both approximately thirteen times over the controls. These amino acids were placed in Group I. The next most stimulatory amino acids were found to be DL-serine and L-aspartic acids. These gave yields of mycelium which were between six and one-half and seven times greater than the controls. These make up Group II. In Group III are the amino acids which gave yields of approximately 4.9 to 5.3 times the weight of the controls. The amino acids in this group were DL-threonine, DL-alanine, and histidine (free base). Glycine, DL-lysine, and L-proline were found to fall into Group IV. These amino acids gave yields which were from 3.3 to 4 times greater than the controls. The remaining amino acids (Figure 1a) were placed in Group V. These gave yields between 1 and 2.2 times over the control flasks. It is

to be noted that the above results were obtained on a medium to which no trace metals had been added. When the amino acids were added in the presence of trace metals the groupings remained essentially the same with the exception that L-proline, L-phenylalanine, L-arginine, and L-tryptophane proved to be more stimulatory. It is interesting to note that no significant yields were obtained in the presence of cysteine, since this amino acid apparently inhibits the growth of the organism.

The next phase of the amino acid studies of Cephalosporium salmosynnematum 3590A which was investigated was the effect of the presence of two or more amino acids on the growth of the fungus. Medium A (Table I) was used for these studies. Zinc, iron, and copper were added in excess. The L-amino acids were added in a concentration of 1 g per liter, while the DL forms were added in a concentration of 2 g per liter since quite often only one of the optical forms is utilized by a fungus. It is to be noted that the L-asparagine of medium A (Table I) served throughout as one component of the amino acid combinations. The following eleven amino acids gave highest yields: DL-alanine, DL-valine, L-proline, L-tryptophane, L-glutamic acid, DL-aspartic acid, DL-serine, L-arginine, histidine (free base), L-phenylalanine, and glycine. The first five amino acids listed gave markedly higher yields. The results obtained with these five amino acids are shown in Table IV.

TABLE IV

MIXTURES OF PAIRED AMINO ACIDS
GIVING HIGHEST YIELDS OF MYCELIUM

Amino Acid Mixtures	Dry Weight of Mycelium per liter	% Conversion of Added Carbon to Mycelium *
DL-alanine L-asparagine	4.54 g	34.9
DL-valine L-asparagine	4.38 g	33.6
L-proline L-asparagine	3.96 g	33.0
L-tryptophane L-asparagine	4.04 g	33.6
L-glutamic acid L-asparagine	4.00 g	33.3

* Based on combined weight of glucose and amino acids added.

When mixtures of three amino acids were employed in medium A (Table I), the best yields were obtained with the combinations shown in Table V. Again L-asparagine served as one of the amino acids.

TABLE V

MIXTURES OF THREE AMINO ACIDS
GIVING HIGHEST YIELDS OF MYCELIUM

Amino Acid Mixtures	Dry Weight of Mycelium per liter	% Conversion of Added Carbon to Mycelium *
DL-serine L-arginine L-asparagine	5.24 g	37.4
L-tryptophane L-arginine L-asparagine	4.76 g	36.6
DL-alanine L-proline L-asparagine	5.02 g	35.8
L-tryptophane Histidine (free base) L-asparagine	4.60 g	35.3
L-proline L-arginine L-asparagine	4.60 g	35.3
L-proline DL-serine L-asparagine	4.94 g	35.0
DL-valine L-glutamic acid L-asparagine	4.90 g	35.0
DL-alanine L-arginine L-asparagine	4.90 g	35.0

* Based on combined weight of glucose and amino acids added.

The final phase of the amino acid studies occurred when it became apparent that by increasing the concentration of any one of several amino acids to supply the total required amount of nitrogen* as organic

* assuming that 50% of dry weight of mycelium is protein

nitrogen, maximum yields could be obtained. The theoretical amount of nitrogen necessary for maximum yield depends upon the concentration of the carbohydrate employed. If 10 g of glucose is added per liter and sixteen percent of the protein present in the mycelium is nitrogen, the amount of nitrogen necessary is 0.4 g per liter (based on fifty percent conversion of carbohydrate to dry weight of mycelium). Two amino acids, L-glutamic acid and L-proline, were found to be the best sources of carbon and nitrogen. L-glutamic acid was the amino acid of choice because of its availability and lower cost. L-glutamic acid in a concentration of 5 g per liter supplies 0.475 g of nitrogen, which is more than is theoretically required. It may be seen from Table VI that slightly more than three percent of the organic nitrogen added remains after maximum amount of mycelium has been produced.

Gross Mineral Requirements

No attempt was made to purify the media used for the study of the gross mineral requirements.

Potassium Requirements

As stated previously, medium B (Table I) was used for these studies. Potassium was added as KCl to both the inoculum and fermentation media. Potassium was added to the inoculum medium at a concentration of 50 mg per liter. To study the potassium requirements for growth, the element was added to the fermentation medium in graded amounts of 0, 5, 10, 25, 50, 100, and 200 mg per liter respectively.

Potassium was found to be essential for the growth of the organism. Its effect on yields of mycelium is shown in Figure 2. The control flasks yielded approximately 0.3 percent of maximum yield. Growth

appears to be a linear function of the potassium levels between 5 and 25 mg per liter. Maximum yield was attained when potassium was added in a concentration of 100 mg per liter. Beyond this level no significant increase in mycelial weight was obtained. The final fermentation medium contains 200 mg of potassium per liter.

Magnesium Requirements

Magnesium was added as $MgCl_2.6H_2O$ to the inoculum medium in a concentration of 5 mg per liter. It was added to a series of flasks containing the basal fermentation medium at the following levels: 1, 2.5, 5, 10, 20, and 40 mg per liter respectively. Flasks containing no added magnesium were included as controls.

Figure 3 shows the effect produced by the presence of increased levels of magnesium in the final fermentation medium. Magnesium was found to be essential for growth. Growth response was greatest at magnesium levels between 1 and 5 mg per liter. Maximum yield, however, was obtained when the concentration of added magnesium was between 10 and 20 mg per liter. Magnesium was added in the final fermentation medium at a concentration of 40 mg per liter. No difficulty was encountered with precipitation of magnesium salts at this concentration and at the pH employed.

Sulfur Requirements

Sulfur was supplied as Na_2SO_4. This inorganic source of the element proved to be adequate for these studies. Except for the already mentioned fact that L-cysteine inhibited growth of the fungus, the effect of organic sulfur on the growth of Cephalosporium salmosynnematum 3590A was not investigated. The media for the sulfur

studies did not require any purification when all the sulfate salts normally employed in the final fermentation medium were replaced by the chlorides at the equivalent metal levels. Sulfur was added to the inoculum medium at a concentration of 25 mg per liter. It was added to the final fermentation medium at levels of 0, 5, 10, 25, 50, 100, and 200 mg per liter respectively.

Figure 4 shows the effect of added sulfur on the growth of the organism. The flasks containing the lowest level of sulfur (5 mg per liter) showed practically an eight-fold increase in yield of mycelium over the flasks with no sulfur added. Maximum yield occurred when added sulfur was present in a concentration of 50 mg per liter. The element was supplied in the final fermentation medium at a concentration of 100 mg per liter.

Phosphorus Requirements

Phosphorus was added to the inoculum medium at a concentration of 50 mg per liter. $NaH_2PO_4.H_2O$ was the salt used to supply the element. To determine the phosphorus requirements for growth, the element was tested in the following concentrations: 5, 10, 25, 50, 100, and 200 mg per liter respectively. Control flasks without any added phosphorus were included.

