# NITROGEN UTILIZATION AND PRODUCTION OF ANTITUMOR SUBSTANCES BY SUILLUS LUTEUS (FR.) S. F. GRAY AND SUILLUS ACIDUS (PECK) SINGER

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ABSTRACT

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by Keith Howard Erke

The purpose of this study was to determine the nitrogen requirements of Suillus luteus (Fr.) S.F. Gray and Suillus acidus (Peck) Singer and to investigate the production of antitumor substances produced in vitro by these organisms. Nitrates, ammonium compounds and amino acids were used as nitrogen sources with emphasis on the utilization of the amino acids.

The organisms were cultured on a rotary shaker. Both medium A, a modified Czapek's-Dox medium, and a synthetic medium were employed. For the nutritional study the various nitrogen sources were added to the basal synthetic medium. The growth response was measured by determining dry weights of the mycelium at 10 and 15 day

periods. The antitumor activity was determined by testing extracts in female Swiss albino mice inoculated with Sarcoma 180 tumors.

Growth curves were established for S. luteus and S. acidus growing on medium A. Increasing the shaker speed resulted in a reduction of the lag phase of the growth cycle and shortened the length of time to the stationary phase. Growth curves were also established for both organisms when grown in the synthetic medium.

Nitrates were not utilized, except limited growth of S. luteus occurred with potassium nitrate. The ammonium compounds supported a fair amount of growth by each organism. The following amino acids were the best nitrogen sources in the synthetic medium: L-alanine, DL-alanine, L-glutamic acid and L-aspartic acid for the best growth of S. luteus, and L-arginine and L-lysine for S. acidus.

Antitumor substances were produced by both organisms when grown in medium A. Suillus luteus grown in medium A for 15 days showed 70% inhibition of tumor growth. Antitumor substances were likewise produced by both organisms when grown in the synthetic medium containing the amino acids which supported better growth. The best antitumor activity

for both organisms occurred in extracts of 15 day old cultures. None of the extracts tested retarded the growth of the mice during the test period.

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By

Keith Howard Erke

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To my family

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# NITROGEN UTILIZATION AND PRODUCTION OF ANTITUMOR SUBSTANCES BY *SUILLUS LUTEUS* (FR.) S.F. GRAY AND *SUILLUS ACIDUS* (PECK) SINGER

## INTRODUCTION

A recent article by Gregory *et al.*(1966) indicates the interest in recent years in antitumor substances produced by Basidiomycetes. Extracts from more than 7000 cultures produced by submerged fermentation were surveyed for antitumor activity in mice. Fifty extracts from cultures representing 20 genera produced an inhibitory effect on Sarcoma 180, Mammary Adenocarcinoma 755 or Leukemia L-1210.

Antitumor activity in Basidiomycetes was first demonstrated by Lucas *et al.* (1957) in *Boletus edulis*. According to the folklore the inhabitants of a small mountain village in eastern Bavaria ingested *B. edulis* to prevent cancer (Lucas, 1960).

Several *Calvatia* species produced antitumor activity (Lucas *et al.*, 1958-59). Further extensive physiologic

studies of these organisms, especially Calvatia gigantea, led to the development of strains that produced greater yields of the antitumor substance. Beneke (1963) reviewed this work in the Presidential address to the Mycological Society of America.

Extracts of several species of Suillus produced activity against Sarcoma 180 (Beneke, unpublished data).

Majid (1965) studied the carbohydrate requirements and their effect on the production of antitumor substances by two strains of Suillus luteus (Fr.) S.F. Gray.

The purpose of this study was to determine the nitrogen requirements and their effect on the production of antitumor substances by Suillus luteus and Suillus acidus (Peck) Singer when grown in submerged cultures.

## REVIEW OF THE LITERATURE

There has been a vast amount reported on the nutrition of the fungi--even if one considers only nitrogen nutrition. It is not feasible to cover all the papers that have been written on this subject. Steinberg (1939, 1950) has summarized the principal work on the nutrient requirements of fungi in synthetic media.

Two of the more notable reports on the early controversy of whether certain fungi could utilize free nitrogen as could certain bacteria were by Duggar and Davis (1916) and Allison, Hoover and Morris (1934). Duggar and Davis reported that the mycorrhizal species Phoma betae was able to utilize free nitrogen. Allison et al. produced evidence to discount previous reports of nitrogen fixation by certain species of Aspergillus and Cladosporium. Nitrogen fixation did not occur among soil actinomycetes and they concluded that only certain mycorrhizal fungi may be capable of nitrogen fixation. Cochrane (1958) concluded that there was no good evidence for nitrogen fixation by the fungi. Nicholas (1965) credits only the yeasts, Rhodotorula and Pullularia, with being able to fix atmospheric nitrogen.

An extensive study on nitrogen nutrition of fungi was done by Czapek (1902, 1903) with Aspergillus niger. Using almost all possible nitrogen sources known he concluded that proteins were most easily synthesized from amino acids or from substances closely resembling amino acids.

A large number of Mucors were divided into two groups on their ability to assimilate nitrates and nitrites (Hagem, 1910). Nitrogen requirements were not fixed but varied with the source of carbon. A number of species could utilize ammonium or nitrate nitrogen with glucose but required nitrate nitrogen with glycerol.

Peptone, ammonium sulphate, ammonium nitrate and potassium nitrate were used as nitrogen sources for growing Aspergillus niger, Sphaeropsis malorum and Diplodia natalensis in liquid cultures (Klotz, 1923). Peptone supported the best growth in all species. The ammonium compounds and potassium nitrate varied in their order of ability to support growth. Klotz described autolysis which was indicated by a decrease in the mycelial dry weight after the utilization of the available carbon source. During autolysis ammonia was given off and amino acids appeared in the medium.

Treschow (1944) did a complete nutritional study on the cultivated mushroom Psalliota bispora (syn. Agaricus

campestris). Ammonium salts and the amino acids, glutamic acid and asparagine, were good sources of nitrogen. Nitrates did not support growth of the mycelium.

Nine species of Marasmius grown in liquid cultures utilized ammonium nitrogen and asparagine (Lindeberg, 1944). Only one of the nine utilized nitrates.

Twenty-one amino acids were utilized as sources of carbon for Fusarium oxysporum var. lycopersici and Penicillium roqueforti (Gottlieb, 1946). Seventeen could be utilized as carbon sources. The six carbon straight chain amino acids, norleucine and lysine, and the sulfur containing amino acids, cysteine and methionine, did not support growth. Some of the amino acids, including aspartic acid and arginine, supported growth when used as a sole source of both carbon and nitrogen.

Herrick (1940) and Herrick and Alexopoulos (1942) found peptone to be a good source of nitrogen for Stereum gausapatum. When thiamine was added to the medium, asparagine and ammonium nitrogen were good sources of nitrogen. Nitrate nitrogen failed to enhance growth even in the presence of thiamine.

Dimond and Peltier (1945) using Penicillium notatum in liquid cultures studied the relation between pH of the medium and time of incubation as it is affected by varying the source of nitrogen or carbon. With nitrate nitrogen the pH dropped and then gradually rose regardless of the source of carbon. With amino acids the pH curve varied according to the source of carbon. They concluded that by supplying the proper nutrients the pH of the medium can be controlled during growth.

Nineteen species of Coprinus were in some degree heterotrophic for thiamine (Fries, 1955). Asparagine and ammonium nitrogen produced the best growth by most species. Some species gave the best yields on aspartic acid or urea. Potassium nitrate was a good source for a few species but in general was inferior to the other forms of nitrogen.

Steinberg (1937, 1939, 1942) reported on the nitrogen metabolism in Aspergillus niger. Molybdenum was important in nitrogen metabolism, probably playing some role in the reduction of nitrates. Nitrate nitrogen, ammonium nitrogen, urea and certain amino acids were good and equivalent sources of nitrogen. Alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, ornithine and proline were equivalent to inorganic nitrogen. Those amino acids with branched

chains or stable cyclic structures were less effective as nitrogen sources than simple amino acids. Increase in the length of the carbon chain was accompanied by a decrease in assimilability. Sulfur containing amino acids were poor nitrogen sources.

Leonian and Lilly (1940) reported some factors that influence the growth in liquid cultures of various thiamine requiring fungi. Various organic acids were found to increase the availability of less favorable nitrogen sources such as arginine and ammonium nitrate. Growth in some cases was increased one thousand percent. Small quantities of ammonium nitrate added to aspartic acid increased the growth over that induced by either of these sources alone.

