ABSTRACT

CARBOHYDRATE UTILIZATION AND ITS RELATION TO THE PRODUCTION OF ANTITUMOR SUBSTANCE IN *SUILLUS LUTEUS*

by Flora Zaibun Majid

Suillus luteus (Fr.) S.F. Gray. (Syn. *Boletus luteus*) possess antitumor activity against Crocker mouse Sarcoma 180 (Beneke, unpublished data). A review of the literature revealed no reports on the carbohydrate requirements of this fungus.

The main purpose of this investigation was to determine some of the carbon requirements of *S. luteus*, to determine if carbon sources would affect yields of antitumor agent, and also to determine if any morphological changes in the fungal hyphae are induced by different sugars in the medium.

The utilization of the nutrients was determined by the growth response of the organisms and measured on the basis of the average mycelial dry weights after ten and fifteen days of incubation. Morphological characters of the fungi were studied under the microscope. The samples were tested for activity against the Crocker mouse Sarcoma 180. Weights of tumors of test animals were compared with those of control animals. Results were

expressed as percent of control tumor growth.

There was a differential utilization of the various carbohydrates by the two strains of Suillus luteus (B-62-03 and B-64-45). Pentoses in general supported poor growth of the strains. Hexoses were more favorable of which glucose was the best for both strains. Oligosaccharides supported fairly good growth of both strains. Cellobiose was best for the strain B-62-03 and maltose for B-64-45. Polysaccharides in general supported poor growth of both.

Yeast extract in medium A was found to contain a substance stimulatory to the growth of S. luteus when strain B-62-03 was used. Peptone was inferior to yeast extract.

Changes in the hyphae, including protoplasm, were effected by various sugars. There was a correlation of good mycelial growth and dense protoplasm. Brown deposits and clamp connections were found in the hyphae when several sugars were substitutes as the carbon source in the medium. Chlamydospores were induced only by sorbose. B-62-03 and B-64-45 showed similar responses when different sugars were used.

Light as used under the experimental conditions had no significant affect on the growth of B-62-03.

There was a differential production of antitumor substance by S. luteus when the various carbon sources

were used in the medium. Glucose and sucrose in the medium resulted in fairly good antitumor activity in B-62-03, while cellobiose and trehalose produced a substance that stimulated tumor growth. Maltose resulted in better antitumor activity in B-64-45 than the other sugars tested.

The production of antitumor activity appeared to be affected by the type of shaker for submerged cultures. This might have been owing to the duration of incubation period.

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by

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To my family

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Introduction

Cancer is one of the most mystifying diseases, the fatal outcome of which was recognized as early as 300 B.C. The organs in the human body, and the cells that form them, vary widely. In some older individuals and occasionally in the young, these cells, which function regularly and consistently in health, divide and multiply capriciously. The ensuing growth is what we know as tumor (Harris, 1962). There is a high incidence of the disease throughout the world. In spite of great efforts, modern science has not yet been successful in finding a means of prevention or cure for cancer.

Much research has been done in the past and much more is being conducted today, to find a possible prophylactic or therapeutic agent for cancer. Attempts have been made to find chemicals inhibitory against cancer in fungi, especially the Basidiomycetes.

Historically, the interest and possible tumor-inhibitory properties of extracts from mushrooms or other Basidiomycetes date back to an old folklore that was discovered by Lucas when he visited a tiny mountain village in the Bohemian Forest near the Bavarian Border in 1934. According to this folklore (Lucas, 1960), the lumberjacks in this area believed that eating a certain

type of mushroom-like fungus Boletus edulis prevents cancer. In 1948, some dried material of B. edulis was obtained for the preparation of crude water extracts and tested against Crocker mouse Sarcoma 180 by the Sloan-Kettering Institute for Cancer research. Very striking inhibitory effects could be demonstrated by this species of Boletus. However, the mycelium was found to be unable to produce the tumor-inhibiting principle in vitro. This led to a search for other Basidiomycetes with tumor inhibiting property.

Suillus luteus (Fr.) S. F. Gray. (Syn. Boletus luteus) possesses antitumor activity against Crocker mouse Sarcoma 180 (Beneke, unpublished data). Further physiological studies of this organism are required to provide a basis for further investigations of the environmental conditions under which S. luteus will be able to produce a greater quantity of the antitumor agent.

Since the nutritional requirements of S. luteus have never been studied, this investigation was concerned with the carbohydrate requirements of the fungus. Not all fungi are able to utilize the same sugars and whether a sugar is utilized or not depends upon both the configuration of the sugar and particular abilities of the specific fungus. While there is an immense amount of information scattered throughout the literature to the effect that a certain sugar is utilized by various species, much of this

information deals with relatively few sugars. Critical studies on the utilization of the sugars are rare (Lilly and Barnett, 1951).

The purpose of this investigation was to determine some of the carbon requirements of S. luteus, to determine if carbon sources would affect yields of antitumor agent, and also to determine if any conspicuous morphological changes in the fungal hyphae are induced by different sugars in the medium.

LITERATURE REVIEW

Carbohydrates as well as lipids and proteins play a fundamental role in the life of animals and plants. They are an important source of energy for living organisms and a means by which chemical energy can be stored. In addition, some carbohydrates are structural units of the cell. Examples of carbohydrates which participate in the energy economy of the cell are the polysaccharides, starch and glycogen. Cellulose and chitin are typical structural carbohydrates (Conn and Stumpf, 1963).

Carbon occupies a critical position among the essential elements required by the living organisms. Almost half of the dry weight of a fungal cell consists of carbon. Carbon compounds are of considerable importance in fungal nutrition, since fungi secure energy by oxidizing organic compounds (Lilly and Barnett, 1951). An extensive literature review on the carbon requirements for organisms in the sub-class Homobasidiomycetidae was made by Stevens (1957), Sedlmayr (1960), and Cook (1962).

Brannon (1923) stated that nearly all fungi which can be cultured are capable of using glucose or fructose as a carbon source.

Swartz (1933) reported maltose as a very favorable sugar for mycelial production in Calvatia saccata, C. caelata, and C. gigantea.

Herrick (1940) reported that Stereum gausapatum used xylose better than arabinose, and raffinose was better than glucose. Glycogen was a good source of carbon for the species.

Johnsson (1941) noted that some of the Coprinus species are able to use raffinose as well as glucose.

Modess (1941) showed the influence of hydrogen ion concentration on the growth of different mycorrhizal fungi in pure culture. Optimum pH for the growth of Boletus bovinus, B. edulis, and B. luteus was found to be 5.5.

Norman & Fuller (1942) stated that the majority of the fungi are capable of utilizing cellulose.

Treschow (1944) observed better mycelial production by Psalliota bispora in galactose or in xylose than in glucose, and oxalic acid was a satisfactory carbon source for the species.

Perlman (1949) found that the use of glucose by Polyporus anceps depended on the proper concentration of thiamine in the medium.

Nokrans (1950) showed that the saprophytic species of Tricholoma utilized galactose better than the mycorrhiza formers. Many species of Tricholoma grew well with cellobiose as a carbon source. Utilization of

cellulose was influenced by the presence of other carbon sources.

Gottlieb et al (1950) reported that some of the wood destroying fungi were able to grow with lignin as a carbon source.

Lilly and Barnett (1951) gave an excellent discussion on carbohydrates as carbon source for fungi. The authors pointed out that not all fungi are able to use exactly the same sugars. Whether a sugar is utilized or not depends upon both the configuration of the sugar and the particular ability of the specific fungi. Glucose favors the growth of more fungi than any other sugar, but some fungi are unable to utilize glucose. The authors further mentioned that the general order of availability of three common disaccharides appears to be maltose, sucrose, and lactose. Cellulose and starch are most abundant polysaccharides which are insoluble and must be hydrolysed or otherwise degraded to low molecular weight compounds. The ability to utilize a certain sugar is dependent on the presence of the necessary enzymes. A fungus uses a compound by a series of stepwise transformations which might differ from fungus to fungus.

Khudiakov and Vozniakovskaia (1951) reported that four different strains of Boletus were able to grow on a medium containing glucose as the sole carbon source.

Siu (1951) suggested that enzymes which attacked cellulose were liberated by microorganisms only in the presence of cellulose.

Humfeld and Sugikova (1952) found that Agaricus campestris was unable to grow on lactose.

Albritton (1952) showed that fewer species of fungi utilize lactose, maltose, sucrose, and raffinose than the monosaccharides (glucose, fructose, and galactose) which these oligosaccharides yield on hydrolysis.

Lilly and Barnett (1953) studied the utilization of 12 sugars (D-glucose, D-fructose, D-mannose, D-galactose, L-sorbose, L-arabinose, D-xylose, maltose, lactose, sucrose, cellobiose, and raffinose), by 57 species of fungi. All the species studied were found to grow on glucose, and usually fructose and mannose. Galactose was used slowly and sorbose poorly. Of the pentoses xylose was the most favorable, while arabinose was best for a few species. Most species utilized cellobiose, and some grew poorly on maltose. Sucrose was used poorly, lactose was unsatisfactory, and there was poor or slow growth on raffinose. Some sugars were favorable for growth only in presence of other utilizable sugars. Sorbose was found to be inhibitory to utilization of maltose, sucrose, and glucose.

Fries (1955) compared the different carbon sources for the growth of Coprinus species. Glucose,

mannose, and fructose were almost equally effective for most of the investigated species. Maltose could be utilized by all species, (but to different degrees), lactose could not be utilized by any, starch supported excellent growth of some, and inulin was not utilized.

Lilly and Barnett (1956) demonstrated that several fungi were able to use L-arabinose and failed to grow or grew poorly on D-arabinose.

Madelin (1956) found that cellobiose was a good source of carbon for most of the Coprinus species investigated.

Bach (1956) studied the physiology of an agaric, Pholiota aurea. Among the hexoses, glucose gave best growth, and among the disaccharides, maltose was the best source of carbon. Starch was the most favorable polysaccharide. The pentose sugars, xylose and arabinose, were unsuitable as sources of carbon.

Wilson and Lilly (1958) studied the utilization of oligosaccharides by some species of Ceratocystis. The identity of the sugars present in the media during incubation was determined by paper chromatography. The original sugars were present in the media at the end of the experiment when the sugars were not used for growth. The expected hydrolytic products of the oligosaccharides were found in the media when the fungi grew well.

Aschan-Aberg (1960) reported that the order of utilization of carbon sources by Collybia velutipes

during the process of fructification was as follows: glucose, sucrose, and maltose.

Robbins and Hervey (1960) showed that Polyporus schweinitzii showed a partial deficiency for at least two unidentified growth promoting substances which were widely distributed in natural products. Malt extract, yeast extract, tomato juice, coconut milk, etc., promoted the growth of the fungus and appeared to contain one or both of the unidentified substances.

Swack and Phillip (1960) determined quantitatively the production of indigotin by a wood-rotting basidiomycete, Schyzophyllum commune. Of the carbohydrates studied, D-xylose gave the highest value for mycelium production, D-fructose and D-glucose gave the highest value for indigotin production.