The effect of phosphorus on growth is shown in Figure 5. The growth in the control flasks was only three percent of maximum. Maximum yield was attained when the level of added phosphorus was between 50 and 100 mg per liter. The element was added to the fermentation medium in a concentration of 200 mg per liter.

Calcium Requirements

No attempt was made to study the calcium requirements of Cephalosporium salmosynnematum 3590A, except that the addition of calcium to the final fermentation medium did not affect yields obtained.

Trace Metal Requirements

Medium B (Table I) was employed for the trace metal studies.

Zinc Requirements

For the determination of the zinc requirements special purification by treatment with dithizone in carbon tetrachloride at pH 5.0 was necessary. The actual purification procedure has already been described in the section under Methods and Materials. Zinc (as $ZnSO_4.7H_2O$) was added to the purified medium in concentrations of 0, 0.05, 0.1, 0.25, 0.5, and 1.0 mg per liter respectively. Iron and copper were added in excess after the purification treatment.

Zinc had a marked effect on growth. The effect of added zinc on yield of mycelium is shown in Figure 6. It is obvious that the level of zinc in the control flasks was effectively reduced, since the yield of mycelium in the flasks without added zinc gave only 0.48 g of mycelium per liter. Maximum yield was obtained when the concentration of added zinc was 0.5 mg per liter. The yield of mycelium at this level was 6.70 g per liter. Zinc was added to the final fermentation medium at a concentration of 1 mg per liter.

Iron Requirements

The iron level was reduced in the test medium through the use of 8-hydroxyquinoline in chloroform as previously outlined. After purification iron was added as $FeSO_4.7H_2O$ to the test medium in concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mg per liter respectively.

Zinc and copper were added in excess. The controls contained no added iron.

The effect of iron on the yield of mycelium is shown in Figure 7. Iron was found to be necessary for growth. The method of purification proved to be effective in that the control flasks yielded only seven percent of maximum yield of mycelium. Maximum yield resulted when the concentration of added iron was 0.5 mg per liter. Iron was added to the final fermentation medium at a concentration of 1 mg per liter.

Copper Requirements

Dithizone and carbon tetrachloride were employed at a pH of 1.5 to reduce the copper content of the media. Copper was added to the purified medium as $CuSO_4.5H_2O$, in concentrations of 0.0005, 0.001, 0.0025, 0.005, 0.01, and 0.025 mg per liter. Zinc and iron were added in excess to the purified medium. Flasks containing no added copper served as controls.

Copper was essential for the growth of Cephalosporium salmosynnematum 3590A. Figure 8 illustrates the effect of added copper on growth. Maximum yield resulted when the level of added copper was 0.01 mg per liter. The element was added to the final fermentation medium at twice the optimum level. It is of interest to note that maximum yields of mycelium obtained in the copper experiments were approximately six percent lower than those obtained in the other trace metal studies.

Other Trace Metal Requirements

No detailed studies of any other trace metal requirements were made. The addition of manganese to the final fermentation medium did not affect cell yields.

Vitamin Requirements

The following vitamins were tested for their individual and combined effects on the growth of the fungus: thiamine, riboflavin, niacinamide, pyridoxine, inositol, choline, calcium pantothenate, para-amino benzoic acid, folic acid, and biotin. The vitamins other than biotin were added singly to medium A (Table I) in graded amounts of 0.005, 0.05, 0.5, 5, and 50 mg per liter. Biotin was added at levels of 0, 0.0005, 0.005, 0.05, 0.5, and 5 mg per liter. The vitamins were also added to medium B (Table I) at two levels, one and ten mg per liter. A mixture of all the vitamins was also tested.

In both experiments, the addition of vitamins did not result in any increase in yield of mycelium over the controls which contained no added vitamins.

pH Requirements

The effect of initial pH on the yield of mycelium was marked. This study was conducted on medium B (Table I). pH determinations were made by means of a glass electrode.

At pH 3 and 4 growth was completely inhibited. At pH 5 approximately eleven percent of maximum yield was obtained. Greatest yield of mycelium resulted at an initial pH of 7, which was found to drop to 6.3 after autoclaving. By lowering the pH one unit below this value, a decrease of 34.5 percent of maximum yield resulted. At an initial pH of 8 a decrease of 9.7 percent of maximum was obtained. pH was adjusted in all media before autoclaving to a value of 7.

Chemical Changes During Growth of Cephalosporium salmosynnematum 3590A

Figure 9 shows the chemical changes occurring in shake flask fermentations with Cephalosporium salmosynnematum 3590A in the final fermentation medium.

Glucose is shown to be rapidly utilized after the first twenty-four hours. By sixty hours, only 1.2 percent of the glucose originally present remained in solution. After seventy-two hours no glucose remained.

The ammonia nitrogen present in the medium initially came from the NH_4OH used to adjust the pH. This level remained fairly constant for the first twenty-four hours, but at thirty-six hours the ammonia nitrogen content of the medium was increased by ten percent over the zero hour flasks. This increase may have been due to concentration of the medium and also to the breakdown of some of the L-glutamic acid with liberation of ammonia. Between thirty-six and sixty hours the level of ammonia nitrogen fell until it reached a value which was 81.6 percent of the original concentration. After this time the ammonia nitrogen level rose until it attained a concentration which was fifteen percent higher than the zero hour level. The increase is attributed to autolysis of the mycelium. Thus it seems that, while the fungus prefers an organic nitrogen source, under some conditions utilization of ammonia nitrogen may occur.

The level of total soluble nitrogen began to decrease after thirty-six hours. The lowest concentration was at sixty hours when only 37.1 percent of the initial concentration remained in solution. As the autolytic process began, the values for total soluble nitrogen rose. At the end of the fermentation period, the soluble nitrogen returned to a level which was 59.3 percent of the zero hour concentration.

Mycelial weight did not increase appreciably during the first twelve hours. By twenty-four hours, the organism had entered a period of rapid

mycelium formation. At forty-eight hours the yield of mycelium reached a value which was 67 percent of maximum. Maximum yield was obtained at sixty hours, when the glucose was practically gone and very little organic nitrogen remained. Autolysis began between sixty and seventy-two hours and proceeded rapidly until at one hundred and eight hours only 38 percent of the mycelium present at sixty hours remained.

The pH was 6.3 at the beginning of the fermentation. It began to increase at thirty-six hours and rose sharply between forty-eight and sixty hours. The sharp increase demonstrates that the buffering capacity of the medium is not very high. The pH rose steadily and reached a high of 8.85 at one hundred and eight hours.

The actual values obtained during the fermentation period are shown in Table VI.

TABLE VI

DATA ON CHEMICAL CHANGES OBTAINED DURING GROWTH OF CEPHALOSPORIUM SALMOSYNNEMATUM 3590A IN THE FINAL FERMENTATION MEDIUM *

Hours	Residual Glucose g/liter	Ammonia Nitrogen mg/ml	Total Soluble Nitrogen mg/ml	Mycelial Weight g/liter	pH
0	10.92	0.460	1.05	0	6.30
12	10.99	0.466	1.06	0.02	6.21
24	10.84	0.457	1.03	0.22	6.23
36	8.85	0.506	1.05	0.84	6.36
48	3.84	0.432	0.747	4.52	6.45
60	0.129	0.371	0.390	6.78	8.22
72	0.102	0.376	0.406	6.40	8.55
84	0.102	0.465	0.530	4.74	8.66
96	0.102	0.521	0.620	3.86	8.77
108	0.102	0.529	0.623	2.58	8.85

* The curves for Figure 9 were derived from these values.