A number of individual amino acids added to a basal medium containing ammonium tartrate stimulated the growth of Cenococcum graniforme (Melin and Mikola, 1948). Melin and Norkrans (1948) reported that a nitrogen source of eighteen amino acids supported good growth of Lactarius deliciosus.

A number of Tricholoma species assimilated ammonium nitrogen and organic nitrogen (Norkrans, 1950, 1953). Nitrate nitrogen was utilized by only one species. Glutamic acid and aspartic acid, their corresponding keto acids, and

also glutamine and asparagine stimulated growth when added to a basal medium already containing an ammonium nitrogen source.

Twenty-five species of fungi including fourteen Basidiomycetes were grown on three sources of nitrogen (Hacskaylo, Lilly and Barnett, 1954). Potassium nitrate was utilized slowly by the Basidiomycetes and very slowly if at all by the other fungi. Asparagine and ammonium sulfate supplemented with fumarate were about equal in supporting growth and better than ammonium sulfate or potassium nitrate.

Bach (1956) reported the agaric Pholiota aurea grew best on surface cultures without agitation. P. aurea utilized ammonium salts, especially ammonium tartrate, asparagine, glutamic acid, arginine, alanine, and urea. Better growth was on combinations like asparagine-glutamic acid or asparagine-yeast extract. Yeast extract, peptone, melon juice and casein hydrolysate were better nitrogen sources than the individual amino acids. The organism failed to utilize nitrates.

Fraser and Fujikawa (1958) found that the amino acids phenylalanine, methionine and proline when added to a basal medium already containing asparagine stimulated the growth of three strains of Agaricus bisporus. The presence of

thiamine was necessary for this growth response to occur. Optimal amounts of glucose, asparagine and inorganic salts for maximum growth of A. bisporus in liquid cultures were determined.

Two strains of the dry rot fungus Merulius lacrymans var. domesticus utilized glycine, alanine, glutamic acid, aspartic acid, asparagine, oximide, acetamide, urea, uric acid and allantoin (Lamprecht, 1958).

Ascobolus immersus could assimilate nitrogen in the form of potassium nitrate but optimum yields were obtained with asparagine, aspartic acid, glutamic acid or urea (Yu-Sun, 1964).

Kluyver and Perquin (1933) enunciated the principles involved in studying mold metabolism by the submerged culture methods. Foster (1949) discussed the merits of this method and the techniques for obtaining submerged growth.

Humfeld (1948) successfully used the submerged culture method to grow mycelium of the common cultivated mushroom, Agaricus campestris, in a small scale fermenter. Humfeld and Sugihara (1949, 1952) described a method for growing A. campestris on shake cultures. A synthetic medium for obtaining good growth was determined. Urea was the nitrogen source used.

Forty-two species of wood-rotting Basidiomycetes were grown in submerged cultures (Jennison, Newcomb and Henderson, 1955). Thirty-eight species were deficient in thiamine. Ammonium salts, urea, casein hydrolysate and some of the amino acids supported growth. The differences in amino acid utilization were related to molecular structure. The simple monoamino, monocarboxy amino acids of short chain length averaged significantly greater yields than those of longer chain length. Short chain amino acids with hydroxyl groups had similar yields to those simple ones of longer chain length. Sulfur containing amino acids and those with aromatic rings were poorly assimilated. The heterocylic forms varied from good to poor. Monoamino, dicarboxy acids were in general good sources of nitrogen.

Morton and Macmillan (1953) in their classic study reported on the assimilation of ammonium salts and nitrates by Scopulariopsis brevicaulis. The failure of ammonium sulfate to be completely assimilated was due to a drop in pH to an inhibitory level. Various organic acids added to the medium acted as buffers to prevent this pH drop and allowed complete utilization of ammonium sulfate. Under corresponding favorable conditions ammonium nitrogen was assimilated faster than nitrate nitrogen. When ammonium and nitrates were available in the medium simultaneously, as with

ammonium nitrate, the ammonium was preferably assimilated until it drops to a very low level and then the nitrate is assimilated. This was due to the ammonium blocking the reduction of nitrate to nitrite.

The best single nitrogen sources for Collybia radicata var. furfuracea grown on shake cultures were: yeast extract, dried skim milk, malt extract, DL-serine and L-asparagine (Stevens, 1957).

A low-temperature Basidiomycete utilized a number of amino acids and urea (Ward, 1964). Ammonium salts were well utilized if the pH was controlled by titration or by inclusion of organic acids in the medium for buffering. Nitrates were not utilized.

Casein hydrolysate, peptone and asparagine were excellent sources of nitrogen for the rust hyperparasite Darluca filum in submerged cultures (Nicolas and Villanueva, 1965). Glutamic acid, glycine, aspartic acid and alanine were also good sources.

The literature reveals little information concerning comparative nitrogen studies of the boletes. Melin (1922, 1923a, 1923b) succeeded in growing several species of Boletus in a glucose-agar medium containing ammonium chloride as a nitrogen source.

Boletus elegans was grown on agar medium by How (1940). Ammonium compounds were the best nitrogen sources. Potassium nitrate, asparagine and peptone were also utilized but growth was poor.

Modess (1941) grew a number of hymenomycetes and gasteromycetes in liquid cultures using asparagine and peptone as nitrogen sources. The effect of the hydrogen ion concentration on growth was studied extensively and the pH that supported optimum growth was determined. The optimum pH for growth of the Boletus species was about 5. The pH that resulted in the optimum growth of Boletus luteus (syn. Suillus luteus) was 5.5.

Reusser, Spencer and Sallans (1958) grew Boletus indecisus in submerged cultures on a medium containing molasses and waste sulfite liquor supplemented with ammonium compounds. Falanghe (1962) grew this same organism in a medium of vinasse with ammonium sulfate added. Falanghe, Smith and Rackis (1964) grew Boletus indecisus in submerged cultures on a medium of soybean whey supplemented with ammonium sulfate, ammonium tartrate and ammonium acetate.

Several reports on utilization of D-amino acids by fungi have appeared in the literature. Regnery (1944), Srb and Horowitz (1944) and Demain (1963) all reported that

mutant strains of Neurospora crassa could utilize certain D-amino acids.

Emerson, Purziss and Knight (1950) found a strain of Penicillium chrysogenum able to utilize D-methionine and D-alanine as sole sources of nitrogen. D-amino acid oxidase activity was found in cell free extracts.

Aspergillus flavus and A. parasiticus were able to assimilate several D-amino acids (Chibata, Tosa and Sano, 1964). Cell free extracts were shown to have D-amino acid oxidase activity.

Blumer and Shopfer (1940) reported that Ustilago scabiosae assimilated both L and D isomers of certain amino acids.

Ward (1964) found a low-temperature Basidiomycete able to utilize D-alanine as well as L-alanine.

Jennison and Perritt (1960) studied the utilization of optical isomers of amino acids by several wood-rotting Basidiomycetes. They found no utilization of D-amino acids when these were the sole source of nitrogen.

# MATERIALS AND METHODS

## Collection and Maintenance of Cultures

Two species of *Suillus* were employed in this study: *Suillus luteus* (Fr.) S.F. Gray collected in the Lansing area in 1962 and designated as B-62-03, and *Suillus acidus* (Peck) Singer collected at the University of Michigan Biological Station near Burt Lake, Pellston, Michigan in 1963 and designated as B-63-50. The taxonomic characteristics of these two organisms are given by Smith and Thiers (1964).

Original isolations were made from the basidiocarps. Bits of mycelium from inside the pileus were removed with a sterile needle and placed on agar slants. Cultures were maintained on bolete medium agar slants.

## Media Employed

Bolete medium for maintenance of cultures:

| | |
|---|---|
| Malt Extract | 5.0 g |
| Glucose | 5.0 g |
| $KH_2PO_4$ | 0.5 g |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g |

| | |
|---|---|
| $NH_4Cl$ | 0.5 g |
| $FeCl_2$ (1% solution) | 0.5 ml |
| Agar | 15.0 g |
| Distilled $H_2O$ | 1000.0 ml |

Medium A, a modified Czapek's-Dox medium, for growing the inoculum (Stevens, 1957):

| | |
|---|---|
| Glucose | 15.0 g |
| Sucrose | 15.0 g |
| Bacto Peptone | 5.0 g |
| Bacto Yeast | 5.0 g |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g |
| KCl | 0.5 g |
| $KH_2PO_4$ | 1.0 g |
| $FeSO_4 \cdot 7H_2O$ | 0.01 g |
| Agar (if solid medium required) | 15.0 g |
| Distilled $H_2O$ | 1000.0 ml |

The basal synthetic medium used in this study was the same as that used by Treschow (1944) and modified by Fraser and Fujikawa (1958). Fraser and Fujikawa found that a medium with 3 or 4 grams of L-asparagine and 30 or 40 grams of glucose gave optimum yields of mycelium, which were higher than the yields produced when the medium contained 1 gram of L-asparagine and 10 grams of glucose.