Martin (1961) showed that the mycelium of a fertile strain of Psalliota hortensis, used for the preparation of commercial spawn, could not grow in absence of calcium.

Sedlmayr, Beneke, and Stevens (1961) studied the vitamin requirement of Calvatia gigantea. The strains investigated were totally heterotrophic with respect to thiamine, and autotrophic with respect to biotin, pyridoxin, and inositol. A partial deficiency was established for an unidentified growth factor(s), present in the Bacto-peptone and Bacto-yeast extract.

Sedlmayr, Beneke, and Stevens (1961) showed that Calvatia species were able to utilize a variety of carbon sources. The amount of growth varied with the carbon source.

Pantidou (1961) described the techniques for culturing and studying species of Boletaceae in culture. A few species, Gyrodon merulioides, Boletinus cavipes, B. pictus, B. spectabilis and B. grisellus, which had not been previously described in culture were described.

Jayko, et al (1962) studied nutritional requirements and fermentative products of some Lactarius species. Glucose, maltose, and dextrin were found to be the best carbon sources.

Santoro and Casida (1962) grew pure cultures of some mycorrhizal fungi (Amanita Caesaria, A. rubescens, A. muscaria, Boletus luteus, and B. bicolor) in a liquid medium with various concentrations of gibberellin. Gibberellin inhibited the growth of all the organisms.

Cook (1962) observed that calvacin production in some strains of Calvatia gigantea varied depending on the carbohydrates available in the culture medium.

Molitoris (1963) studied the growth of three strains of Beauveria tenella in relation to carbon source and concentration, and presence of inorganic salts. The synthesis of cell material and lipid increased with higher concentration of carbohydrates, and a high carbohydrate nitrogen ratio favored fat production.

Negrutskii (1963) studied the effect of vitamin B_1 and H, and gibberellin on the growth of Fomitopsis annosa. The fungus synthesized vitamin B_1 and H from nutritive substances in the medium. Gibberellin in any concentration hindered growth.

Robbins, et al. (1963) in their study of growth factors for Polyporus schweinitzii identified ferulic acid as a new cofactor. The beneficial effect on the growth of the fungus of natural materials in a basal medium was mainly due to the presence of ferulic, oleic, linoleic, palmitic, and stearic acids in the natural materials.

Lingappa, et al. (1963) showed that polysaccharides like dextrin or starch, were the best carbon sources for the growth of Aureobasidium pullulans. Light increased growth markedly when polysaccharides were the carbon sources. Variation in cell morphology had been described in response to sugars and light.

Tsu-Ning (1963) found that Pleurotus ostreatus, if grown in submerged shaker cultures, produced oxalic acid from simple carbohydrates as efficiently as did Aspergillus niger.

Robbins and Hervey (1963) studied unidentified filtrate growth substances for several fungi. Tomato juice, or a water extract of beech wood improved growth of several fungi.

Beneke (1963) summarized all of the work done at that time on Calvatia gigantea in his Presidential address

to the Mycological Society of America. The best carbon sources for the mycelial growth of the fungus were glucose, cellobiose, dextrin, glycogen and maltose. Cellobiose appeared to be the best for calvacin production. Calvacin is an antitumor agent or substance that inhibits 13 out of 24 types of cancer in the laboratory animals.

Falanghe, et al. (1964) tested various soy bean whey media as substrate for several species of fungi in submerged culture. Tricholoma nudum and Boletus indecisus had the greatest rate of growth and production of mycelial protein, with much shorter incubation period compared with those of other species.

Ward (1964) showed the difference in growth of a low temperature basidiomycete, on filter-sterilized as opposed to autoclaved medium. Growth on the filter-sterilized medium was only about one-fourth of that on the autoclaved medium, and growth was also greatly reduced when glucose or KH_2PO_4 was autoclaved separately. This suggested that interaction between glucose and KH_2PO_4 was responsible for the stimulatory effect after autoclaving. This was confirmed by an experiment in which the medium was (a) autoclaved, (b) filter-sterilized, or (c) filter-sterilized without glucose and KH_2PO_4, which were autoclaved separately and added to the sterile medium. The results obtained clearly demonstrated that interaction between glucose and KH_2PO_4 when autoclaved,

accounted for the stimulation of growth.

Robbins and Hervey (1965) reported the growth of Morchella crassipes was enhanced by the addition of natural materials to a medium containing mineral salts, dextrose, vitamins of the B complex, and purine and pyrimidine bases. The enhancement was due to the ash in the natural products, primarily manganese and calcium.

Wilson (1965) studied various factors affecting the germination of Calvatia gigantea. Gibberellic acid and furfural had no effect on germination. Light was slightly inhibitory to germination. Although many carbohydrates supported germination, maltose, soluble starch, and sucrose were the best.

A general picture regarding the utilization of various sugars by several fungi has been provided by Cochrane (1958) and is summarized here.

Monosaccharides: Of the hexoses D-Glucose is biologically most important and is utilized for growth by virtually all fungi. A few forms however, have been reported not to grow with glucose. D-fructose and D-mannose are equivalent to glucose for growth for a large number of fungi. D-galactose is used by most fungi but is not usually so good a source of carbon as glucose. L-sorbose supports normal growth of a few fungi, however it appears not to be utilizable by most forms and it is definitely toxic to some. D-xylose is the most utilizable of the pentoses generally, and has been reported to be

superior to glucose for some fungi. It however supports very poor growth of several fungi. In most studies L-arabinose is inferior to xylose and glucose as a carbon source. Rhamnose, a methyl pentose, has been reported to be used only by a few organisms. D-ribose supports growth of some fungi. The L-isomers of the naturally occurring aldohexoses and of xylose do not support growth of fungi, nor do those monosaccharides which do not occur in nature (Cochrane, 1958).

Oligosaccharides: The following 5 disaccharides maltose, cellobiose, trehalose, sucrose, and lactose and one trisaccharide, raffinose, is important in the nutrition of some fungi. Maltose is utilized virtually by all fungi which have been tested. Cellobiose is almost as widely utilizable as maltose. Trehalose have been found to support growth of a large number of fungi including even some rather fastitious fungi. Sucrose is generally a good source of carbon for many fungi. Lactose is used by far fewer fungi than any of the disaccharides so far mentioned and may therefore be considered as a poor carbon source. Melibiose is not utilized by many fungi. Majority of the fungi utilize raffinose but non-utilization is also common. The utilization of an oligosaccharide is believed to be preceded by and dependent on its conversion to hexoses or, possibly hexose phosphates (Cochrane, 1958).

Polysaccharides: Among the polysaccharides starch, dextrin, inulin, glycogen, and cellulose are considered to act as carbon source for many fungi. Starch is an excellent carbon source for most fungi, even for rather fastidious forms. Dextrins, which are chemically or enzymetically modified starches of uncertain structure, are generally satisfactory source of carbon for many fungi. Inulin, a polymer of D-fructose has been found to be a good source of carbon for many but not all fungi. Glycogens are quite generally available to fungi of widely different ecological groups. Those fungi unable to use starch can be expected not to use glycogen, since so far as is known, the same enzymes attack both polysaccharides. Cellulose is by far the largest natural reservoir of biologically utilizable carbon. It is an excellent source of carbon for many fungi, but the species within a given genus are not all necessarily alike in their response to cellulose. Insolubility of cellulose however makes the nutritional study of the compound difficult. Whether a polysaccharide is used or not is depended on the ability of the fungus to produce the necessary hydrolytic enzyme and also on the configuration of the sugar.

MATERIALS AND METHODS

The cultures of Suillus luteus were maintained on a Bolete medium which had the following composition:

Malt Extract	5 grams
Dextrose	5 grams
KH_2PO_4	0.5 grams
$MgSO_4.7\ H_2O$	0.5 grams
NH_4Cl	0.5 grams
$FeCl_3$ (1% solution)	0.5 ml
Agar	15 grams
Distilled water	1000 ml

Stock cultures were originally obtained by removing small pieces of tissue (pseudo-parenchyma) under aseptic conditions, from inside the cap or stipe of an immature basidiocarp. Two strains were employed for the carbohydrate studies. The collection data of which are given below:

Strain B-62-03 collected near Lansing, Michigan. It was brought in for identification to the Biology Research Center, East Lansing, Michigan, in 1962.

Strain B-64-45 collected by Dr. E. S. Beneke from the Rose Lake area, near East Lansing, Michigan, on October 5, 1964.

PLATE I

Fruiting body of Suillus luteus strain, B-62-03.

PLATE II

Fruiting body of Suillus luteus dark yellow strain, B-64-45.

PLATE III

Fifteen day old culture of Suillus luteus strains, B-62-03 (on the right) and B-64-45 (on the left)

The taxonomic characteristics of S. luteus has been described, by Smith (1965). Plates I and II show some of the morphological characters of this mushroom-like fungus. The pores are deeper yellow in B-64-45 (Plate II) than in B-62-03 (Plate I). The mycelial culture of B-62-03 on medium A, produces a ridged, compact colony, with tan color on the back, in two weeks. (Plate 3). The Sarcoma 180 tumor retarding property of this strain is about 72%, as compared to the control tumor in mice, from extracts of S. luteus (Beneke, unpublished data). The mycelial culture of B-64-45 on medium A produces a slightly more cottony colony with a tan to buff color on the back, in two weeks (Plate 3). Sarcoma 180 tumor retarding property was about 62%, as compared to the control tumor, with extracts of S. luteus (Beneke, unpublished data).

Experimental Media Used:

A synthetic basal medium of Lindeberg's supplemented with thiamine hydrochloride (Sedlmayr, 1960), further modified by replacing ammonium tartarate with ammonium chloride, was used for the carbohydrate study. A modified Czapek's-Dox's medium, designated by Stevens (1957) as medium A, was used for comparison of growth, since the fungus grew much better on medium A than on Lindeberg's medium. The composition of these two media used are as follows:

Modified Lindeberg's Medium:

Glucose	20 grams
NH_4Cl	5.0 grams
KH_2PO_4	1.0 grams
$MgSO_4.7H_2O$	0.5 grams
$FeCl_3$ (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
Thiamine hydrochloride	100 γ/liter
Distilled water	995.0 ml

Medium A:

Glucose	15.0 grams
Sucrose	15.0 grams
Bacto peptone	5.0 grams
Bacto yeast	5.0 grams
$MgSO_4.7H_2O$	0.5 grams
KH_2PO_4	1.0 gram
KCl	0.5 gram
$FeSO_4.7H_2O$	0.01 gram
Distilled water	1000 ml

Apparatus and Sterilization Procedures:

Pyrex Erlenmeyer flasks of 250 ml capacity were used as culture vessels. Some 500 ml Florence flasks were also used for certain experiments. Disposable Petri dishes

(Falcon) were used for growing inocula. Waring blendors were used to blend inocula. The density of the inocula was determined with a Klett-Summerson photoelectric colorimeter using the green filter (540 mμ). Pipettes were used to dispose the inocula. The cultures were grown in liquid media on a rotary shaker. A reciprocal shaker was also employed for certain experiments. The culture flasks were capped with aluminum foil (Cook, 1962).