DISCUSSION

When a new medium is devised, the purpose for which it is to be used should be kept in view. A natural medium has many uses, and is especially suitable for routine maintenance of cultures, for isolations, and for preliminary investigations. In recent years natural media have been employed extensively for the production of antibiotic substances by various organisms. Frequently, natural substrates may be fortified with one or more pure chemical compounds.

When it is desired to investigate the nutritional requirements of a given organism, synthetic media are far superior to natural media. As previously stated, the essential components of a synthetic medium are carbon and nitrogen in utilizable forms, minerals in gross and trace amounts, and accessory growth substances. The specific carbon and nitrogen sources to be employed can be determined only by experimentation. The development of a suitable synthetic medium for a specific organism may require considerable investigation. The use of appropriate analytical procedures in conjunction with the synthetic medium make it possible to determine the exact concentrations required by the organism.

The medium devised by the study of the nutritional requirements of Cephalosporium salmosynnematum 3590A gave good yields of mycelium. Foster[23] has pointed out that available data show that fungi in surface culture may convert 25 to 50 percent of the carbohydrate into dry cell substance. Based on glucose and asparagine added, Olson and Johnson[69] have found this figure to be as high as 53 percent for Saccharomyces cerevisiae grown in submerged culture. Jarvis and Johnson[40]

obtained yields of approximately 40 to 44 percent with Penicillium chrysogenum Q176. In the present study a value of 45 percent was obtained for Cephalosporium salmosynnematum 3590A based on the glucose and L-glutamic acid added.

The nitrogen requirement of Cephalosporium salmosynnematum 3590A was of interest. Although the organism was able to utilize inorganic nitrogen under the conditions of these experiments, the yields obtained were poor. Several amino acids supplying organic nitrogen were found to be preferred as nitrogen sources. Best results were obtained with L-proline and L-glutamic acid. In a publication on vitamin deficiencies in the filamentous fungi, Robbins[81] made the statement that the addition of amino acids or yeast extract improved the growth of Cephalosporium sp. in a mineral-sucrose solution. A diligent search of the literature has produced no other references to the nutritional requirements of members of this genus.

It is possible that the actual amounts of the minerals found necessary for growth will vary with the medium and conditions employed. The fact that the necessary nature of these minerals for the growth of Cephalosporium salmosynnematum 3590A could be demonstrated is more important than the values obtained. In the case of each mineral, the level contained in the final fermentation medium was effectively reduced to the point which showed that growth of the organism was very limited in the flasks without the addition of the element under investigation. When the element was added to the medium in graded amounts, growth proceeded as a function of the concentration of the metal until a maximum was reached as shown by the growth curves obtained.

The fact that Cephalosporium salmosynnematum 3590A is able to reach maximum yield without the addition of vitamins is not surprising. Many of the filamentous fungi have been reported as being "self-sufficient" with respect to vitamins. This observation means that these fungi are able to synthesize from a vitamin-free medium all vitamins in sufficient quantities to meet their needs. The possibility exists that the fungus may require some as yet uncharacterized growth factor. If this supposition is true, the yields obtained in the present study indicate that it was present in an adequate amount in the other constituents added to the medium. A similar interpretation could be advanced for the trace metals which were not investigated.

Finally, it may be pointed out that recent advances in aeration and fermentation procedures have increased the facility in making studies of the kind herein reported. Because of these improvements it is likely that more information concerning the nutrition of fungi and related organisms may be expected in the near future.

SUMMARY

A chemically-defined medium was developed which supplied the nutritional requirements of Cephalosporium salmosynnematum 3590A. A yield of dry mycelium of 6.78 g was obtained from 10 g of glucose and 5 g of L-glutamic acid present in one liter of this medium.

Glucose was readily utilized by the fungus and it served as the major carbon source. The organism required organic nitrogen to attain maximum yield. A variety of amino acids served as suitable nitrogen sources as well as carbon sources. L-proline and L-glutamic acid were the best.

Studies were made on the potassium, magnesium, sulfur, phosphorus, zinc, iron, and copper requirements of Cephalosporium salmosynnematum 3590A for growth. Maximum yields were obtained when these minerals were added to the test medium at the following levels listed in mg per liter: potassium 100, magnesium 20, sulfur 100, phosphorus 100, zinc 0.5, iron 0.5, and copper 0.01.

Under the conditions of these experiments the addition of vitamins to the medium did not affect cell yields.

An initial pH of 6.3 proved to be optimum for the growth of the fungus.

Analytical data compiled showed that practically no glucose remained at sixty hours, by which time the greatest yield of mycelium had been produced. It was further demonstrated that while organic nitrogen was preferred, some utilization of ammonia nitrogen did take place.