For this nitrogen study it was decided to use 40 grams of glucose and an amount of nitrogen equivalent to the amount found in 4 grams of L-asparagine. To determine how much growth could be attributed to carry over of nutrients with inoculum, a control was used leaving the nitrogen source out of the synthetic medium. This was designated as N-free medium. The ingredients of the basal synthetic medium were as follows:

| | |
|---|---|
| Glucose | 40.0 g |
| KCl | 0.2 g |
| $MgSO_4 \cdot 7H_2O$ | 0.2 g |
| $KH_2PO_4$ | 0.14 g |
| $Na_2HPO_4 \cdot 12H_2O$ | 1.9 g |
| $CaCl_2 \cdot 2H_2O$ | 0.27 g |
| $FeCl_3 \cdot 6H_2O$ | 0.017g |
| $H_3BO_3$ | 0.01 mg |
| $CuSO_4 \cdot 5H_2O$ | 0.1 mg |
| $MnSO_4 \cdot 4H_2O$ | 0.02 mg |
| $ZnSO_4 \cdot 7H_2O$ | 2.0 mg |
| $MoO_3$ | 0.02 mg |

The pH was adjusted to 5.5 by addition of 1N NaOH or 6N HCl. The pH was determined by a Coleman pH meter.

The inorganic mineral elements were added from stock solutions. These stock solutions were prepared at the following concentrations (the amount of stock solution to make 1 liter of medium is indicated in parenthesis): 2.0 g KCl in 100 ml distilled water (10 ml); 1.4 g $KH_2PO_4$ in 100 ml distilled water (10 ml); 2.7 g $CaCl_2 \cdot 2H_2O$ in 100 ml distilled water (10 ml); 9.5 g $Na_2HPO_4 \cdot 12H_2O$ in 100 ml distilled water (20 ml); 0.17 g $FeCl_3 \cdot 6H_2O$ in 100 ml distilled water (10 ml); 2.0 g $MgSO_4 \cdot 7H_2O$ in 100 ml distilled water (10 ml). The trace elements were combined in one solution: 0.0001 g $H_3BO_3$; 0.001 g $CuSO_4 \cdot 5H_2O$; 0.0002 g $MnSO_4 \cdot 4H_2O$; 0.02 g $ZnSO_4 \cdot 7H_2O$; and 0.0002 g $MoO_3$ in 100 ml distilled water. Ten ml of the trace element solution were used to make 1 liter of medium.

Sterilization and Cleaning

All flasks and pipettes were washed in a hydrochoric-nitric acid cleansing solution, rinsed with tap water and distilled water.

All media were sterilized at 121°C except urea. The appropriate amounts of a Seitz filtered urea solution were added to the sterile basal synthetic medium.

Mice and Tumor Maintenance

The mice were Female Swiss albinos between 18 and 22 grams in weight. The tumor was Crocker Mouse Sarcoma 180 which was maintained by weekly transfers to different mice.

Experimental Procedure

To prepare for the differential nitrogen tests, Petri dishes with medium A were inoculated at 4 points and incubated for 15-20 days at 25°C. The individual colonies of Suillus luteus reached a diameter of about 20-30 mm; those of Suillus acidus about 15-20 mm. Twelve colonies were cut out and transferred with a sterile needle to a sterilized Waring blendor, 60 ml of sterile distilled water added and the mixture blended for 30 seconds. With a sterile 5 or 10 ml pipette 5 ml of the blended mycelium were transferred to each 250 ml Erlenmeyer flask containing 50 ml of medium A. The mycelium was grown on a rotary shaker for 10-13 days. The mycelium from these flasks was transferred to the synthetic medium.

The synthetic medium was generally inoculated from medium A cultures that had been grown for 12 days. The mycelium in one flask was sufficient to inoculate a maximum of 10 flasks containing synthetic medium. The medium A was decanted from the mycelium. The mycelium was then washed

with 20 ml of sterile distilled water and the water decanted. This was repeated twice making a total of three rinsings to reduce the nutrients on the surface of the mycelium. Sixty ml of sterile distilled water were added to the washed mycelium, poured into a sterile Waring blendor, and blended for 25 seconds. Five ml of the homogenate were transferred with pipettes to the 250 ml Erlenmeyer flasks containing 50 ml of synthetic medium. All flasks were capped with 2 layers of sterilized aluminum foil. The flasks were rotated in a constant temperature room at 25°C.

For each nitrogen source mycelial dry weights from 3 flasks were averaged at 10 and 15 day intervals. An additional flask containing medium A was inoculated with mycelium from the same source as a growth control.

The amount of growth was determined by the dry weights of the mycelium. Sargent filter papers (# 500) were numbered and dried in the oven for 5 to 6 hours at 85°C. The weight of each paper was taken immediately upon removal from the oven and recorded. The contents of the experimental flasks were filtered through these papers using a Buchner funnel and vacuum pressure. The mycelium on the filter paper was washed several times with distilled water and dried at 85°C for 12 hours. The weight of the filter

paper and mycelium was determined immediately and recorded. The weight of the mycelium was determined by subtracting the weight of the filter paper.

Growth curve experiments were established for both organism when grown on medium A and the synthetic medium. Amino acids that supported good growth were used as nitrogen sources in the synthetic medium: L-glutamic acid for growing S. luteus and L-arginine for growing S. acidus. Intervals of 5, 10, 15, and 20 days were usually used for growth curve experiments utilizing medium A. Averages were taken at 10, 15, 20, and 25 days for growth curve experiments utilizing the synthetic medium. Shaker speed was 150 rpm except for one growth curve experiment on medium A which was run at 120 rpm.

Antitumor Activity Tests

Several crude extracts were tested for antitumor activity in the mice. The trials were divided into two different experiments. In the first experiment extracts from the synthetic medium were tested. These extracts were from cultures with nitrogen sources giving better mycelial growth. L-glutamic acid and DL-alanine were used for growing S. luteus. L-arginine was used for growing S. acidus. Extracts of S. luteus grown with L-glutamic acid were taken from 10,

15, and 20 day cultures. Extracts of S. luteus grown with DL-alanine and S. acidus grown with L-arginine were taken from 10 and 15 day cultures.

In the second experiment extracts were taken from cultures of S. luteus and S. acidus that had been grown in medium A for 10 and 15 day periods.

To prepare extracts for testing the mycelium in the medium was blended for 60 seconds in a Waring blendor. This mixture was filtered under vacuum with a Buchner funnel using Sargent filter paper (# 501). The filtrate was filtered through a sterilized Seitz filter under vacuum. The resulting filtrate was dispensed into sterile serum bottles which were capped with sterile rubber caps. Extracts from two flasks were sufficient for testing a single group of mice for one week. An 0.85% saline solution was used as a control.

Groups of 6 mice were used for the control and for each extract tested for antitumor activity. Tests were over a 9 day period. On Day 0 the mice were inoculated subcutaneously with the tumor in the axillary region of the mouse with a 16 gauge trocar. Tumors were cut with a sterile scalpel into uniform pieces approximately 1 mm cubed in a Petri dish containing a solution of 0.85% saline and 0.1% chloramphenicol.

The mice were weighed on Day 1 and Day 8. The mean weight per mouse was calculated.

Injections of the test material were made daily beginning on Day 1 and continuing through Day 7. One ml in a disposable syringe was injected per mouse intraperitoneally. On Day 8 the mice were killed and the tumors removed and weighed. Evaluation of the data was based on the protocol of the Cancer Chemotherapy Reports (1962).

# RESULTS

## Growth Curves on Medium A and Effect of Shaker Speed

The earlier experiments established growth curves of Suillus luteus and Suillus acidus when the two organisms were grown in medium A. The first growth curves were determined when the organisms were grown with a shaker speed of 120 rpm, and subsequently at a speed of 150 rpm. The results are shown in Table I, Fig. 1 and Fig. 2.