The glassware was treated with hydrochloric-nitric acid cleansing solution, rinsed with tap water and finally with distilled water. Acid-washed pipettes were sterilized with dry air at 165°-180° C for three hours. The media were sterilized by autoclaving at 121°C, 15 pounds pressure, for 15 minutes.

Sargent No. 500 filter paper and Buchner funnel were used for filtering contents of each flask to determine the dry weight of mycelia.

Experimental Procedure:

Fifty ml of modified Lindeberg's medium was placed in a 250 ml Erlenmeyer flask and capped with aluminum foil as suggested by Cook (1962). The pH was checked and then each flask was autoclaved for 15 minutes at 15 pounds pressure at 121°C.

The various carbohydrates were added to the basal medium in quantities which supplied 8 grams of carbon per liter (Sedlmayr, Beneke and Stevens, 1961). The following

heat stable sugars were autoclaved with basal medium for 15 minutes at 15 pounds pressure at 121°C (Lilly and Barnett, 1958): glucose, sucrose, galactose, arabinose, lactose, inulin, dextrin, starch, glycogen, cellulose and cellobiose.

To avoid the breakdown of sugars during autoclaving the heat sensitive sugars (raffinose, melibiose, maltose, trehalose, fructose, mannose, rhamnose, ribose, sorbose, and xylose) were sterilized by Seitz-filter and added individually and aseptically to the autoclaved basal medium in 250 ml flasks.

Transfers made from stock cultures were maintained in Bolete medium. Transfer cultures were seeded at four points into Petri dishes containing medium A, to prepare the inoculum of an experiment. To prepare the inoculum for an experiment five discs (diam. Ca 2.2 cm each) were removed from a 15 day agar culture in a Petri dish and blended with 50 ml of sterile distilled water for 30 seconds in a sterile 360 ml semimicromonel metal Waring blendor (Sedlmayr 1960). The density of the inoculum was determined with a Klett-Summerson photoelectric colorimeter using the green filter (540 mμ). The blended suspension gave a reading of 15-20% transmission under the above described condition.

Five ml of the blended mycelium was added to 50 ml of the modified Lindeberg's medium in a 250 ml Erlenmeyer

flask. The cultures were incubated in the dark at approximately 25°C on a rotary shaker giving 120 excursions per minute. The medium had no additional aeration other than that caused by continuous agitation.

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

Due to limited shaker space and cost of media, in every experiment 4 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 from quadruplicate flasks.

The amount of growth was determined by mycelial dry weight. The contents of each flask were filtered on Sargent No. 500 filter paper in a Buchner funnel. The mycelial pellets were washed with distilled water to remove any excess medium, and dried at 100°C in an oven for 18 hours. The replicates in all instances were approximately equal on the dry weight basis indicating that sufficient inoculum was used. In all the experiments pH was checked at the conclusion.

For the study of any conspicuous structural change induced by the different carbohydrates, slides were prepared (water mount) from ten day-old cultures grown on rotary shaker, and observed under the microscope. General characters of the hyphae were drawn with the aid of a camera lucida.

Four sugars, which supported good growth of B-62-03 and B-64-45 respectively were selected to observe their effect on production of the antitumor substances by the fungi. Since medium A supported much better growth of Suillus luteus than Lindeberg's medium, the fungi were grown on Medium A. The various sugars selected were added to the basal medium in quantities equivalent to 12 grams of carbon per liter, which corresponded with the original carbon content of the carbohydrates in Medium A.

The contents of the flask that was tested for antitumor agent production were blended for 60 seconds in a sterile Waring blendor and the solid portion was separated from the liquid by suction flask filtration. One hundred ml of the liquid and 1 ml of 1% merthiolate (1 gram merthiolate, 14 grams of borax per 100 ml of distilled water) were placed in a sterile rubber capped serum bottle (Cook, 1962). According to Stevens, the merthiolate solution does not affect the results of the tumor assays (Cook, 1962).

The samples were tested for activity against the Crocker mouse Sarcoma 180 as follows. Female Swiss albino mice weighing between 18 and 22 grams were used for Sarcoma 180 test (Cancer Chemotherapy reports, 1962). To grow the tumor, uniformly cut pieces (large enough to fit into a 16 guage trocar) of tumor were implanted sub-cutaneously in the axillary region. These were allowed to

grow for 7 days. The mice were then sacrificed by cervical separation and the tumors were removed. The tumors were then cut into equal pieces of approximately 2-4 mm in size. The mice that were used in the experiment were implanted with one of these squares of tumor on the right axillary region where they were allowed to grow for 7 days. The treatment (injections of the extract) began 24 hours after the implant. The mice were injected in the interperitoneal region, with 1 ml daily for 7 days. The control mice were injected with the same volume of an .85% saline solution at the same time. After 7 days the mice were sacrificed and tumors removed. Weights of tumors of test animals were compared with those of control animals. Results are expressed as percent of control tumor growth. Each testing group consisted of 6 mice.

RESULTS AND DISCUSSION

Carbon is one of the essential elements required by living organisms. Almost half of the dry weight of fungal cells consists of carbon. Protoplasm, enzymes, the cell wall, and reserve nutrients stored within the cells have compounds containing carbon. Carbon compounds are equally important in fungal nutrition. Fungi secure energy by oxidizing organic compounds. Organic compounds differ in composition, structure and configuration. These are key factors which must be considered in relation to utilization of organic compounds by fungi. (Lilly and Barnett, 1951).

The main purpose of this work was to determine the carbohydrates which support good growth of the test organism (Suillus luteus), to observe if any morphological change is produced in the fungal hyphae by different carbohydrates, and to determine if any significant change in antitumor activity of the fungus is caused by various carbohydrates.

The sugars that were selected in this experiment were the ones studied by Sedlmayr (1960) in her carbohydrate study of Calvatia species.

Table I

The Utilization of Various Monosaccharides by Suillus luteus

C-sources	B-62-03				B-64-45			
	10 days		15 days		10 days		15 days	
	mg*	pH+	mg*	pH+	mg*	pH+	mg*	pH+
PENTOSES								
l(+) arabinose	24.2	4.4	24.7	4.8	10.4	4.6	13.0	4.55
d(+) xylose	22.3	4.87	26.0	4.85	13.1	5.15	20.6	5.15
d(-) ribose	19.4	5.5	19.6	5.5	17.3	5.7	18.6	5.7
HEXOSES								
d(+) glucose	105.3	3.0	106.4	2.6	68.5	2.8	88.5	2.49
d(+) galactose	48.7	3.6	49.5	4.1	32.6	4.1	35.3	4.0
d(+) mannose	51.8	3.1	84.8	2.5	21.3	3.3	80.5	2.3
d(-) fructose	32.3	3.55	100.6	2.25	22.5	4.1	23.4	3.4
l(-) sorbose	18.2	4.99	18.8	4.98	11.9	5.19	14.4	5.7
l(+) rhamnose	11.4	5.8	16.2	5.7	29.3	5.7	30.5	5.4
CONTROL								
C-free	18.5	5.5	26.4	5.8	19.7	4.7	33.5	5.4
Medium A	442.5	6.1	459.2	8.2	297.2	7.0	297.3	7.85

*Average mycelial dry weight of four replicates.

+Average of four replicates.

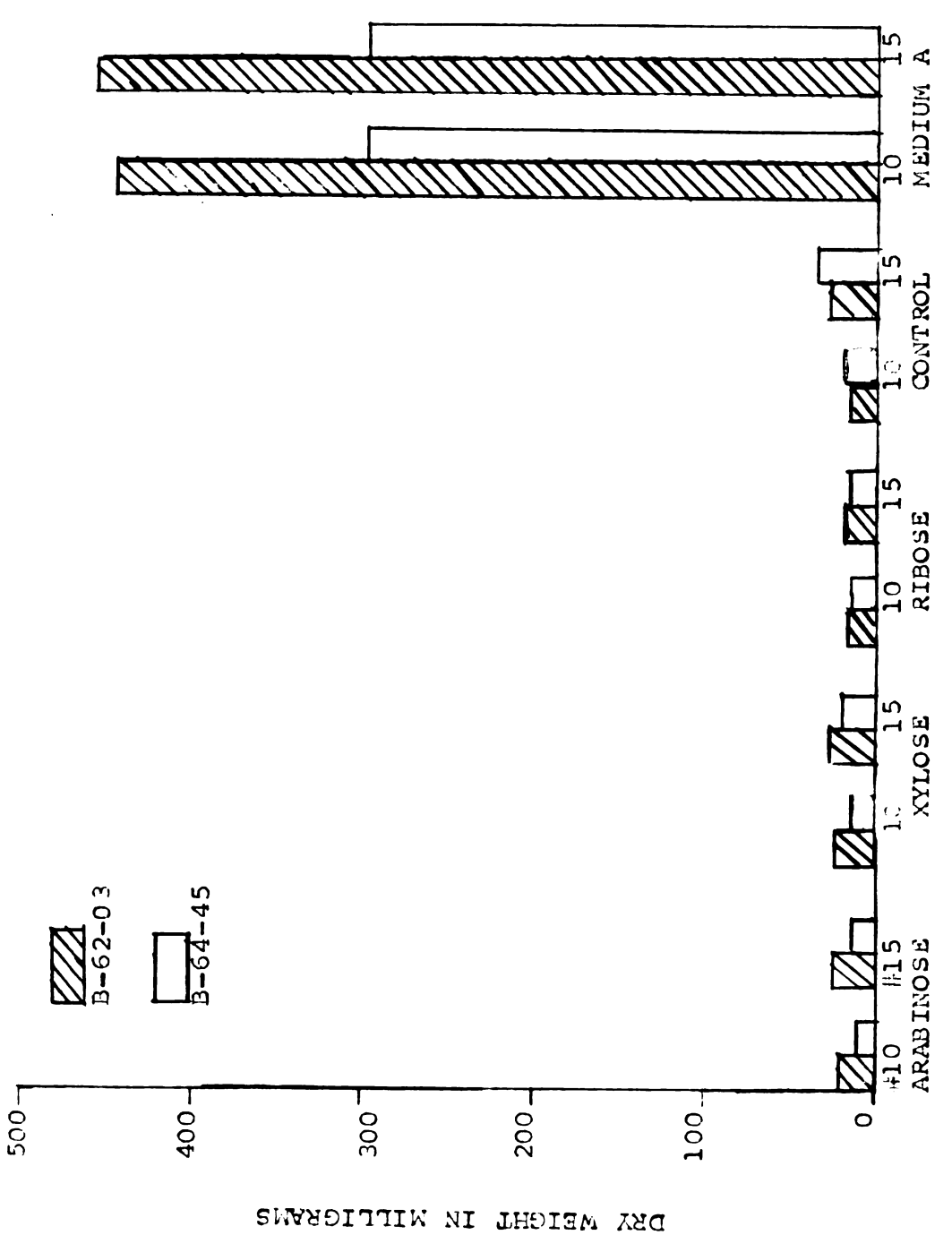


Figure 1 - Utilization of various monosaccharides by Sulfolus luteus strains, B-62-03 and B-64-45.