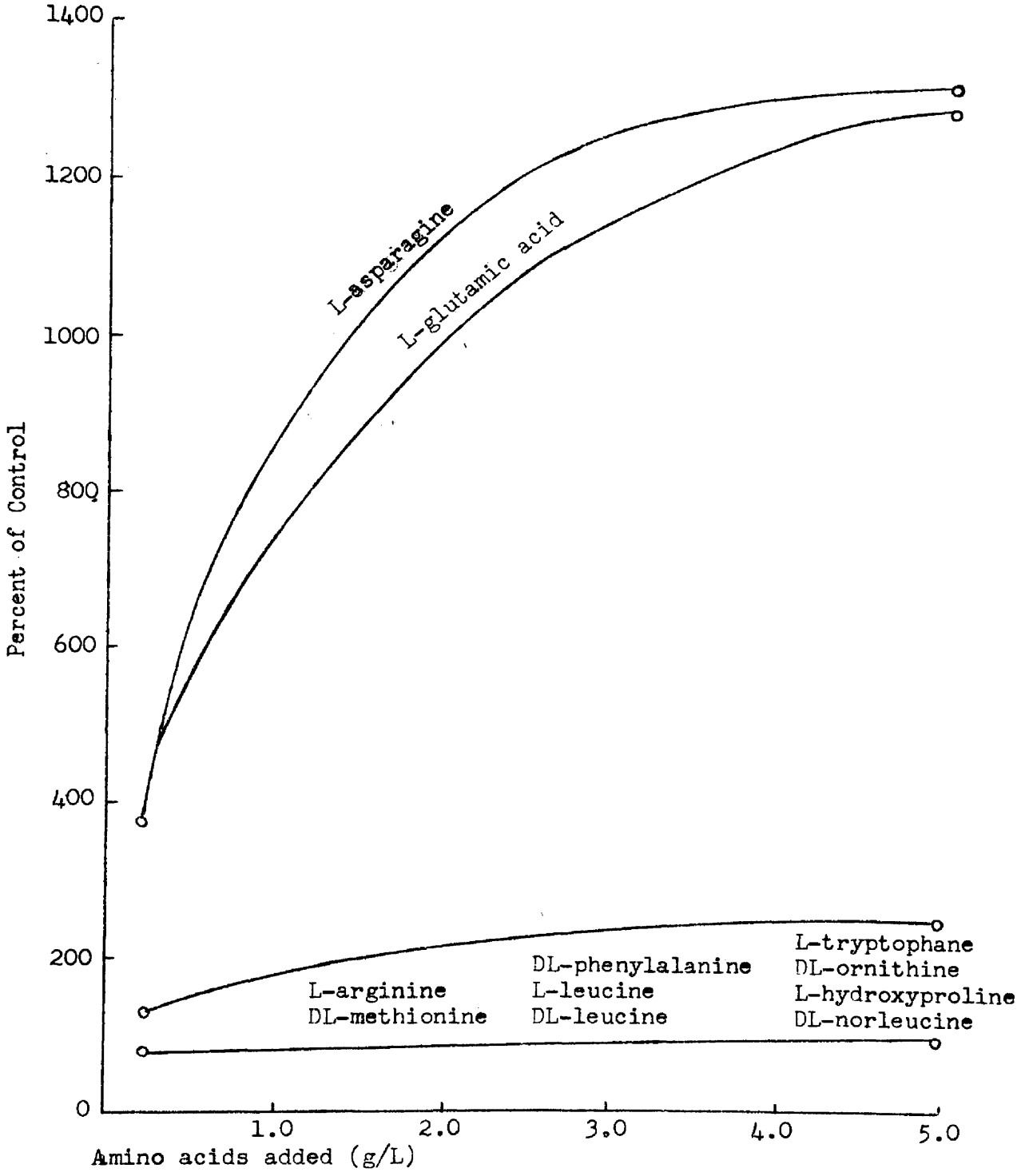


Fig. 1a Effect of concentration of various amino acids on yield of mycelium

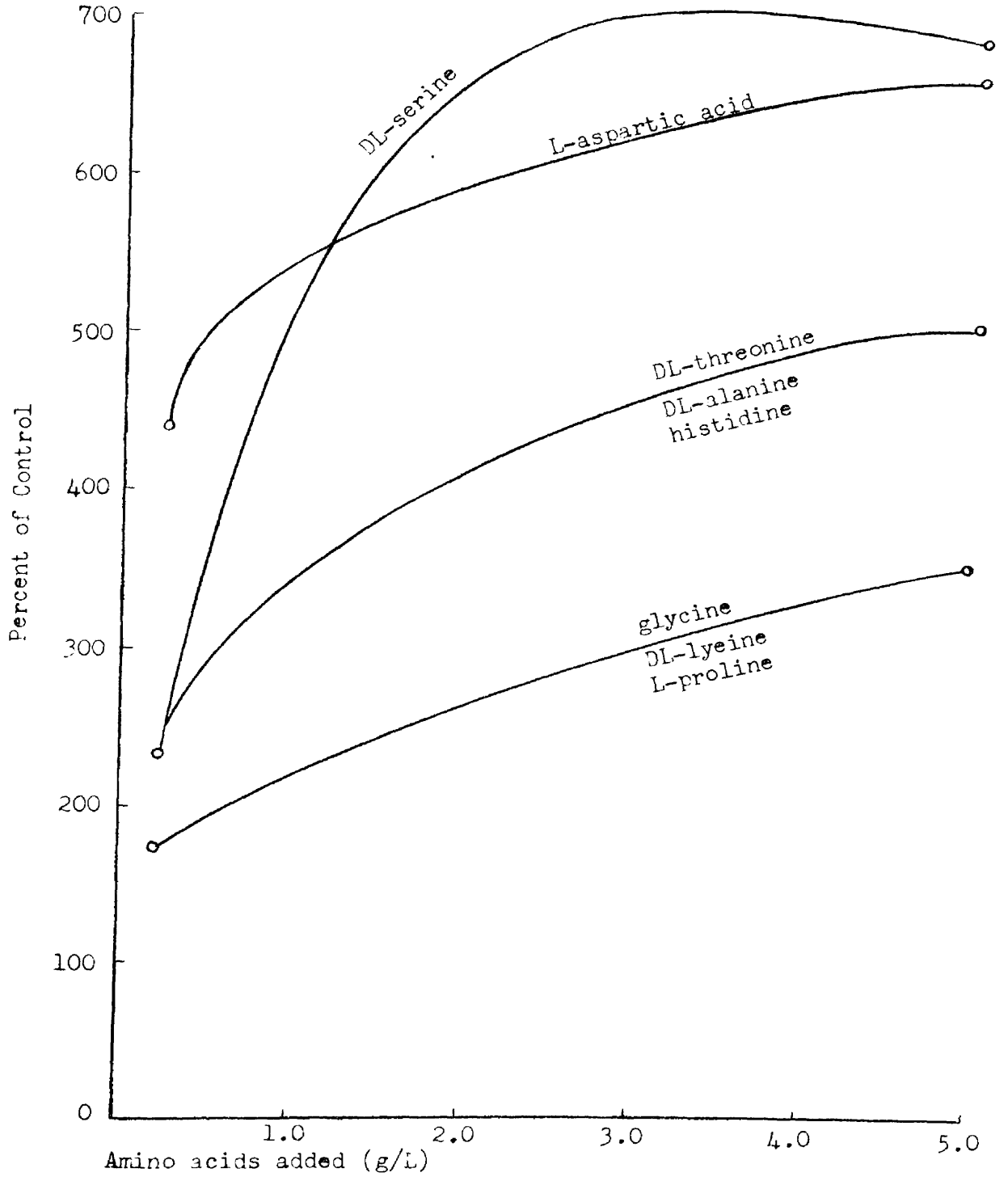


Fig. 1b Effect of concentration of various amino acids on yield of mycelium