Suillus luteus and Suillus acidus both have the four general phases of growth (Cochrane, 1958; Mandels, 1965): 1) The lag phase with relatively little increase in the amount of mycelium; 2) The exponential or logarithmic phase where the mycelium is increasing at a uniform rate; 3) The stationary phase where no new mycelium is being formed or if it is being formed is balanced by autolysis; and 4) The autolytic phase characterized by the breakdown of carbohydrates and proteins.

Increasing the shaker speed reduced the lag phase, the time from inoculation to the beginning of the exponential growth. With the shaker speed at 120 rpm relatively little

growth has occurred for either organism at the end of the five day period. At 150 rpm at least three-fourths of the total growth had occurred in five days.

The results in Table I indicate that the stationary phase was reached more rapidly when the time of lag phase was reduced. With the shaker speed at 120 rpm, the highest recorded mycelial dry weights are at the end of the 15 day period. The highest recorded mycelial dry weights at the 150 rpm shaker speed occurred at the end of the 10 day period.

TABLE I

GROWTH OF SUILLUS LUTEUS AND SUILLUS ACIDUS IN MEDIUM A AT TWO SHAKER SPEEDS

| | Dry Weight of Mycelium in mg | | | | | |
|---|---|---|---|---|---|---|
| 120 rpm | 5 days | 10 days | 12 days | 15 days | 20 days | 25 days |
| Suillus luteus | 31 | 376 | - | 381 | 243 | 232 |
| Suillus acidus | 15 | 108 | - | 423 | 345 | 289 |
| 150 rpm | | | | | | |
| Suillus luteus | 287 | 351 | 315 | 226 | 216 | - |
| Suillus acidus | 300 | 380 | 359 | 346 | 326 | - |

Each figure is an average of four replicates

Figure 1.--Growth curve of Suillus luteus in medium A at two shaker speeds.

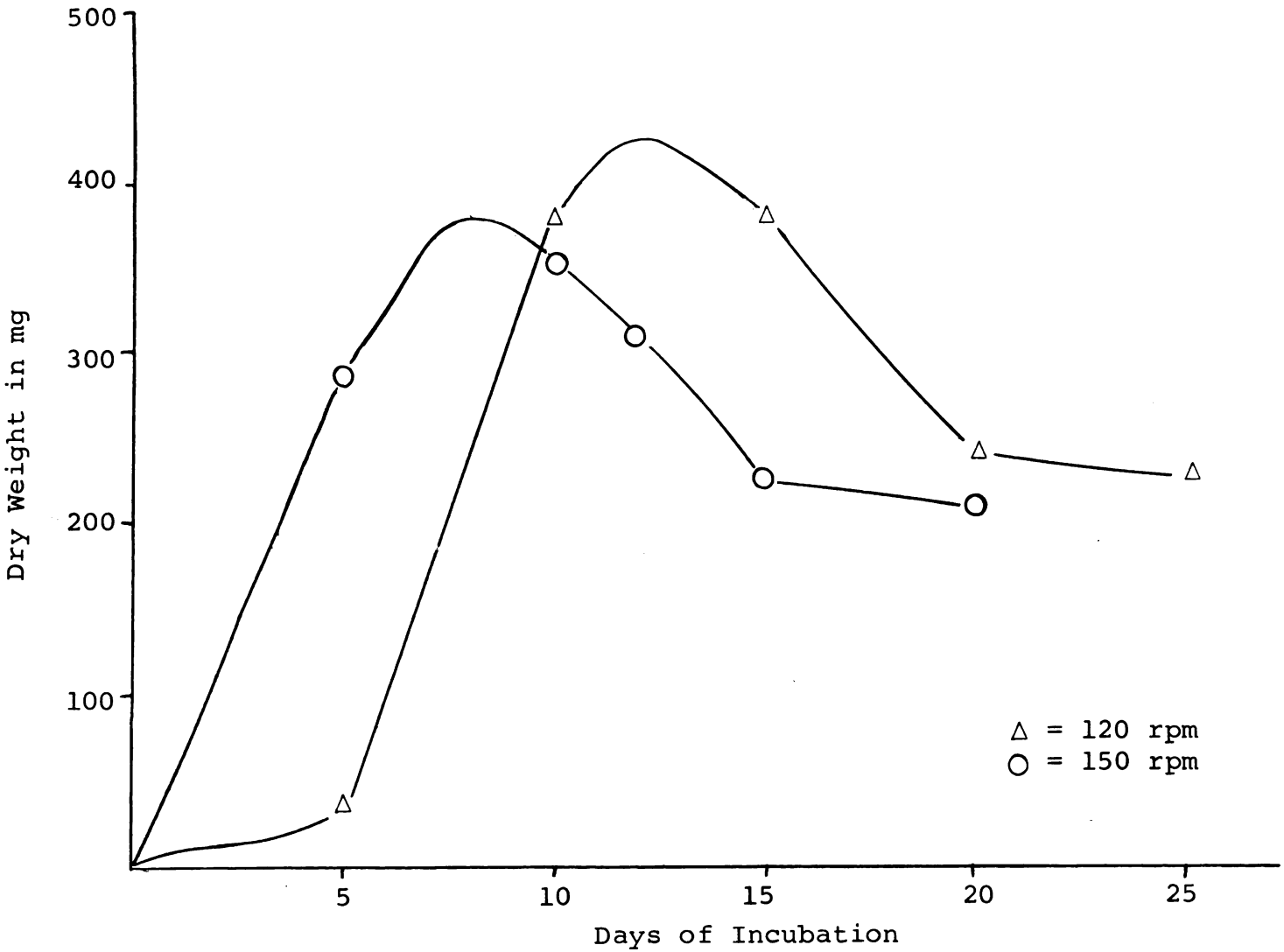
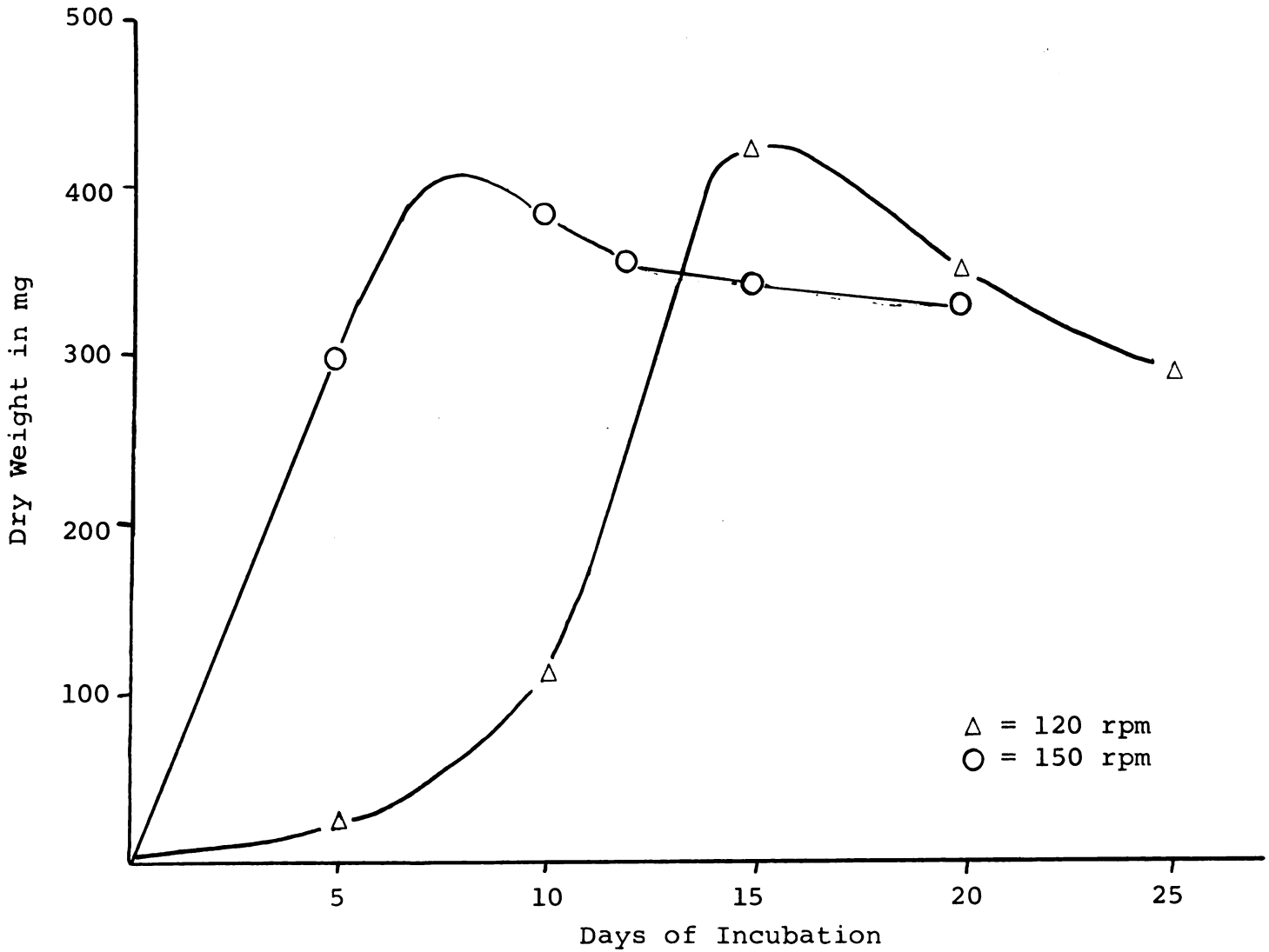


Figure 2.--Growth curve of Suillus acidus in medium A at two shaker speeds.