Figure 2 - Utilization of various monosaccarides by Sullus luteus strains, B-62-03 and B-64-45.

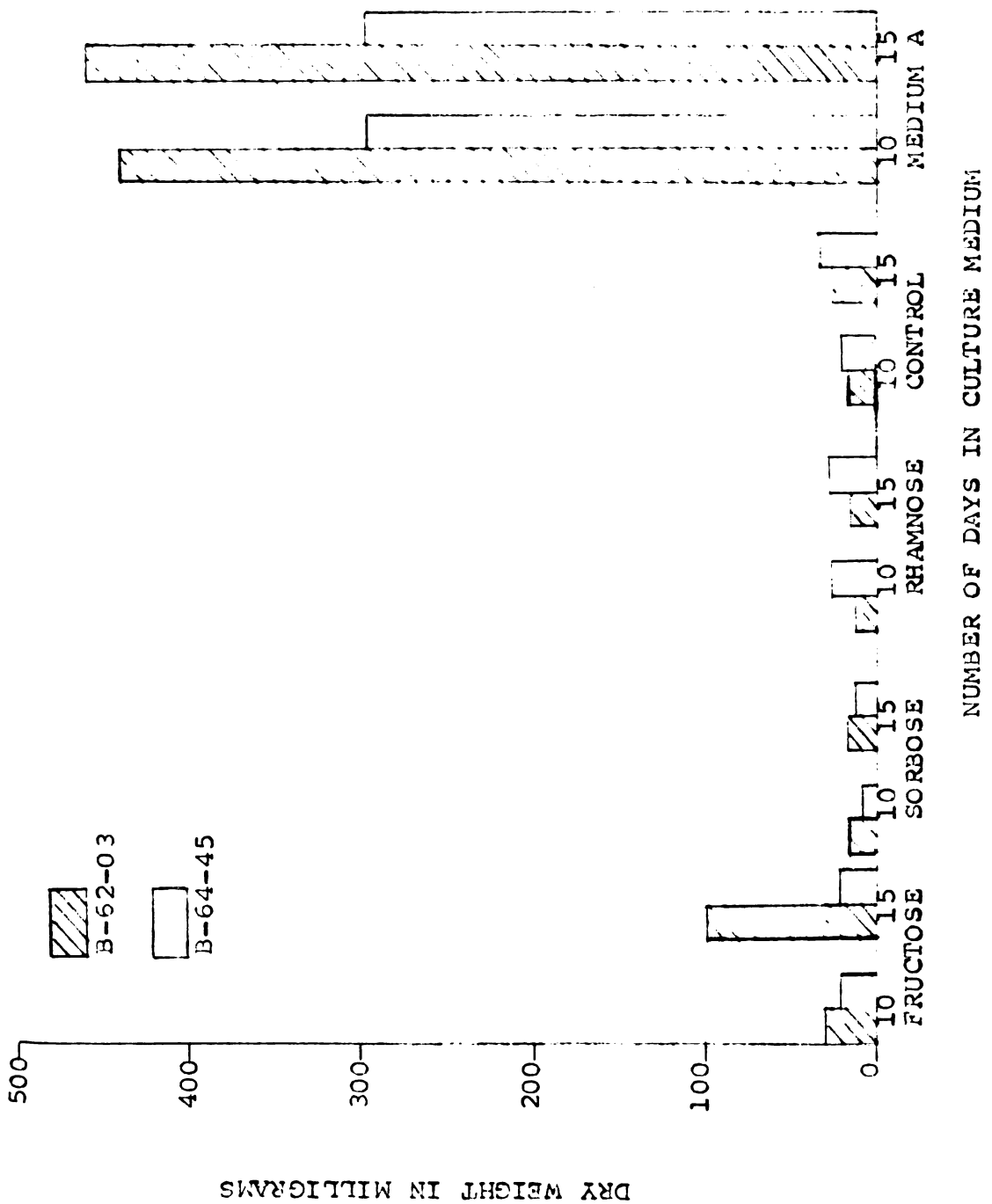


Figure 3 - Utilization of various monosaccarides by Suillus luteus strains, B-62-03 and B-64-45.

Carbon Utilization. 1. Monosaccharides

Table I contains the data depicting the utilization of various monosaccharides by the two strains of Suillus luteus (B-62-03 and B-64-45). The pentoses used in this experiment were utilized less readily than most of the hexoses by S. luteus with the exception of l-Sorbose in case of B-62-03, and B-64-45, which supported poorer hyphal growth than some of the pentoses (Table I). The two strains used in this experiment showed some variation in their ability to utilize different sugars as expressed by their difference in dry weight when grown under the same nutritional and environmental condition (Tables I, II & III).

The best carbon source among the pentoses for both strains was xylose (Table I).

Mycelial growth of B-62-03 and B-64-45 at the end of 15 days appeared to be less in most of the pentoses studied than in the control medium. This may indicate that most of the pentoses were not readily utilized by the organisms. Some fungal growth might have been due to carry over of nutrients by inocula, when it was equal to that in control medium. (xylose and arabinose in B-62-03 and xylose in B-64-45, Table I). B-62-03 grew more poorly in ribose than in C-free medium and B-64-45 showed much poorer growth in arabinose and ribose than in control medium. There might have been more autolysis in the

mycelium, thus reducing the weight as evidenced by morphological and cytological observation of the hyphae.

The hexoses employed in this study were found to be better carbon sources than most of the pentoses. Glucose and fructose were the best carbon sources for B-62-03 and glucose and mannose supported the best growth of B-64-45.

Of the hexoses D-glucose is biologically most important and is utilized for growth by virtually all fungi. A few forms, however, have been reported not tc grow with glucose (Cochrane, 1958). If an attempt is made to record on a single chart all the known biochemical fates of glucose and all the compounds derived therefrom, the result is a complex network. A study of this network reveals numerous possible "pathways" whereby carbon atoms, initially contained in glucose, may ultimately appear in CO_2, other hexoses, pentoses, lipids, amino acids, purines, pyrimidines, etc. (White, Handler and Smith, 1964). Table I shows that glucose was the best carbon source for both B-62-03 and B-64-45 among the hexoses. D-fructose and D-mannose are equivalent to glucose for growth for a large number of fungi (Cochrane, 1958). Table I shows that for B-62-03 fructose is almost as good as glucose and for B-64-45 mannose is almost equivalent to glucose. Mannose was found to support considerable fungal growth of B-62-03. It can be noted in Table I that glucose was

readily used by both strains whereas the rate of fructose utilization was rather slow for B-62-03 during the first 10 days of incubation but later it became faster. A similar situation occurred with mannose utilization by B-64-45.

Both B-62-03 and B-64-45 grew rather poorly on galactose. Growth of B-64-45 was almost equal to that on the control medium at the end of 15 days. 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, a form able to enter the main respiratory pathways.

Rhamnose has been reported to be used only by a few organisms (Cochrane, 1958). It supported no mycelial growth for B-62-03 (less than carbon free medium), while B-64-45 was slightly better than the control medium. Since rhamonose supported no growth, autolysis may have occurred in the hyphae of B-62-03, while any growth of B-64-45 might have been due to a carry over of nutrients with the inoculum.

Sorbose supports normal growth of a few fungi; however it appears not to be utilizable by most forms and it is definitely toxic to some (Cochrane, 1958). Growth on sorbose at the end of 15 days appeared to be less than the control medium in both the strains. This might support the above view of toxicity of sorbose. The

following statement of Lilly and Barnett gives a general picture of utilization of hexoses by fungi.

Lilly and Barnett (1951, p. 121) stated: "The following generalizations about utilization of the common hexoses may be drawn. (1) There is no single sugar which supports the maximum amount of growth for all the fungi. (2) Most fungi utilize glucose, although the maximum amount of growth was not always attained on this sugar. (3) The more closely the configuration of another sugar approaches that of glucose, the more fungi utilize it. It is believed that these generalizations are valid for all fungi which utilize sugars."

2. Oligosaccharides

Table II indicates that among the oligosaccharides cellobiose supported the best growth of B-62-03 and maltose supported the best growth of B-64-45. It was remarkable that cellobiose supported very poor growth of B-64-45 while maltose did not turn out to be a very good carbon source for B-62-03. Maltose is utilized virtually by all fungi which have been studied. Cellobiose is almost as widely utilizable as maltose (Cochrane, 1958). In analyzing the data on cellobiose and maltose utilization (Table II) it appears that maltose utilization increased considerably for strain B-62-03 in 15 days of the incubation in comparison to the 10 day culture. The same observation appears to hold true for B-64-45 in the

case of cellobiose. This might indicate that the involved enzyme was inducible. In spite of the fact that both cellobiose and maltose on 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 by an α-glucosidic linkage. Consequently, they require different enzymes for cleavage (Sedlmayr, 1960). The difference between the utilization of these two carbohydrates by the Suillus strains was probably due to the difference in abilities to produce the required enzymes.

Sucrose was found to be a good carbon source for both B-62-03 and B-64-45 (Table II). This was expected in B-62-03 since both components of this disaccharide (glucose and fructose) produced a satisfactory growth when used separately (Table I). Although one of the components, fructose, was not very favorable for the growth of B-64-45, utilization of sucrose by this strain probably indicates that the organism produces the hydrolyzing enzyme (as also probably does B-62-03) and the glucose liberated acts as a carbon source for the strain. Sucrose is generally a good source of carbon for many fungi (Cochrane, 1958).

It could be assumed from the results shown in Table II that trehalose was utilized satisfactorily by the

Table II

The Utilization of Various Oligosaccharides by Suillus luteus.

C-sources	B-62-03				B-64-45			
	10 days		15 days		10 days		15 days	
	mg*	pH+	mg*	pH+	mg*	pH+	mg*	pH+
OLIGOSACCHARIDES								
d(+) sucrose	85.5	2.5	98.5	2.6	82.2	2.8	91.7	2.5
d(+) maltose	32.7	4.0	66.9	2.9	75.5	2.8	100.1	2.6
d(+) lactose	37.3	5.7	39.3	5.9	26.2	4.75	29.8	5.2
d(+) cellobiose	66.3	2.6	111.0	2.25	15.7	4.4	33.1	4.3
d(+) melibiose	19.6	4.8	21.1	5.0	24.4	5.6	27.6	5.5
d(+) trehalose	82.8	2.8	91.2	2.4	74.7	2.8	82.1	2.55
d(+) raffinose	19.2	5.8	20.7	6.0	28.6	5.6	29.8	5.75
CONTROL								
C-free medium	18.5	5.5	26.4	5.8	19.7	4.7	33.5	5.4
Medium A	442.5	6.1	459.2	8.2	297.2	7.0	297.3	7.85

*Average mycelial dry weight of four replicates.

+Average of four replicates.