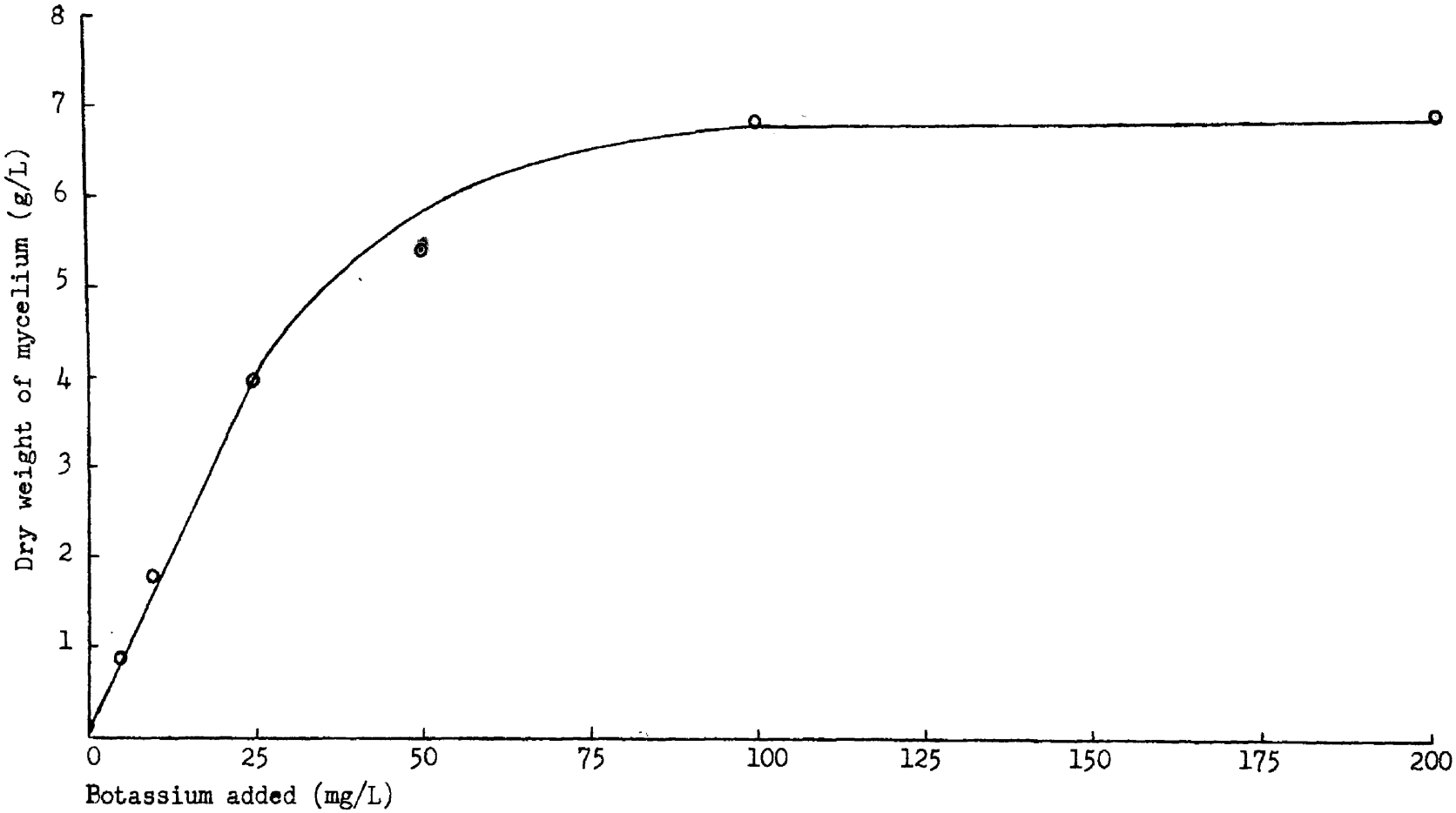


Fig. 2 Effect of potassium on yield of mycelium

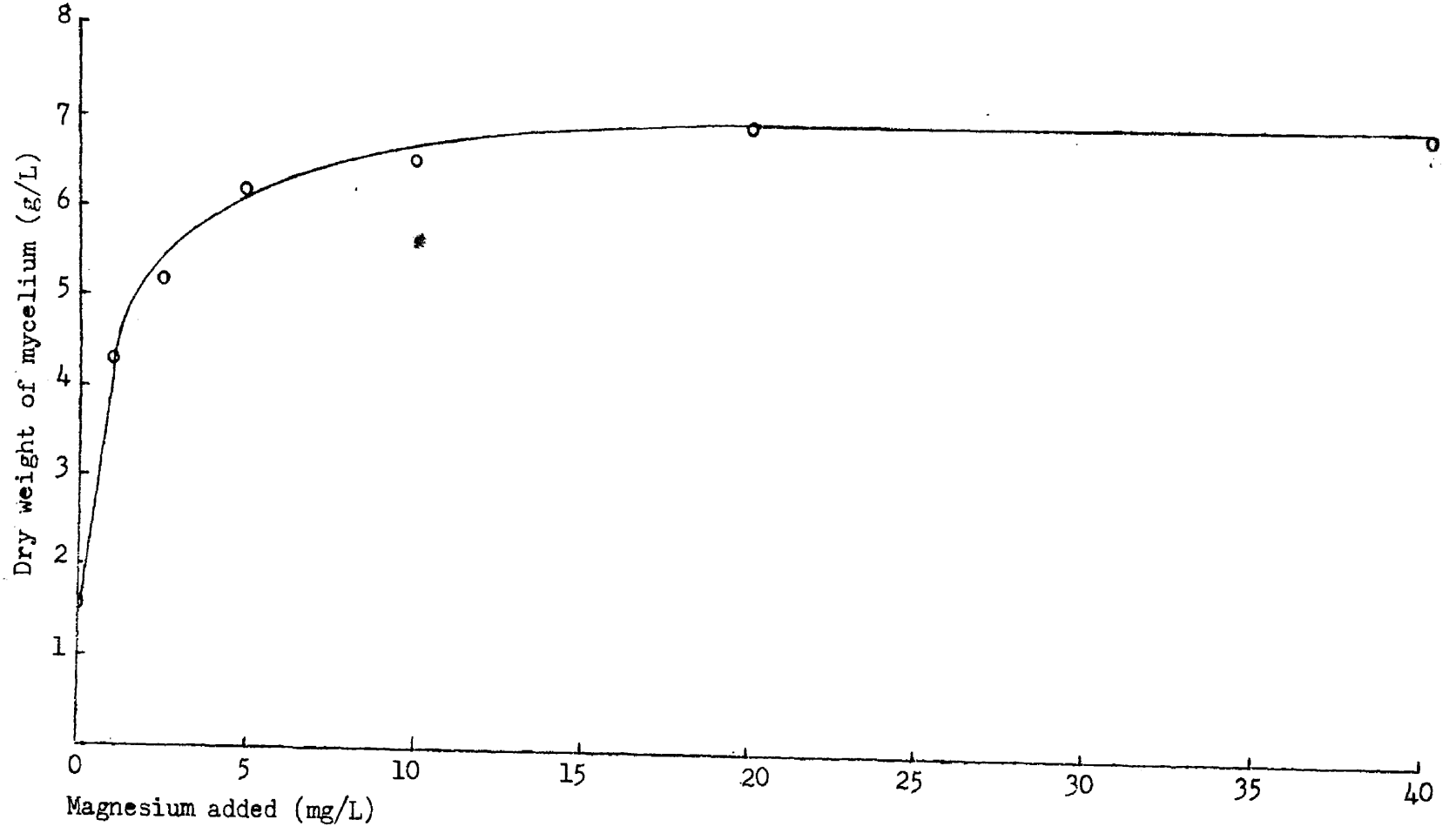


Fig. 3 Effect of magnesium on yield of mycelium

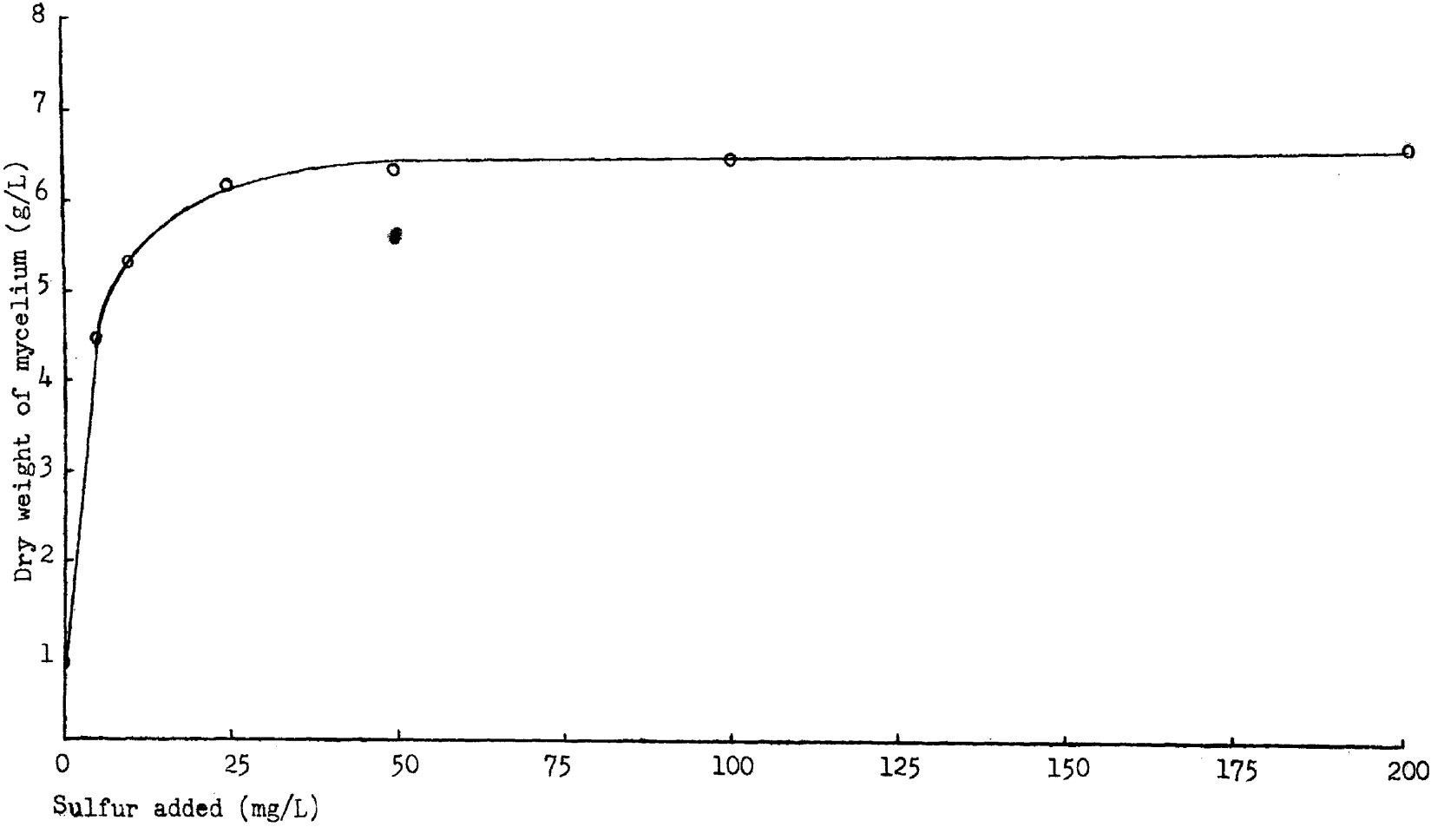