In the experiment with shaker speed at 150 rpm data was taken at the 12 day period. The mycelial dry weight was already lower than that recorded at the 10 day period. By the general appearance of the curves in Figures 1 and 2 the stationary phase probably occurred about 1 or 2 days before the 10 day period.

In the experiment with the shaker speed at 120 rpm the stationary phase for Suillus acidus probably occurred at or very near the 15 day period (Fig. 2). For Suillus luteus the situation was somewhat more complex. The dry weight recorded for the 10 day period is nearly the same as that recorded for the 15 day period. It is hard to conceive that the stationary phase of growth extended over this 5 day interval. It seems more likely that the value at the 10 day period represents a point in the exponential phase of growth and that the value at the 15 day period represents a point in the autolytic phase. The stationary phase probably occurred at around 13 days.

Nitrogen Requirements in Synthetic Medium

The results of the growth promoting effect of the various nitrogen sources on Suillus luteus and Suillus acidus in the synthetic media are given in Table II. Mycelial dry weights of at least three replicates were taken at 10 and 15

day periods. PH readings were also an average of not less than 3 replications and were taken at 10 and 15 day periods. The shaker speed was 150 rpm. Also included in Table II are the mycelial dry weights of the medium A flasks used to check the viability of the inoculum.

Accurate mycelial dry weights of S. luteus and S. acidus grown on cupric nitrate, L-tyrosine, L-cysteine and L-cystine could not be determined because of insoluble compounds in the medium at the time for taking dry weights. The growth of both organisms on these four nitrogen sources was poor.

The two nitrates listed in Table II, potassium nitrate and calcium nitrate, supported very little or no growth of S. luteus or S. acidus. Calcium nitrate shows no appreciable difference from the N-free medium for growth of either species. Potassium nitrate did not support growth of S. acidus, but supported some growth of S. luteus, since there was an increase of 24 mg over the dry weight of the N-free medium at the 15 day period.

The ammonium compounds used supported growth of both organisms. Growth of S. acidus was enhanced more than growth of S. luteus. Ammonium chloride supported better growth of both organisms than did ammonium nitrate. Ammonium nitrate

is considered a form of ammonium nitrogen because it has been shown that when both forms are present in the medium at the same time the ammonium form is preferably used (Morton and MacMillan, 1953; Foster, 1949).

Urea did not support growth in either species. The mycelial dry weights failed to reach the level of the N-free medium.

Certain of the amino acids supported the best growth of S. luteus and S. acidus. The growth of S. luteus will be considered first. Comparisons of the dry weights will be made at the 15 day period. Of the sixteen amino acids used, seven did not enhance any growth above the N-free medium. These were DL-methionine, DL-phenylalanine, L-leucine, glycine, DL-valine, L-histidine, and L-proline. The other nine resulted in growth of S. luteus. L-alanine was the best nitrogen source followed by DL-alanine and L-glutamic acid. The others listed in decreasing order of ability to support growth were L-aspartic acid, L-arginine, DL-serine, L-asparagine, L-lysine, and DL-isoleucine. DL-isoleucine was about equal to ammonium chloride in its ability to support growth.

TABLE II

GROWTH OF SUILLUS LUTEUS AND SUILLUS ACIDUS IN SYNTHETIC MEDIUM WITH VARIOUS NITROGEN SOURCES

| Nitrogen Source | Suillus luteus | | | | | Suillus acidus | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 days | | 15 days | | Medium A | 10 days | | 15 days | | Medium A |
| | wt* | pH* | wt* | pH* | wt** | wt* | pH* | wt* | pH* | wt** |
| N-free | 75 | 4.9 | 85 | 4.6 | 249 | 46 | 4.6 | 56 | 3.8 | 436 |
| $KNO_3$ | 76 | 5.6 | 109 | 5.9 | 254 | 28 | 5.4 | 31 | 6.1 | 322 |
| $Ca(NO_3)_2$ | 56 | 4.7 | 81 | 5.5 | 286 | 43 | 5.1 | 49 | 6.1 | 321 |
| $NH_4NO_3$ | 88 | 2.4 | 114 | 2.4 | 232 | 124 | 2.7 | 156 | 2.1 | 338 |
| $NH_4Cl$ | 113 | 2.5 | 163 | 2.2 | 394 | 165 | 2.3 | 234 | 2.0 | 350 |
| Urea | 45 | 8.1 | 50 | 8.3 | 297 | 50 | 7.9 | 37 | 8.5 | 413 |
| L-asparagine | 143 | 4.4 | 296 | 4.2 | - | 35 | 5.7 | 38 | 5.5 | 384 |
| DL-methionine | 46 | 3.8 | 57 | 3.9 | 228 | 16 | 5.8 | 15 | 6.0 | - |
| DL-phenylalanine | 27 | 4.8 | 32 | 4.3 | 223 | 19 | 5.5 | 16 | 5.5 | 351 |
| L-glutamic acid | 406 | 6.7 | 546 | 6.3 | 246 | 134 | 7.0 | 131 | 7.3 | 289 |
| DL-alpha alanine | 395 | 5.4 | 539 | 5.7 | 227 | 123 | 4.9 | 255 | 5.3 | 292 |
| L-alanine | 608 | | 741 | | 226 | | | | | |
| L-leucine | 47 | 3.9 | 45 | 5.0 | 244 | 23 | 5.3 | 22 | 5.4 | 282 |
| glycine | 39 | 4.8 | 80 | 4.6 | 215 | 12 | 5.4 | 13 | 6.3 | 314 |
| L-arginine | 173 | 5.1 | 345 | 4.2 | 366 | 315 | 7.0 | 402 | 7.6 | 313 |
| DL-valine | 57 | 4.2 | 72 | 4.2 | 257 | 42 | 5.0 | 64 | 5.5 | 315 |
| L-aspartic acid | 326 | 6.3 | 409 | 6.2 | 224 | 112 | 6.5 | 186 | 6.5 | 366 |
| L-histidine | 49 | 5.0 | 50 | 5.1 | - | 29 | 5.3 | 36 | 5.2 | 362 |
| L-lysine | 178 | 3.0 | 276 | 2.7 | 319 | 140 | 3.0 | 297 | 2.0 | 415 |
| DL-isoleucine | 138 | 3.3 | 176 | 3.2 | 236 | 18 | 4.9 | 23 | 4.5 | 398 |
| DL-serine | 104 | 5.2 | 304 | 4.4 | 258 | 21 | 5.6 | 19 | 5.4 | 373 |
| L-proline | 35 | 4.9 | 38 | 4.6 | 324 | 41 | 4.9 | 53 | 4.7 | 380 |

- Contaminated.

* Average of at least three replicates.

**Weight of one flask of medium A having same source of inoculum as that used in synthetic medium for a particular nitrogen source.

The number of amino acids supporting good growth of S. acidus was less than for S. luteus. Ten of the fifteen amino acids used that were not utilized were: L-asparagine, DL-methionine, DL-phenylalanine, L-leucine, glycine, DL-valine, L-histidine, DL-isoleucine, DL-serine, and L-proline. This list includes all those that were not good nitrogen sources for S. luteus. L-arginine was the best nitrogen source for S. acidus followed by L-lysine, DL-alanine, L-aspartic acid and L-glutamic acid. The growth in DL-alanine was roughly comparable to that in ammonium chloride.