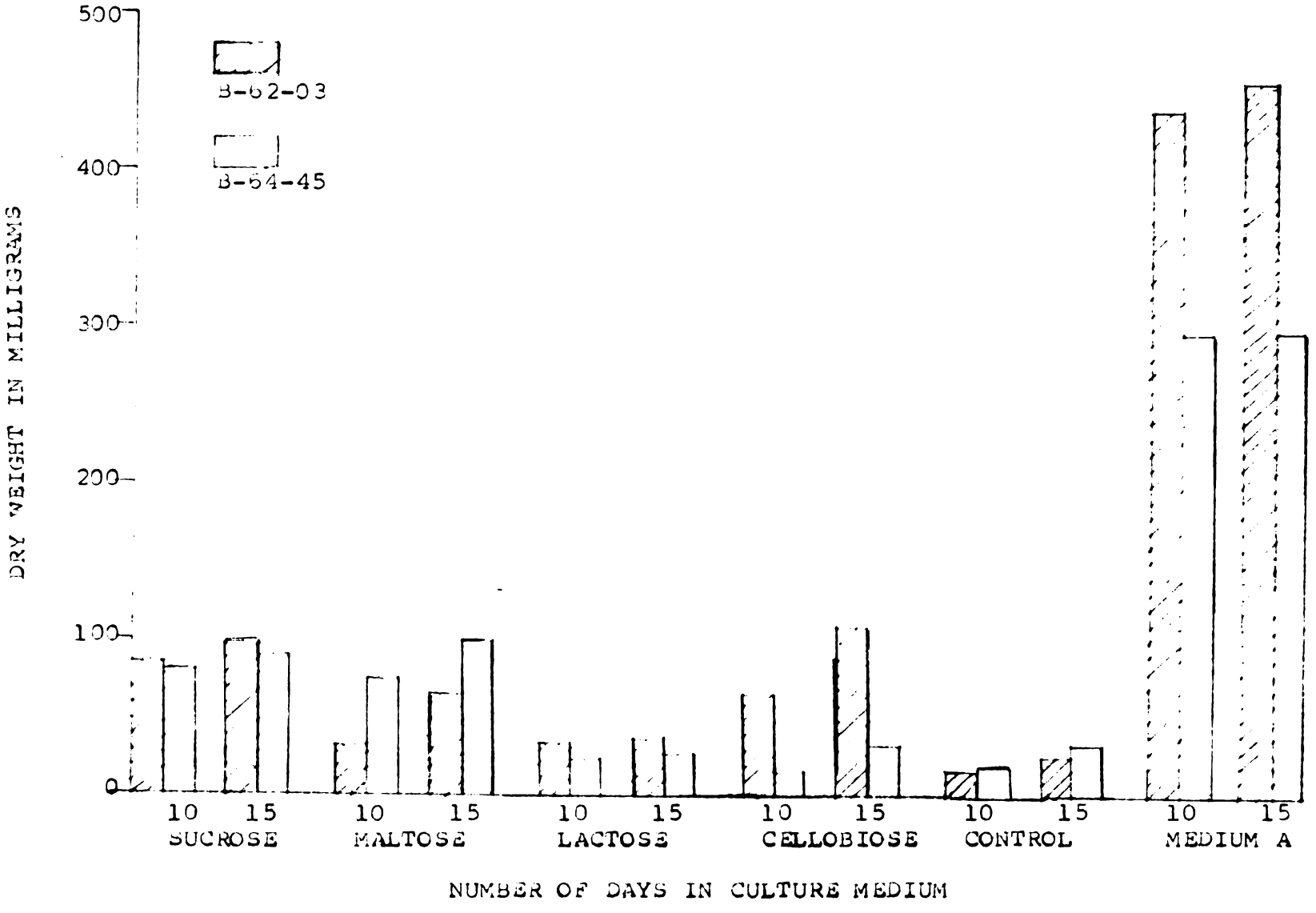


Figure 4 - Utilization of various oligosaccharides by Suillus luteus strains, B-62-03 and B-64-45.

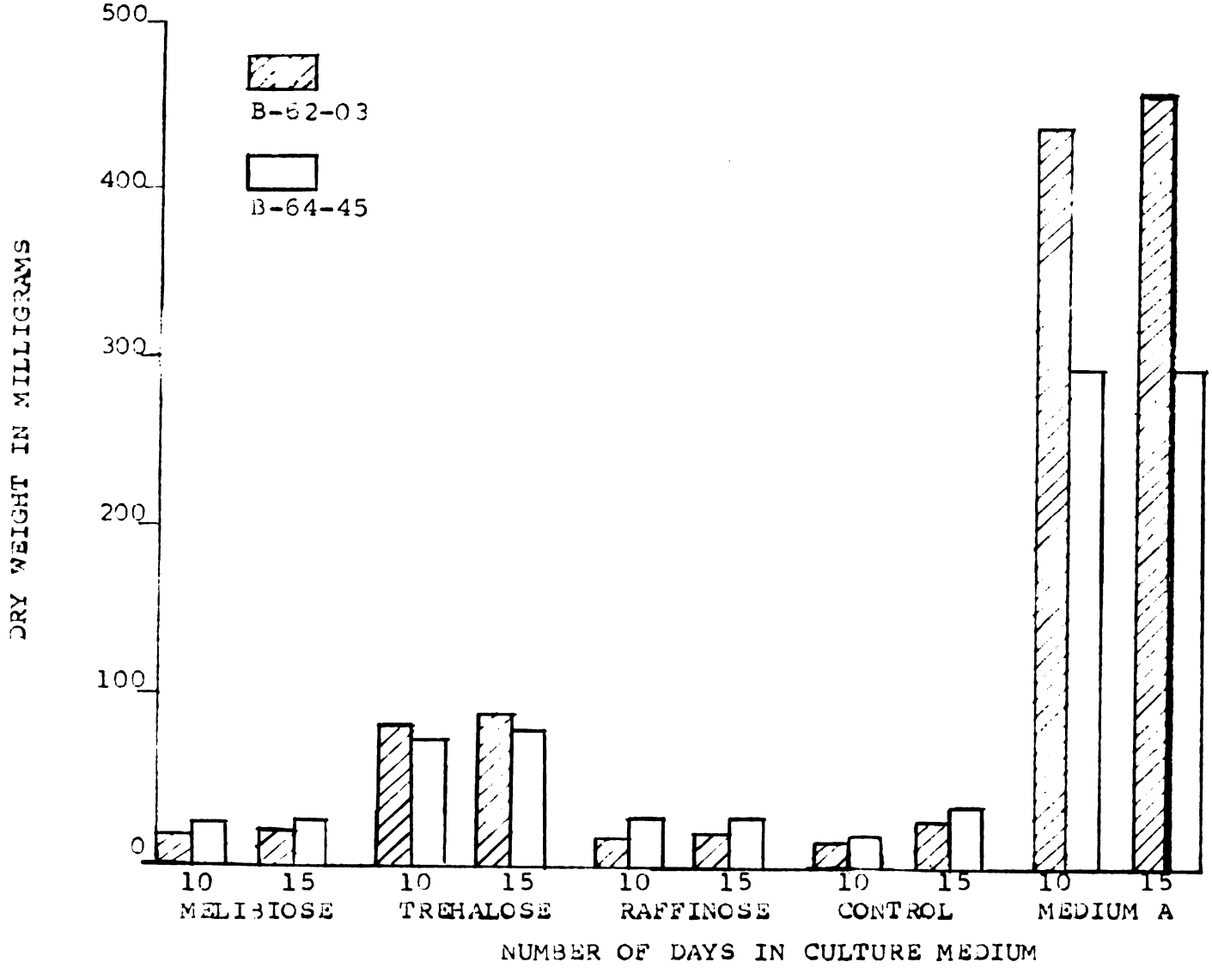


Figure 5 - Utilization of various oligosaccharides by Suillus luteus strains, B-62-03 and B-64-45.

strains of Suillus. Trehalose has been found to support growth of a large number of fungi including some that are relatively fastidious. This disaccharide is formed early in the metabolism and used up later. However, an adequate understanding of trehalose metabolism is not yet available (Cochrane, 1958).

Lactose supported poor growth of both strains of Suillus luteus. Mycelial growth was found to be more than that in the control medium in B-62-03 and slightly less in B-64-45. Lactose is used by far fewer fungi than any of the disaccharides so far mentioned and may therefore be considered as a poor carbon source (Cochrane, 1958). This might be due to the lack of the necessary hydrolytic enzyme, lactase, or due to the inability of the fungi to utilize one of the component hexoses, galactose (Sedlmayr, 1960).

Mycelial growth in 15-day cultures in melibiose appeared to be less than that on control medium in both the strains. This might be due to the lack of the hydrolytic enzyme melibiase or the inability of the fungi to utilize one of the component hexoses, galactose. Since growth was slightly less than that in C-free medium it might indicate that autolysis had occurred in the mycelium. Melibiose is not utilized by many fungi (Cochrane, 1958).

Raffinose, a trisaccharide which contains one linkage as in sucrose and another as in melibiose was utilized poorly by both the strains of Suillus luteus. Since the growth on raffinose in both the strains was less than that of carbon free medium, autolysis may have occurred in the mycelium of these strains. The majority of fungi utilize raffinose but non-utilization is also common (Cochrane, 1958).

The utilization of an oligosaccharide is believed to be preceded by and dependent on its conversion to hexoses or possibly hexose phosphates (Cochrane, 1958). The above statement appears to be true from the standpoint that the best utilized oligosaccharide for B-62-03, cellobiose, consisting of glucose, appeared to be the best utilized monosaccharide for the strain. The best utilized oligosaccharide for B-64-45 was maltose consisting of glucose which also seemed to be the best monosaccharide for that strain. Inability of B-62-03 to utilize maltose and B-64-45 to utilize cellobiose satisfactorily probably was due to the inability of the strains to produce the necessary hydrolytic enzymes, maltase and cellobiase respectively.

3. Polysaccharides.

Table III indicates that none of the polysaccharides used in this experiment supported good growth of the fungi, particularly in comparison to the monosaccharides and the

Table III

The Utilization of Various Polysaccharides by Suillus luteus

C-sources	B-62-03				B-64-45			
	10 days		15 days		10 days		15 days	
	mg*	pH+	mg*	pH+	mg*	pH+	mg*	pH+
POLYSACCHARIDES								
Dextrin	23.0	4.0	46.8	3.8	14.3	4.85	39.2	4.9
Starch	36.5	3.8	39.9	3.6	47.8	3.0	48.1	2.75
Inulin	17.0	4.8	19.3	4.5	18.5	3.7	18.6	4.5
Glycogen	20.5	3.68	20.7	3.68	25.7	3.65	32.5	4.3
CONTROL								
C-free medium	18.5	5.5	26.4	5.8	19.7	4.7	33.5	5.4
Medium A	442.5	6.1	459.2	8.2	297.2	7.0	297.3	7.85

*Average mycelial dry weight of four replicates.