Fig. 4 Effect of sulfur on yield of mycelium

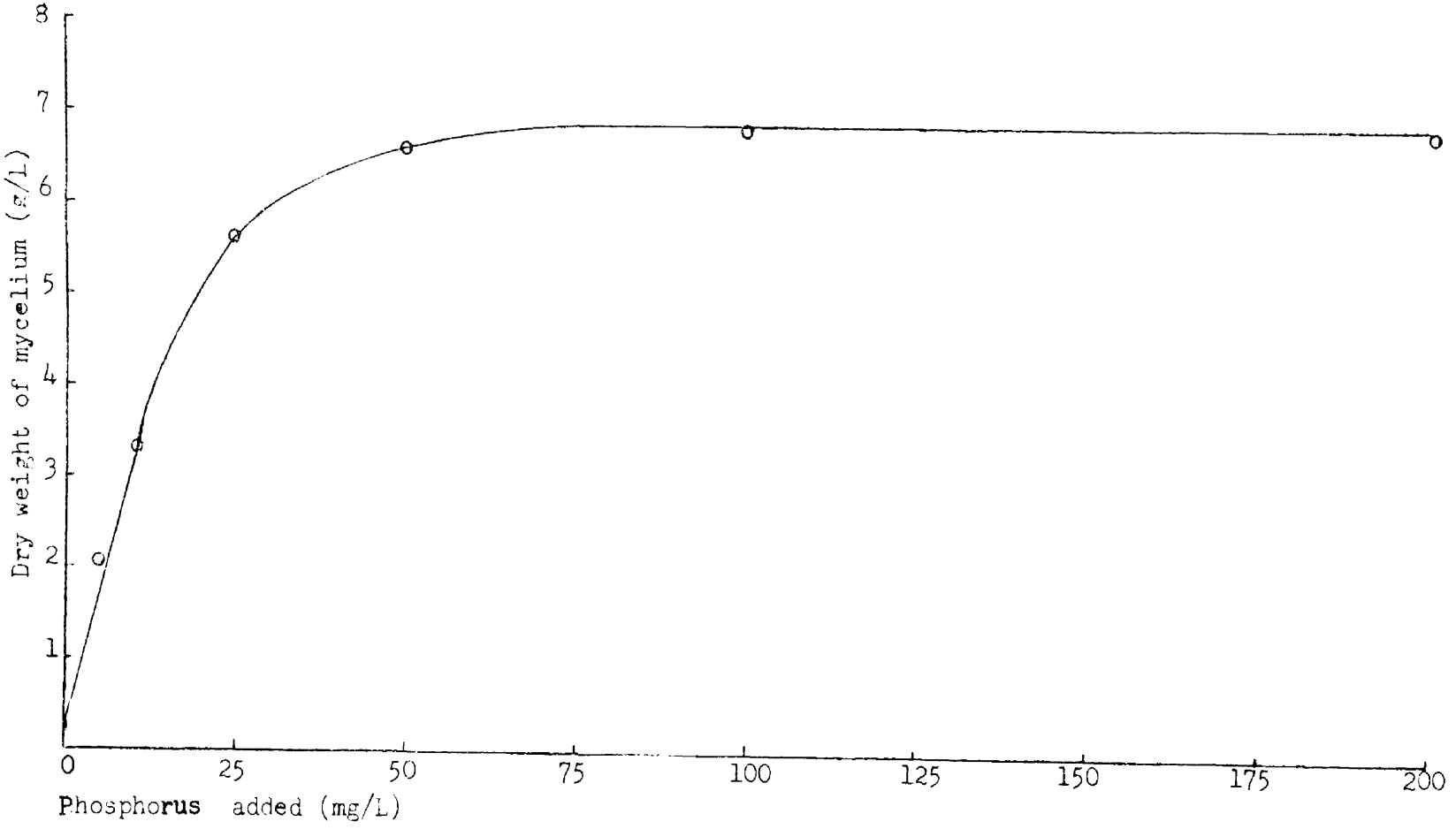


Fig. 5 Effect of phosphorus on yield of mycelium

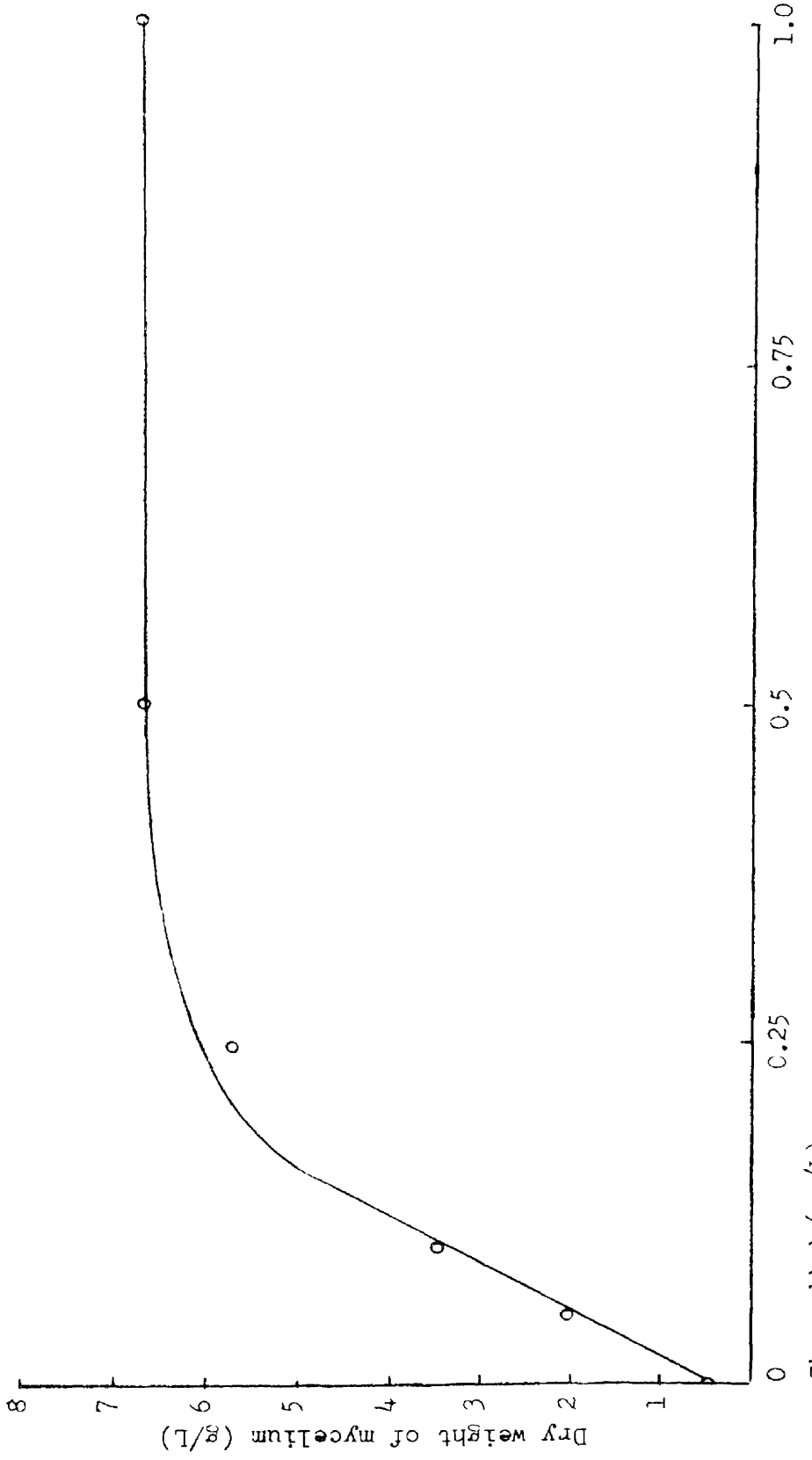


Fig. 6 Effect of zinc on yield of mycelium