The pH for the 10 and 15 day periods are recorded in Table II. The initial pH of 5.5 in the medium should not have retarded mycelial growth. PH at the 10 and 15 day periods in most cases stayed within the range favorable to growth for both species. The ammonium compounds showed a considerable drop in pH. Urea had a pH near 8 at the 10 and 15 day periods.

Utilization of Glutamic Acid Optical Isomers by Suillus luteus

The results of the experiment to test the ability of S. luteus to utilize the different optical isomers of glutamic acid as nitrogen sources are given in Table III. L-glutamic acid and DL-glutamic acid supported growth of S. luteus equally well. D-glutamic acid did not support the growth of S. luteus.

TABLE III

GROWTH OF SUILLUS LUTEUS IN SYNTHETIC MEDIUM WITH OPTICAL ISOMERS OF GLUTAMIC ACID

| Isomer | Dry Weight of Mycelium in mg | |
|---|---|---|
| | 10 days | 15 days |
| D-glutamic acid | 27 | 26 |
| DL-glutamic acid | 402 | 524 |
| L-glutamic acid | 392 | 523 |

Each figure is an average of at least 3 replicates.

Growth Curves on Synthetic Medium

The results of the growth curve experiments when Suillus luteus and Suillus acidus were grown on the synthetic medium are given in Table IV, and Fig. 3 and Fig. 4.

TABLE IV

GROWTH OF SUILLUS LUTEUS AND SUILLUS ACIDUS IN SYNTHETIC MEDIUM WITH SELECTED NITROGEN SOURCES

| | Dry Weight of Mycelium in mg | | | |
|---|---|---|---|---|
| | 10 days | 15 days | 20 days | 25 days |
| Suillus luteus with L-glutamic acid | 430 | 540 | 481 | 430 |
| Suillus acidus with L-arginine | 250 | 441 | 591 | 577 |

Each figure is an average of four flasks.

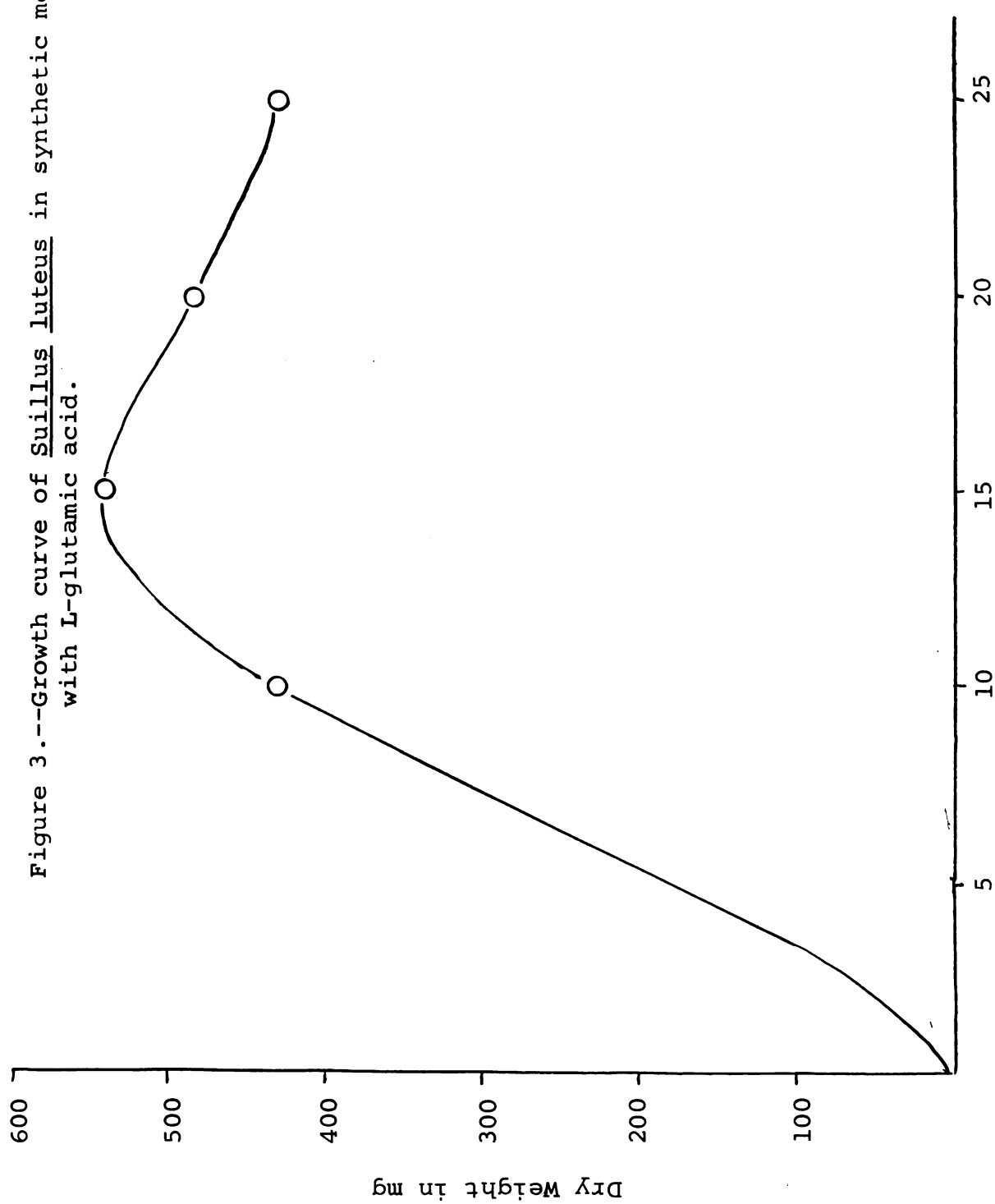
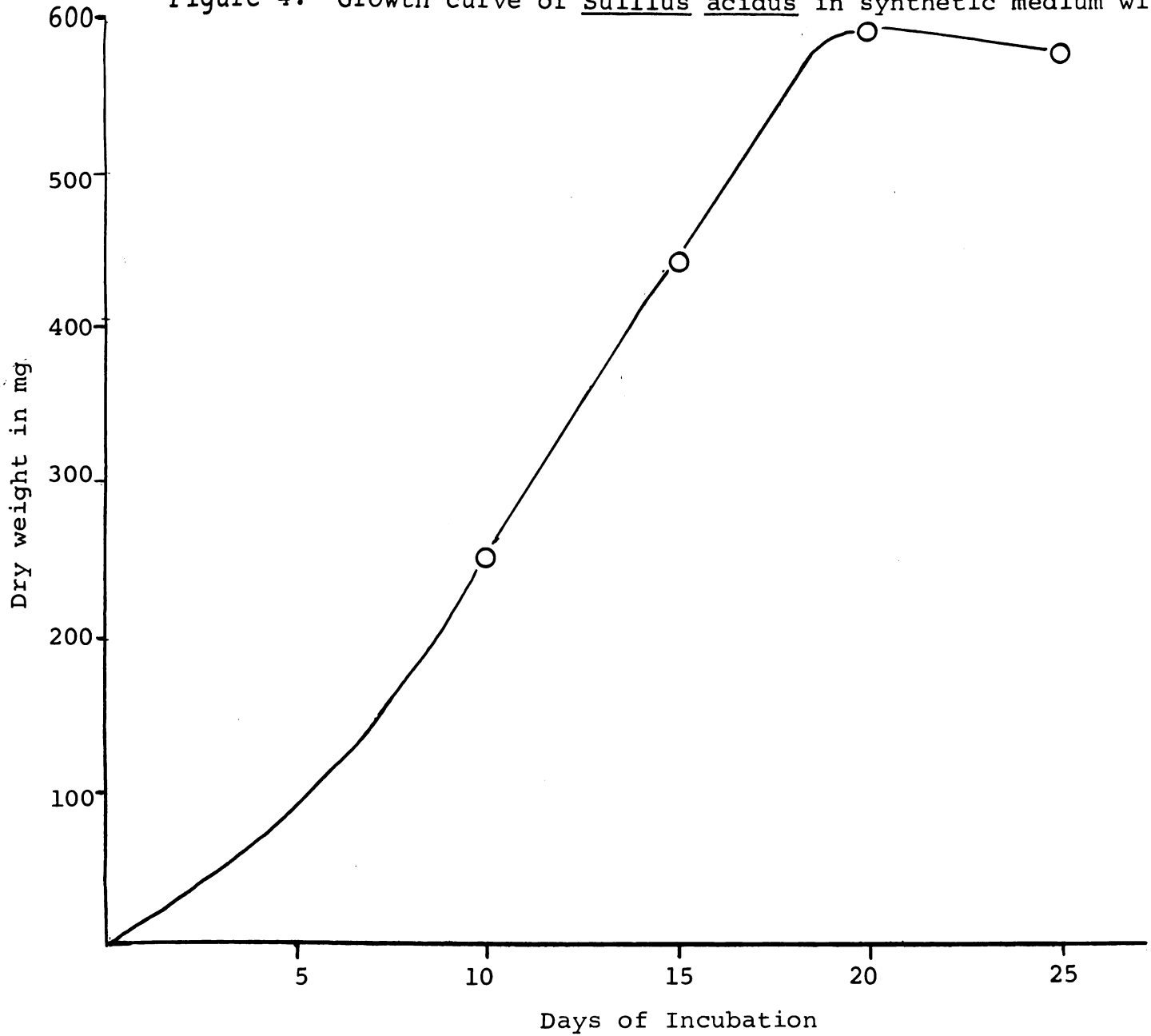