+Average of four replicates.

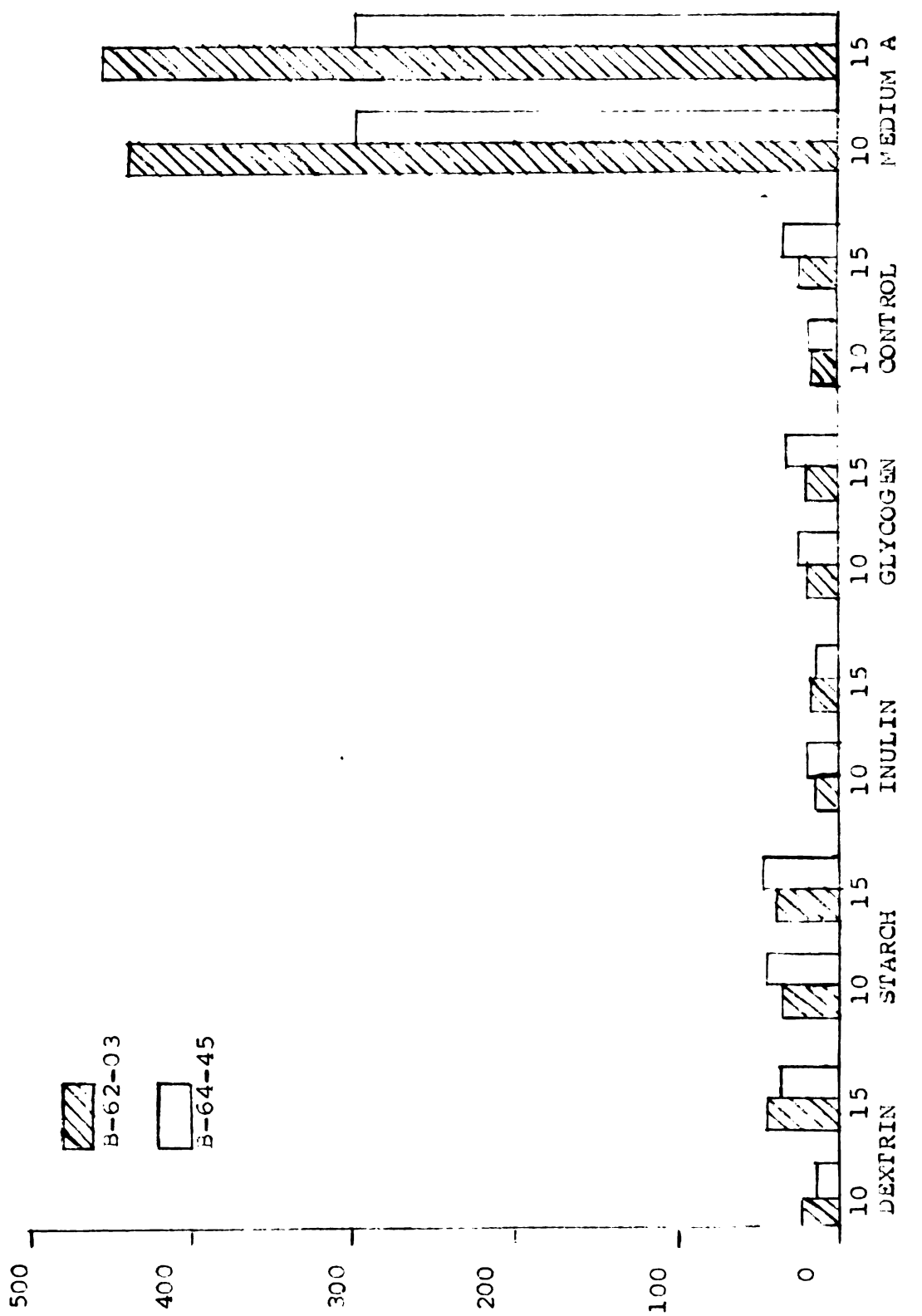


Figure 6 - Utilization of various polysaccharides by Sullus luteus strains, B-62-03 and B-64-45.

oligosaccharides. This probably indicates the inability of the fungi to produce the necessary hydrolytic enzymes sufficiently, since the component of the majority of the polysaccharides used in this experiment (Dextrin, starch, glycogen) was glucose, the most favorable monosaccharide for both the strains. Inulin, a polyfructoside, did not support good growth of B-62-03, although fructose was a good carbon source for this strain (Table I).

Dextrin was the best carbon source among the polysaccharides for B-62-03 and starch was the most favorable polysaccharide for B-64-45. Starch supported the next best growth of B-62-03 and Dextrin of B-64-45. This was expected since according to Cochrane (1958) Dextrins are chemically or enzymatically modified starches of uncertain structure, and are generally satisfactory sources of carbon for fungi.

Starch is composed of a mixture of two different polysaccharides, amylose and amylopectin. The glucose units of amylose are bound to each other in α-1,4-glucosidic linkage. Amylopectin contains chains of glucose units like those of amylose but it also has branches of these glucose chains linked through an α-1,6-glucosidic linkage (Fairley and Kilgour, 1963). Starch is an excellent carbon source for most fungi, even for rather fastidious forms. Dextrins are also generally a satisfactory source of carbon for many fungi (Cochrane, 1958).

Glycogen is a glucose polysaccharide similar to the amylopectin fraction of starch. It supported better growth of B-64-45 than B-62-03, which corresponds with the observation that starch favored the growth of B-64-45 more than it did B-62-03. Mycelial growth in glycogen however was slightly less than in the control medium in B-62-03 and equivalent to that in B-64-45. Glycogen is quite generally available to fungi of widely different ecological groups. Those fungi unable to use starch can not be expected to use glycogen, So far as is known, the same enzymes attack both polysaccharides (Cochrane, 1958).

Inulin, a polyfructoside appeared to be a poor carbon source for both B-62-03 and B-64-45. In case of B-62-03, it was probably due to the inability of the fungus to produce the necessary hydrolytic enzyme inulase, since fructose was found to be a good carbon source for this strain. Inulin, a polymer of D-fructose, has been found to be a good source of carbon for many but not all fungi (Cochrane, 1958). Hawker (1950) suggested that the inability of fungi to use inulin for growth was due to the failure to secrete the enzyme, rather than to any inhibitory effect of the inulin.

Cellulose is by far the largest natural reservoir of biologically utilizable carbon. It is an excellent source of carbon for many fungi, but the species within a given genus are not all necessarily alike in their response

to cellulose. Insolubility of cellulose, however, makes the nutritional study of the compound difficult. (Cochrane, 1958). Cellulose was a poor carbon source for both B-62-03 and B-64-45. Since no satisfactory technique could be devised to test the cellulose utilization by the fungi, owing to the insolubility of cellulose, the data obtained were considered to be inaccurate. Poor mycelial growth was obtained in all experiments. Hence the data were not included in Table III. Cellulose is a linear polymer of D-glucose, where the glucose molecules are joined together through β-1,4-glucosidic linkage. The poor growth obtained in cellulose, especially of B-62-03, was probably due to the lack of cellulase formation. However, cellobiose, the intermediate product of this polysaccharide, was one of the best carbon sources for the organism.

In order of utilization, cellobiose was the best carbon source for B-62-03 followed by glucose, fructose, sucrose, trehalose and mannose. For B-64-45 maltose was the best carbon source followed by sucrose, glucose, trehalose and mannose.

The pH of the Lindeberg's medium and medium A was adjusted to 5.5 for the nutritional studies. It was noted that in Lindeberg's medium good mycelial growth was mostly associated with a lowering of the pH of the medium (Tables I and II). The pH remained about the same when

the sugars were not well utilized. The pH is affected during growth by metabolic activities, raised by absorption of anions or production of ammonia from nitrogen compounds, lowered by formation of organic acids or absorption of cations. These effects of growth on pH complicate results, particularly in poorly buffered media commonly employed (Cochrane, 1958). The hydrogen ion concentration of a nutrient solution may change 10,000-fold during a few days as a result of the metabolic activities of a fungus. These changes in pH are due to changes in the relative amounts of acids and bases formed or withdrawn and to the ionization constants of these compounds (Lilly and Barnett 1951).

Fungi produce acids from non-acidic nutrients such as the carbohydrates. Various organic acids such as pyruvic, citric and succinic are also produced. Pyruvic acid accumulates in the nutrient solution in which many fungi are grown, and in some instances the formation of this acid accounts for a considerable part of the early lowering of pH. The eventual utilization of pyruvic acid causes the pH of the nutrient solution to rise. Other metabolizable acids behave similarly (Lilly and Barnett, 1951). This probably is a good explanation of the rapid lowering of pH in media where the carbohydrate was well utilized (Tables I and II) and also for a rise of pH after a lowering at first (e.g. sucrose in B-62-03, Table II).

Growth in medium A, however, was found to be associated with a rise of pH, which was greater in B-62-03 showing a more rapid growth than B-64-45 (Tables I, II and III). Ammonia is the most common basic substance produced by fungi. The production of ammonia results from the deamination of amino acids and proteins (Lilly and Barnett, 1951). In medium A ammonia supply is greater due to peptone and yeast extract. (See appendix for their composition.) In Lindeberg's medium, on the other hand, ammonium chloride is the only N_2 source. According to Lilly and Barnett (1951) the process which produces acid usually dominates during early fungal growth, especially when ammonium nitrogen is used.

It was further observed that good hyphal growth and low pH in the Lindeberg's medium was associated with a change of color of the nutrient solution. It was bright orange when the growth of the fungus was good and the pH of the medium was low. When the nutrient solution appeared to be pale colored, the growth was poor and consequently there was slight change of pH of the medium.

At no time did any of the strains show mycelial growth in test media as great as that in medium A (Tables I, II, and III), indicating that growth factors must be present in Medium A that were not present in the modified Lindeberg's medium (Cook, 1962). B-62-03 showed greater mycelial growth on medium A in comparison to B-64-45

during the 15 days incubation period. The length of incubation necessary for optimum growth for each strain was determined previously by running a series of growth curves.

Medium A supported better growth than Lindeberg's medium, because it contained peptone and yeast extract. (See appendix for their composition.)

Utilization of Peptone and Yeast Extract

A study was made on the effect of peptone and yeast extract separately and together in the medium A on Suillus luteus B-62-03. The fungus was grown in medium A with the usual combination of 5 grams of peptone and 5 grams of yeast extract/liter. Then it was also grown on medium A with ten grams of peptone in one case and with ten grams of yeast extract in the other case.

Table IV

Utilization of Peptone and Yeast Extract by B-62-03*

Medium	20 days	
	mg	pH
"A" (with original combination of peptone and yeast)	310.0	7.0
"A" (with yeast)	770.2	5.3
"A" (with peptone)	256.0	4.7

*Average mycelial dry weights of four replicates grown on reciprocal shaker.

The results in Table IV indicate that the yeast extract supported better growth of B-62-03 than peptone.