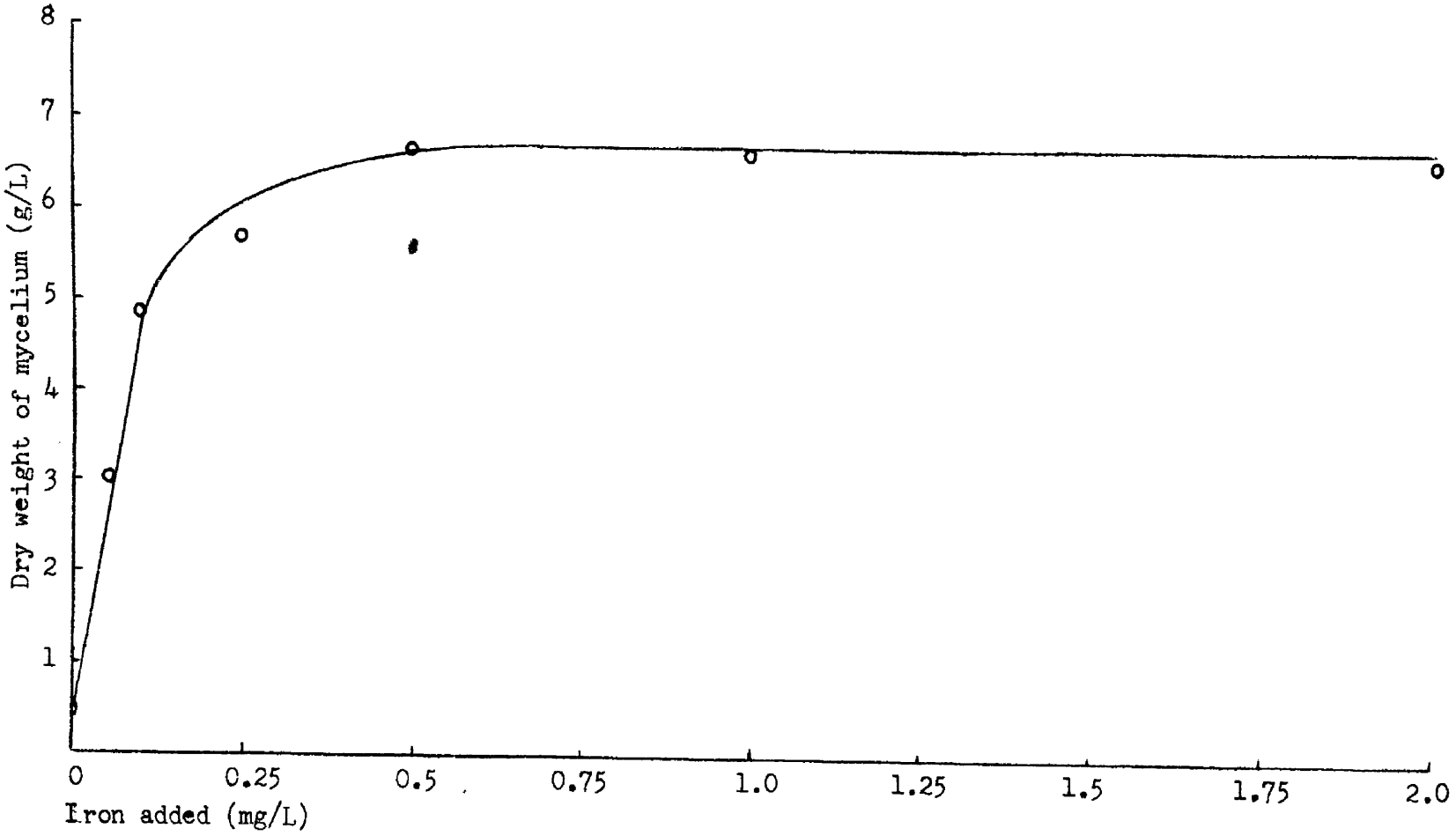


Fig. 7 Effect of iron on yield of mycelium

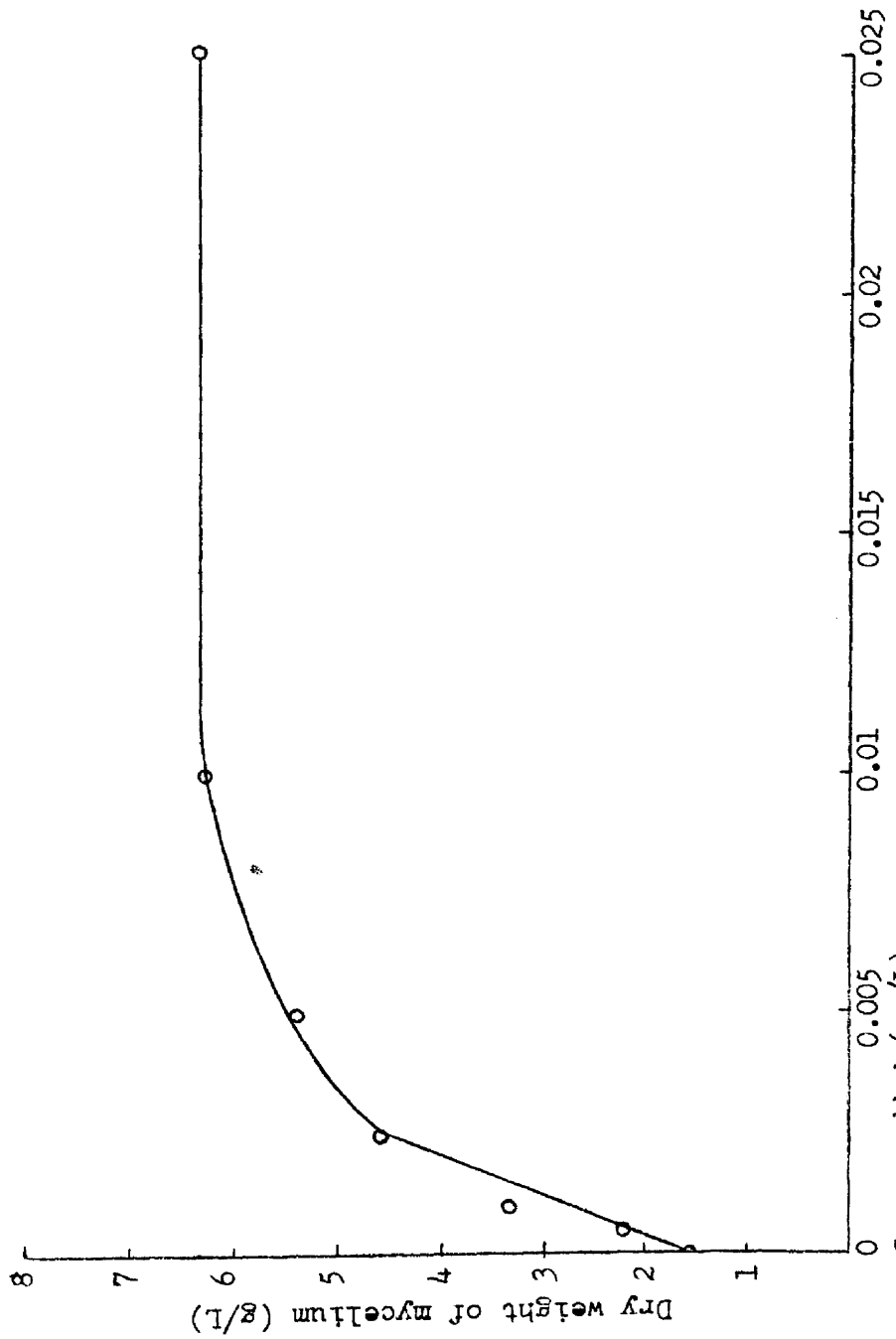


Fig. 8 Effect of copper on yield of mycelium

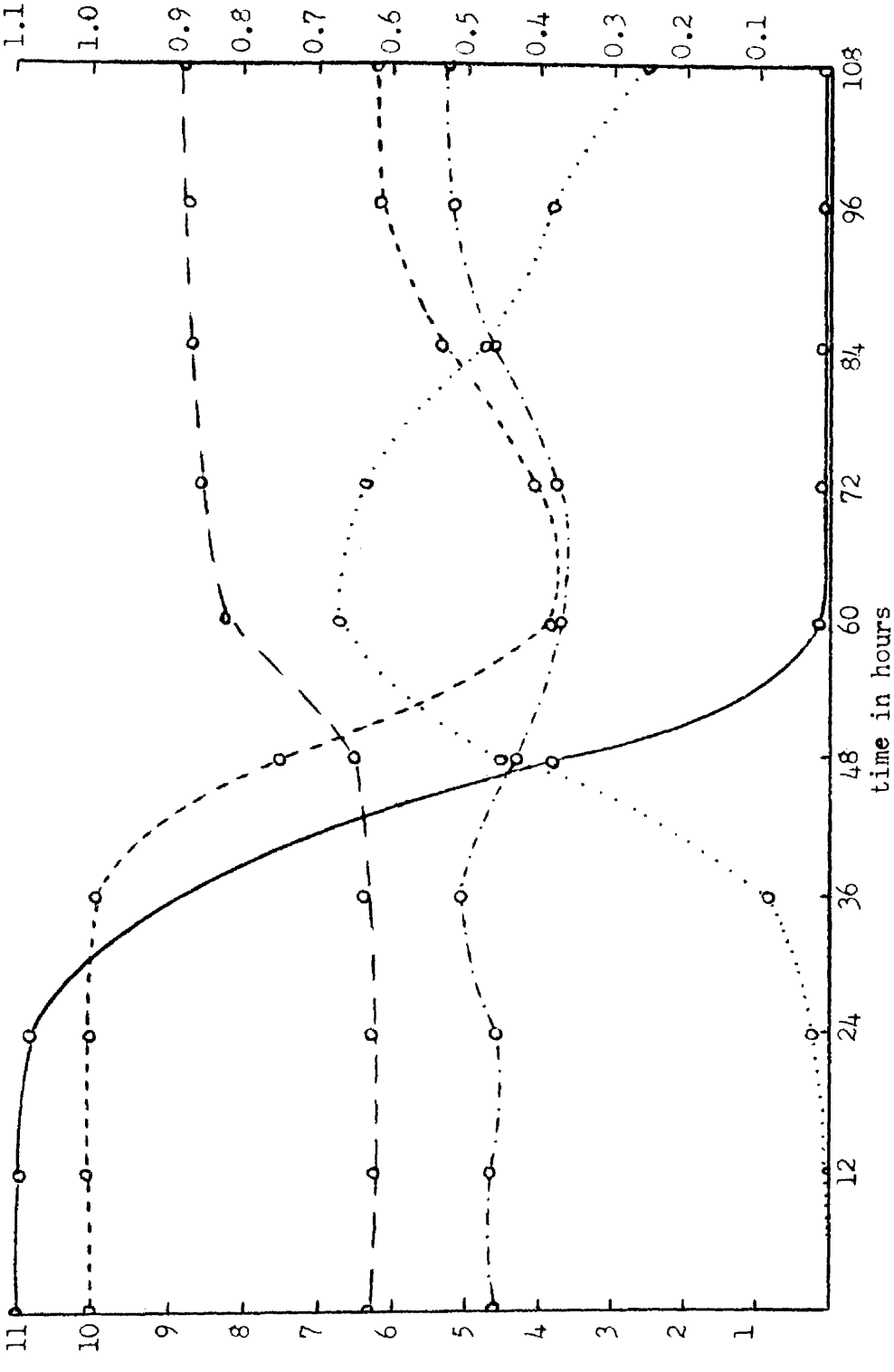


Fig. 9 Chemical changes during growth of Cephalosporium salmosynnematum 3590A

——— glucose g/L
— — — — pH
..... dry weight of mycelium g/L
- - - - - total soluble nitrogen mg/ml
-·-·-·-·- ammonia nitrogen mg/ml

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