Figure 3.--Growth curve of Suillus luteus in synthetic medium with L-glutamic acid.

Figure 4.--Growth curve of Suillus acidus in synthetic medium with L-arginine



The greatest mycelial dry weight for Suillus luteus was recorded at the 15 day period on synthetic medium, while on medium A at the same shaker speed the peak weight was at the 10 day period, indicating that the stationary phase of growth was reached later on the synthetic medium than on medium A. The greatest dry weight recorded for the synthetic medium using L-glutamic acid as the nitrogen source was roughly 200 mg more than for medium A. (See Fig. 3)

The greatest dry weight recorded for S. acidus grown in synthetic medium with L-arginine occurred at 20 days as compared to 10 days when grown in medium A. The dry weight at 20 days on the synthetic medium exceeded the dry weight at 10 days on medium A by about 200 mg. The stationary phase of growth for S. acidus is reached later on the synthetic medium than on medium A. Growth in terms of total dry weight is better on the synthetic medium than on medium A. (See Fig. 4)

## Antitumor Activity of Suillus luteus and Suillus acidus

Several extracts of both Suillus luteus and Suillus acidus, grown on synthetic medium and medium A, were tested for antitumor activity in the mice. The results of these experiments are given in Table V.

TABLE V

ANTITUMOR ACTIVITY OF *SUILLUS LUTEUS* AND *SUILLUS ACIDUS*

| Organism and Nitrogen Source | Days of Incubation | Mean Tumor wt mg | T/C | Mean Animal Weight Gain gm |
|---|---|---|---|---|
| **Experiment #1: Synthetic media extracts** | | | | |
| *Suillus luteus* | | | | |
| L-glutamic acid | 10 | 128 | 59 | 5.0 |
| | 15 | 102 | 47 | 4.1 |
| | 20 | 103 | 47 | 3.8 |
| DL-alanine | 10 | 129 | 59 | 4.3 |
| | 15 | 117 | 54 | 5.2 |
| *Suillus acidus* | | | | |
| L-arginine | 10 | 138 | 63 | 4.8 |
| | 15 | 115 | 53 | 4.4 |
| Control* S.D. = 153 | | 218 | | 2.4 |
| **Experiment #2: Medium A extracts** | | | | |
| *Suillus luteus* | 10 | 317 | 94 | 2.4 |
| | 15 | 101 | 30 | 3.0 |
| *Suillus acidus* | 10 | 184 | 55 | 3.7 |
| | 15 | 336 | 100 | 1.8 |
| Control* S.D. = 208 | | 336 | | 1.8 |

*0.85% saline solution.

T/C = mean tumor weight of treated group/ mean tumor weight of control group.

S.D. = standard deviation of control group.

The best antitumor activity of the synthetic medium is given by Suillus luteus grown on L-glutamic acid. Extracts of the 15 and 20 day cultures have a T/C ratio of 47%. In all cases there was an increase of antitumor activity in 15 day cultures over 10 day cultures. Extracts of S. luteus grown on DL-alanine and S. acidus grown on L-arginine were about equal in tumor inhibition.

The mean animal weight gains showed that the extracts from the synthetic medium did not affect the growth of the test animals. All of the test group animals showed a greater mean weight gain than the control group. In three cases this gain was twice or more than the gain of the control group.

The data for the antitumor activity of the medium A extracts indicate greater production at a different phase. Extracts of S. luteus grown in medium A for 10 days showed only slight inhibition. At the 15 day period the T/C ratio was 30%, the best inhibition in this data. S. acidus shows a T/C ratio of 55% at the 10 day period but failed to show any inhibition at the 15 day period.

The mean weight gains of the mice tested with medium A extracts were all as good or better than the mean weight gain of the control group. There was no inhibitory effect on the growth of the mice by the extracts of medium A.

# DISCUSSION

It was reasonable to expect that good growth of Suillus luteus and Suillus acidus should occur in medium A. In addition to glucose, sucrose and certain inorganic elements necessary for growth, the medium contains peptone and yeast extract. Analysis of peptone and yeast extract is given in the appendix. Many of the naturally occurring amino acids are present which would provide suitable nitrogen sources. Several vitamins are present, including thiamine which was required by Suillus luteus (syn. Boletus luteus) and was required or enhanced the growth of four other species of Boletus (Melin and Nyman, 1940). A number of inorganic elements are present that may be required by the organisms for good growth.

When analyzing the results of any nutritional study the conditions under which the experiment was done must be considered. The nitrogen sources supporting the best growth under one set of conditions may vary under another set of conditions. Hagem (1910) demonstrated that the nitrogen requirements of a number of Mucors was not fixed but varied

according to the source of carbon. Christie (1958, 1959) found that the best nitrogen source for Phytophthora cactorum was dependent upon the initial pH and also the carbon source. In assigning a rank order to the nitrogen sources according to their ability to support growth it is recognized that these results may have shown some variation if the experiments had been carried out under other conditions.

It is notable that the synthetic medium was not supplemented with any growth factors or vitamins. It is well known that many Basidiomycetes require thiamine for growth (Jennison et al., 1955; Melin and Nyman, 1940; Yusef, 1953; Sedlmayr, 1960; Fries, N. 1950; Fries, L. 1955). As stated earlier Melin and Nyman (1940) reported specifically that Suillus luteus (syn. Boletus luteus) required thiamine for growth.

The amount of thiamine required to promote maximum growth is in the magnitude of 1 microgram per liter (Fries, 1965). Thiamine and any other growth factors required for growth could have been carried over in large enough quantities with the inoculum which was grown in medium A.

Nicholas (1965) states that "most groups of fungi utilize nitrate nitrogen although some do not, e.g., some members of the Saprolegniaceae, Blastocladiales, and some

higher basidiomycetes." There are numerous reports in the literature that demonstrate the inability of many Basidiomycetes to utilize nitrate nitrogen. The nitrate compounds in this study failed to support growth of the organisms studied with the possible exception of potassium nitrate which may have supported a little growth of Suillus luteus.

The ammonium compounds supported fair growth by each organism. These compounds showed a considerable drop in pH, a characteristic of ammonium salts of strong acids resulting from unequal utilization of the cation $NH_4^+$ (Cochrane, 1958). In many instances of nitrogen nutrition the decrease in pH was sufficient to reduce growth (Cochrane, 1958) and could have prevented larger yields of mycelium by S. luteus and S. acidus.

Ammonium chloride was a better nitrogen source for both species than was ammonium nitrate. The organisms were unable to utilize nitrate so only half the nitrogen in the ammonium nitrate was available. Both compounds had equivalent amounts of total nitrogen. Essentially ammonium chloride had twice the amount of available nitrogen as ammonium nitrate.