Morphological Studies of Suillus luteus Mycelium in Submerged Culture

Pantidou (1961) described cultural characters of several species of Boletaceae and reported on formation of the fruiting bodies by the genera Boletinus and Suillus in culture. In microscopic characteristics most of the species of Boletaceae showed a uniformity. The differences in hyphal characteristics such as coloring, septation, and branching were more in the nature of degree than of kind.

The majority of the species possessed hyphae of an ordinary type, appearing cylindrical, hyaline with homogeneous or granular contents in the living cells or hyaline and empty in the non-living. Several species of Boletaceae however, could be differentiated from each other on the basis of their characteristic hyphal modifications.

Hyphae with papillated appearance were found and described in species of Suillus and Xerocomus. Such hyphae occurred in varied frequencies in almost all species studied. In this case the ordinary hyphae were modified by depositions of amorphous yellow to brown material on the surface of the walls. This material became easily detached from the cell wall, floating off when mounted in lacto-phenol mounting medium. The deposits

were either small and rather regular in shape, seen only at the edges of the hyphae, or they were large, irregular, and covered the whole surface. In some species they were more firmly attached to the walls and gave an ornamented appearance to the hyphae. It is likely that this material contributed largely to the color of both sides of the mats. In some isolates where papillated hyphae were less numerous or absent, the mats and their reverse sides were lighter colored (Pantidou 1961).

For the study of any morphological change induced by the different carbohydrates, slides were prepared (water mount) from ten-day old cultures grown on rotary shaker, and observed under the microscope. Changes in the forms of the hyphae were drawn with the aid of a camera lucida.

The morphological changes that were induced by the use of the different sugars as carbon sources are indicated below. Strain B-62-03 and B-64-45 showed similar responses in the different sugars used.

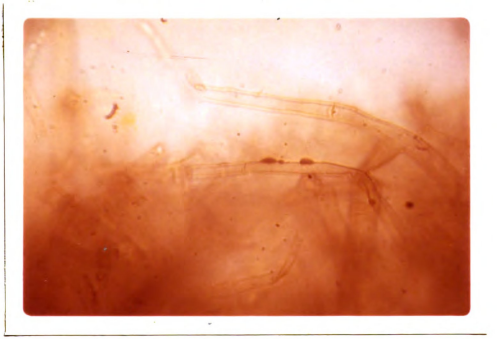
Monosaccharides

Pentoses

Arabinose:

Both in B-62-03 and B-64-45 protoplasm was scarce, shrunken in some, with papillated hyphae. Brown deposits were abundant in B-64-45 but scarce in B-62-03.

PLATE IV



Mycelium of Suillus luteus strain, B-62-03, grown in submerged culture with ribose as carbon source.

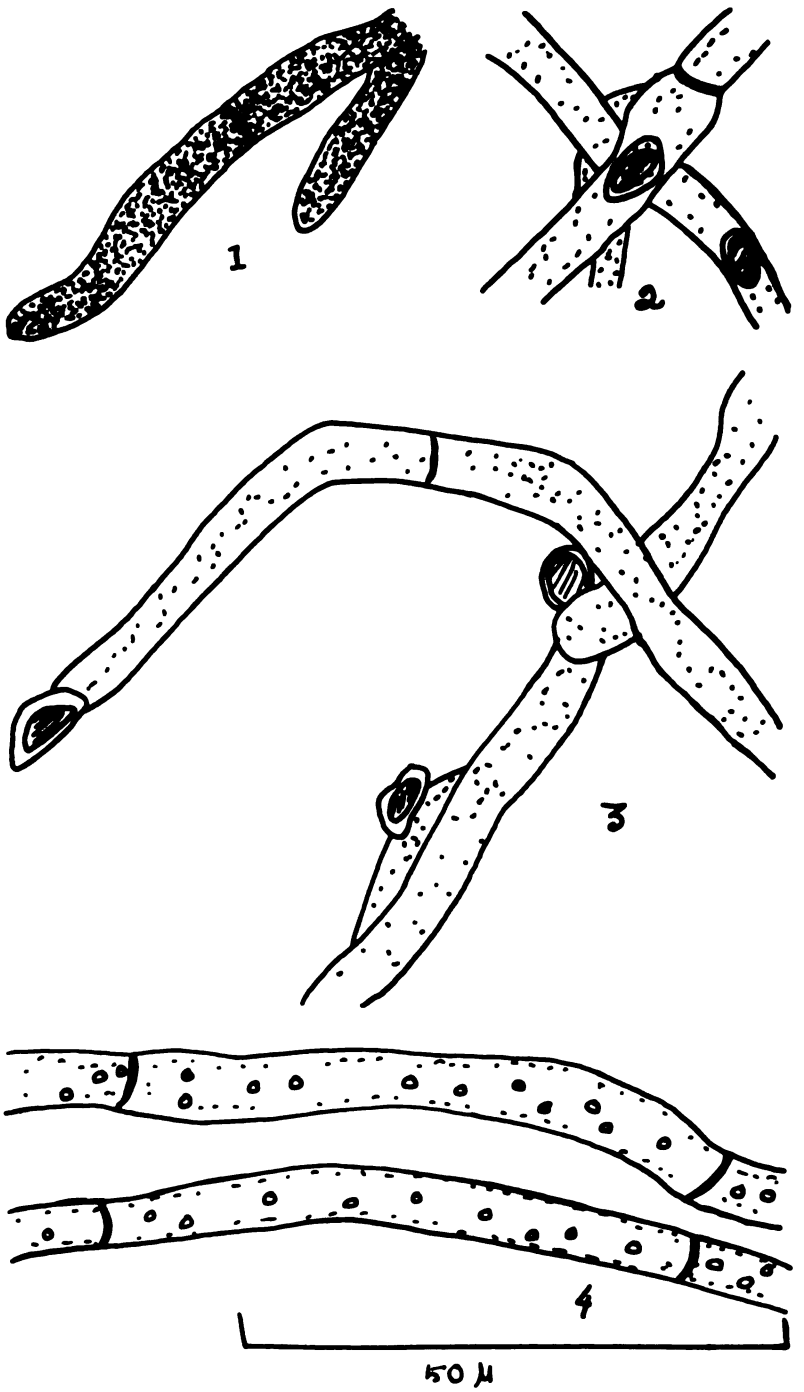


Plate V. Mycelium of Suillus luteus strain, B-62-03, grown in submerged culture with various sugars as carbon source: 1, glucose; 2, xylose; 3, ribose; 4, control (- carbon).

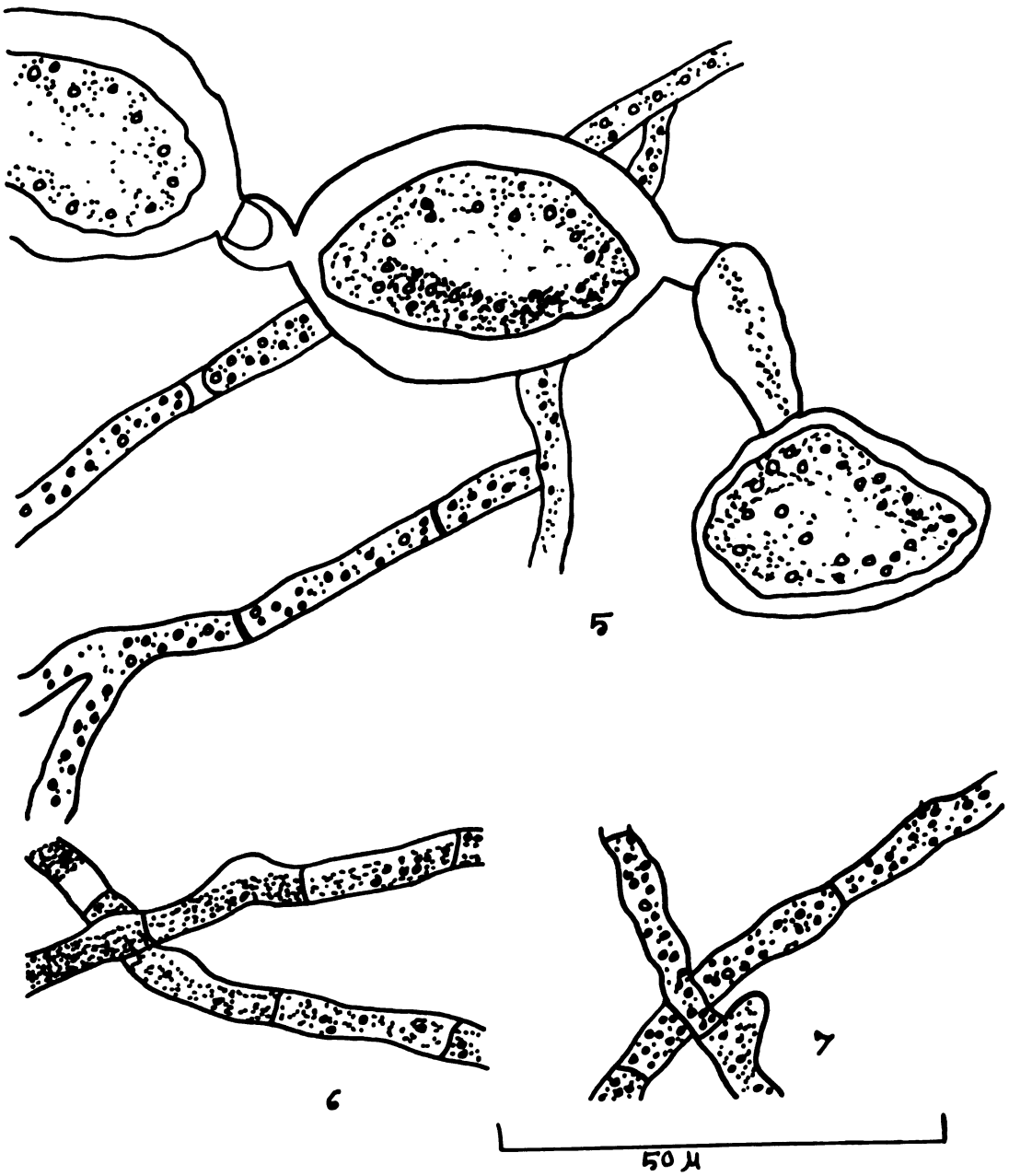


Plate VI. Mycelium of Suillus luteus strain, B-62-03, grown in submerged culture with various sugars as carbon source: 5, sorbose; 6, mannose; 7, raffinose.

Xylose:

Protoplasm was not dense and brown deposits and clamp connections were present.

Ribose:

Protoplasm was thin, and brown deposits were abundant (Plate V, Fig. 3).

Hexoses:

Rhamnose:

Protoplasm is scarce in the cells and the hyphae were papillated.

Glucose:

Protoplasm was very dense. In some of the older cells it was shrunken from the wall surface which might indicate the beginning of autolysis. There were clamp connections present but brown deposits were not observed (Plate V, Fig. 1).

Galactose:

Protoplasm was not very dense in the hyphae. Large oil droplets and clamp connections were present. Brown deposits were rare on the cell wall.

Mannose:

Protoplasm was usually dense but may be rather thin in some hyphae. The hyphae were slightly wavy (Plate VI, Fig. 6), but brown deposits were not observed.

Fructose:

Protoplasm was very dense in hyphae, but brown deposits were not observed.

Sorbose:

Protoplasm was thin with large oil droplets present in the hyphae. Large conspicuous chlamydospores were present, being more abundant in the strain B-62-03 than in B-64-45 (Plate VI, Fig. 5). Brown deposits were present.

Oligosaccharides:

Sucrose:

Protoplasm was very dense. Autolysis began in some hyphal cells which might be due to the low pH of the medium (Table II). Brown deposits were not observed.

Maltose:

Dense protoplasm, shrunken from the cell wall in some hyphae. Brown deposits were not observed.

Lactose:

Thin protoplasm in hyphae and brown deposits were more abundant in B-64-45 than in B-62-03.

Cellobiose:

Protoplasm in the strain B-62-03 was very dense whereas that in B-64-45 was scarce and shrunken. Brown deposits were present in both strains.

Melibiose:

Protoplasm was more dense in B-62-03 than in B-64-45. Brown deposits were more abundant in B-64-45.

Trehalose:

Hyphae with very dense protoplasm and clamp connections were present.

Brown deposits were not observed.

Raffinose:

Hyphae had shrunken or thin protoplasm in the cells with large oil droplets. Hyphae were wavy and contained clamp connections (Plate VI, Fig. 7). Brown deposits were not observed.

Polysaccharides:

Dextrin:

Hyphae had fairly dense protoplasm with large oil droplets. Clamp connections were observed. Brown deposits were not found.

Starch:

Hyphae showed fairly dense protoplasm, some cells were shrunken and vacuolated. Clamp connections were observed, but no brown deposits were found.

Inulin:

Protoplasm was thin and shrunken in some hyphal cells. Brown deposits were abundant in strain B-64-45 and scarce in B-62-03.

Glycogen:

Protoplasm was less dense, large oil droplets were present in protoplasm. No brown deposits were observed.

Cellulose:

Protoplasm was found to be fairly dense in the cells and no brown deposits were found.

Control Medium (without carbon):

Protoplasm of the elongated hyphae was very thin with large oil droplets. Brown deposits were more abundant in the strain B-64-45 than in B-62-03 (Plate V, Fig. 4).

Medium A:

Hyphae grown in "medium A" developed very dense protoplasm and no brown deposits were observed.

The descriptions given above indicate that structural changes in the hyphae and protoplasm were directly or indirectly affected by various sugars. It was noted that in most instances there was a correlation between good growth and dense protoplasm. Poor growth in most instances were associated with thin protoplasm. Brown deposits were more abundant in the strain B-64-45 and in sugars which supported poor growth.

It was noted that brown deposits were rare when the hyphae had very dense protoplasm. Since the hyphae were taken from the various sugars under the controlled conditions of these experiments it is possible that the brown deposits may occur in more cases if changes were made in pH, temperature, and length of time for growth of the hyphae in the various sugars. Chlamydospores were found to be induced by only one sugar, Sorbose. Clamp connections were common in most instances. However, an explanation can be given for the cases where no clamp connections were found, as changes may be necessary in the

environmental conditions when a different carbohydrate is utilized to induce clamp formation. Further study of the conditions for formation of clamp connections in submerged culture might indicate the basis for this phenomen.

Effect of Light on Growth of B-62-03

Most reports on the effect of light on the fungi have been concerned with reproduction rather than vegetative growth. However, light has been found to depress or promote growth in some fungi (Lilly and Barnett, 1951).

To study the effects of light, a number of plates (B-62-03 inoculated on medium A) were kept in a constant temperature room (22-24°C) and exposed to the fluorescent lights in the laboratory for the part of the light period mentioned in Table V. These plates were then removed to the constant temperature dark room for the remaining period. Growth was measured after the total 15 days of incubation was completed.

Table V

Effect of Fluorescent Light on B-62-03

Total days		Diameter of Colony
Light	Dark	
0	15	2.26 cm.
3	12	2.40 cm.
7	8	2.33 cm.
10	5	2.25 cm.
15	0	2.41 cm.

Table V shows that light does not have any significant effect on the growth of B-62-03. It appeared to be slightly stimulatory in some instances (3 days and 15 days). Results obtained from plates exposed to light for 7 and 10 days were almost equivalent to that kept in the dark all the time. This may indicate that slight variation in growth might have been due to the variation in size of inocula, and individual variability, which is not significant.

Production of antitumor substance

Stevens (1957) showed variations in tumor-retarding activity of cultures of Collybia radicata, when different carbon sources were utilized. Cook (1962) reported that there was a differential production of Calvacin (an inhibitor of Crocker mouse Sarcoma 180) by Calvatia gigantea, when the various carbon sources were used. Cultures grown in cellobiose generally produced the most Calvacin. There was no correlation between the amount of growth and the amount of Calvacin produced when the selected carbon sources were used.

In this study an attempt was made to determine if variation in antitumor activity was evident in Suillus luteus when other carbon sources were substituted in the basal medium.

Table VI indicates the results of the Crocker mouse Sarcoma 180 tumor tests using six mice in each test.

The designation T/C in Table VI was determined by taking the average tumor weight of the experimental mice, injected with the extract of Suillus luteus culture grown in different carbon sources, and dividing this figure by the average tumor weight of the control mice, which were injected with normal Saline(.85% Saline).

This method of determining tumor retardation is followed by the Cancer Chemotherapy National Service Center.

Table VI indicates that glucose (in rotary shaker) and sucrose (in reciprocal shaker) as a carbon source result in the greatest antitumor activity in strain B-62-03. Whereas antitumor activity was much reduced when this strain was grown on a reciprocal shaker with glucose as a carbon source. When grown on a rotary shaker with sucrose as a carbon source it stimulated, rather than retarded the growth of the tumor.

Lucas, et al., (1953-59) reported that compounds in the filtrate of Calvatia species which stimulated tumor growth appeared in some instances where the T/C was reported to be 100 or more. This was found to be due to the production of these substances in younger cultures prior to the development of Calvacin. Calvacin once produced in vitro, could be lost with further mycelial growth or that more time may be needed for Calvacin production (Stevens, 1957).

Table VI

Antitumor Activity of Cultures of *Suillus luteus* Strains B-62-03 and B-64-45 against Crocker Mouse Sarcoma 180

Carbon Source	Days of Incubation	Shaker	Strain B-62-03 T/C	Strain B-64-45 T/C
Glucose	15	rotary	66	81
	20	reciprocal	92	124
Maltose	15	rotary	-	84
	20	reciprocal	-	60
Cellobiose	15	rotary	102	-
	20	reciprocal	93	-
Trehalose	15	rotary	145	95
	20	reciprocal	83	120
Sucrose	15	rotary	128	78
	20	reciprocal	69	112

T/C = Experimental tumor weight divided by the control tumor weight.

The strain B-62-03 when grown on a rotary shaker with cellobiose as a carbon source showed some stimulation of tumor growth, whereas it showed very slight retardation of tumor growth when grown on a reciprocal shaker with the same carbon source.

When trehalose was used as a carbon source the strain B-62-03 showed some antitumor activity when grown on a reciprocal shaker. But it stimulated tumor growth when grown on a rotary shaker with the same carbon source.

Maltose was the best sugar for the antitumor activity of the strain B-64-45 (when grown on a reciprocal shaker). The strain still showed antitumor activity when grown on a rotary shaker with maltose as a carbon source, but the activity was reduced.

Glucose supported some antitumor activity of B-64-45, when grown on a rotary shaker. The fungus however stimulated tumor growth when grown on a reciprocal shaker with glucose as a carbon source.

When trehalose was utilized as a carbon source, B-64-45 showed slight antitumor activity when grown on a rotary shaker, but it stimulated tumor growth when grown on a reciprocal shaker.

Some retardation of tumor growth was obtained when B-64-45 was grown on a rotary shaker with sucrose as a carbon source. But the fungus stimulated tumor growth when grown on a reciprocal shaker with the same carbon source.

It was evident that the two strains B-62-03 and B-64-45 showed some variation in their antitumor activity in different sugars and also in different shakers which might have been due to the duration of incubation period.

None of the sugars used in this experiment however, seemed to be as effective as having both sugars present in medium A, as Suillus luteus when cultured in medium A, retarded tumor growth up to 72% of the controls (Beneke unpublished data). Greater antitumor activity in medium A probably is due to the presence of two different sugars (glucose and sucrose) in the medium and also due to difference in experimental condition.

SUMMARY

Two strains of Suillus luteus (B-62-03 and B-64-45) were used to determine utilization of carbon sources and the effect of some of the carbon sources on the production of antitumor activity. Morphological changes induced by different carbon sources were studied under the microscope.

There was a differential utilization of the various carbohydrates by the two strains of Suillus luteus. Pentoses in general supported poor growth of the strains. Hexoses were more favorable, of which glucose was the best for both strains.

Oligosaccharides supported fairly good mycelial growth of both. Cellobiose was the best oligosaccharide for the strain B-62-03 and maltose was best for B-64-45.

Polysaccharides in general supported poor growth of both strains.

Yeast extract in medium A contained substances stimulatory to the growth of the strain B-62-03.

Structural changes in the hyphae and protoplasm were affected by various sugars. There was a correlation of good mycelial growth with dense protoplasm. Brown

deposits and clamp connection were found to develop when certain sugars were placed in the medium. Chlamydospores were induced only by sorbose. B-62-03 and B-64-45 showed similar response structurally in different sugars used.

Light as used under the conditions of this experiment had no effect on the growth of B-62-03.

Antitumor activity of S. luteus was affected by various carbon sources used under these experimental conditions. Of the sugars tested glucose and sucrose resulted in the greatest production of antitumor activity of B-62-03 while cellobiose and trehalose produced a substance(s) which stimulated tumor growth. Maltose supported best antitumor activity of the strain B-64-45, while trehalose induced the production of a stimulatory substance for tumor growth in the fungus. Type of shaker used affected the antitumor activity of the fungus. This might be however owing to the duration of incubation period.

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APPENDIX

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
Manganese	7.80
Zinc	83.00
Copper	19.00
PER CENT	
Phosphorus	0.89
Iron	0.028
Si02	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
PER CENT	
Glycine	2.4
Histidine	0.94
Isoleucine	2.9
Leucine	3.6
Lysine	4.0
Methionine	0.79
Phenylalanine	2.2
Threonine	3.4
Tryptophane	0.83
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 Proteosa 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	0.05%
Magnesium	0.056%
Manganese	Nil
Iron	0.0033%
Ash	3.53%
Lead	15.00 ppm

Arsenic	0.09 ppm
Zinc	18.00 ppm
Copper	17.00 ppm
SiO2	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%
MICROGRAMS PER GRAM	
Pyridoxine	2.50
Biotin	0.42
Thiamine	0.50
Nicotinic acid	35.00
Riboflavine	4.00