Urea, which is a utilizable nitrogen source for many fungi (Cochrane, 1958), failed to support any growth of

Suillus luteus or Suillus acidus. The high pH may have been inhibitory to growth. The pH values at the 10 and 15 day periods were near 8 or higher. Modess (1941) found that the Boletus species did not grow in a medium with an initial pH above 6.9. If the high pH developed early enough it could very well have inhibited the growth of the two organisms. It is not known what caused this rise in the pH, especially since very little growth occurred. Humfeld and Sugihara (1952) used urea in their nutrition study of Agaricus campestris because its utilization does not affect the pH of the medium enough to inhibit growth. Cochrane (1958) stated that the pH of a medium can rise either by the absorption of anions or by the production of ammonia. Sudden release of the nitrogen in the form of ammonia from the urea could have been responsible for the rise in pH. It may have been that the urea was not compatible with something in the medium that caused it to break down and release ammonia. Another explanation may involve the presence of urease which Cochrane (1958) says is present in many of the fleshy Basidiomycetes. According to Foster (1949) urease catalizes the following reaction:

$$NH_2CONH_2 \xrightarrow[H_2O]{\text{urease}} 2NH_3 + CO_2$$

It is possible that one of these phenomenon occurred to release ammonia in large enough quantities to raise the pH to a level that inhibited growth.

Steinberg (1942) working with Aspergillus niger and Jennison et al. (1955) working with some wood-rotting Basidiomycetes correlated the ability of these fungi to utilize amino acids with the molecular structure of the amino acids. No absolute set of rules can be set down since variability occurs with every fungal species studied. In general the ability of a particular fungus to utilize amino acids is related to the length of the carbon chain, branching, cyclic structures present and the functional groups present.

Sulfur containing amino acids are usually poor sources of nitrogen. Cysteine, cystine and methionine did not support growth of either S. luteus or S. acidus.

Amino acids having cyclic structures are also generally poor sources of nitrogen. The amino acids used in this study having cyclic structures were: phenylalanine, proline, tyrosine, and histidine. None of these supported growth of S. luteus or S. acidus.

Amino acids that support the best growth are usually found among the simple short chained monoamino monocarboxy forms, the monoamino dicarboxy forms and their amides, or

among the basic amino acids. Amino acids that supported the best growth of S. luteus and S. acidus were from these groups.

Alanine a simple, short chained, monoamino monocarboxylic acid was the best form of nitrogen for S. luteus. Both of the dicarboxylic acids, glutamic acid, and aspartic acid, supported very good growth of S. luteus. The amide of aspartic acid, asparagine, supported growth of S. luteus. The basic amino acids arginine and lysine were reasonably good nitrogen sources. Serine a short chained, monoamino monocarboxylic form with a hydroxyl group supported growth.

The basic amino acids, arginine and lysine, supported the best growth of S. acidus, with arginine being the best nitrogen source. Alanine supported good growth. Aspartic and glutamic acid also supported some growth of S. acidus.

Usually an increase in the length of the carbon chain is accompanied by a decrease in assimilability. Valine a simple monoamino monocarboxylic acid similar in structure to alanine but having an extra carbon did not support growth in either organism studied. Leucine an amino acid with a five carbon chain and a methyl group on the gamma carbon, likewise, did not support growth of either organism. Isoleucine, however, supported some growth of S. luteus.

Glycine the shortest chained monoamino monocarboxylic acid did not support growth of either organism.

The occurrence in nature of the L-forms of the amino acids is more common than the occurrence of the D-forms (Meister, 1965). Reports of utilization of the D-forms of the amino acids by fungi, especially by Basidiomycetes, are likewise considerably less than reports of utilization of the L-forms.

The ability of fungi to utilize D-isomers of amino acids is attributed by Cochrane (1958) and Nicholas (1965) to the presence of D-amino acid oxidase. D-amino acid oxidase deaminates amino acids by the following reaction:

$$R\text{-}CH(NH_2)COOH + 1/2\ O_2 \longrightarrow R\text{-}COCOOH + NH_3.$$

The ammonia released is then incorporated into L-amino acids. Although D-amino acid oxidase is a general term its action is specific and its presence does not confer the ability to utilize all D-amino acids. Blumer and Schopfer (1940) found Ustilago scabiosae able to utilize D-valine, D-isoleucine and D-alanine but not D-leucine or D-phenylalanine. Ward (1964) found a low-temperature Basidiomycete able to utilize D-alanine but not D-asparagine.

Suillus luteus failed to utilize D-glutamic acid and therefore did not have a D-amino acid oxidase specific for

the deamination of this amino acid.

There is some indication in the data that S. luteus was also unable to utilize the D-isomer of alanine. DL-alanine was used as a nitrogen source and gave excellent growth, comparable to that of L-glutamic acid. An equivalent amount of L-alanine increased the growth by about 200 mg, indicating that more nitrogen was available from the L-form than from the DL-form.

Both Suillus luteus and Suillus acidus produced antitumor substances when grown on the synthetic medium and on medium A. When grown on the synthetic medium there appeared to be a correlation between the production of antitumor substances and growth of mycelium. The growth peak for Suillus luteus when grown with L-glutamic acid occurred at or near the 15 day period. The highest antitumor activity occurred from extracts of cultures grown for 15 days. There was no decrease in antitumor activity in the cultures that were grown for 20 days, indicating that the antitumor substances had not broken down with the onset of autolysis of the mycelium. Suillus luteus grown on DL-alanine and Suillus acidus grown on L-arginine showed similar results, the antitumor activity increasing with growth of mycelium.

Suillus luteus had the best inhibition in medium A at the end of 15 days. Inhibition was 70% and correlated with the work of Beneke (unpublished data) who reported Suillus luteus to have inhibition of 72% when grown on medium A. Almost no inhibition occurred at the 10 day period. Suillus acidus extracts reduced the tumor size almost by one half at the end of the 10 day period but failed to show any inhibition at the end of 15 days.

Stevens (1957) working with Calvatia gigantea grown in medium A showed that variation in production of calvacin occurred with the age of the cultures. Young cultures did not produce the tumor inhibiting substances or sometimes produced substances that stimulated tumor growth. Once the antitumor substances were produced they could be lost with increasing age of the cultures. Majid (1965) found considerable variation in the amount of antitumor substances produced by Suillus luteus and acknowledged that this might have been due to the duration of the incubation period. It appears that when the organisms are grown on medium A there is a point in the growth cycle when optimum amounts of the antitumor substances occur. At points before and after this period of optimum yields, less or no antitumor activity occurs.

Stevens (1957) correlated the ability of Collybia radicata var. furfuracea to produce antitumor substances with the source of carbon. He found that although several carbon sources gave good yields of mycelium, glucose, mannose and galactose more consistently caused the organism to produce the tumor retarding principle. He found no relationship between the nitrogen source and the production of the tumor inhibiting principle. Cook (1962) and Majid (1965) also correlated antitumor activity with the carbon source utilized. Majid found utilization of glucose or sucrose to result in the largest production of antitumor activity by Suillus luteus. Glucose was the carbon source used in the synthetic medium of this study and was a favorable carbon source for the production of antitumor substances by both Suillus luteus and Suillus acidus.

The role of the nitrogen source in the production of the antitumor substance is not known. It certainly seems reasonable that with the organism involved in the production of the active substances, nitrogen sources supporting good growth are a prerequisite. The three nitrogen sources used in Experiment 1 on antitumor activity all supported good growth and all were favorable for the production of antitumor activity. Experiments on the exact chemical nature of the

antitumor substances may elucidate further the role of nutrients in its production by Suillus sp.

## SUMMARY

1. The nitrogen requirements of Suillus luteus and Suillus acidus were studied. Growth curves were established for the two organisms when grown on medium A and the synthetic medium containing amino acids that supported good growth.

2. Increasing the shaker speed resulted in the reduction of the lag phase of growth and an earlier stationary phase.

3. Nitrates were not utilized by either species except potassium nitrate which may have supported very limited growth of S. luteus.

4. The ammonium compounds supported a fair amount of growth by both species.

5. Some of the amino acids were the best sources of nitrogen. Amino acids that were utilized best by S. luteus were: L-alanine, DL-alanine, L-glutamic acid, and L-aspartic acid. L-arginine and L-lysine supported the best growth of S. acidus.

6. Although L- and DL-glutamic acid were excellent sources of nitrogen for S. luteus, D-glutamic acid was not utilized by this organism.

7. Antitumor substances were produced by both S. luteus and S. acidus when grown in medium A and the synthetic medium.

8. Extracts of S. luteus grown on medium A for 15 days gave the best tumor inhibition--70% retardation.

9. All extracts of S. luteus and S. acidus grown in synthetic medium showed antitumor activity. In all cases the best antitumor activity occurred from cultures grown for 15 days.

## LITERATURE CITED


Allison, F. E., S. R. Hoover and H. J. Morris. 1934. Nitrogen fixation studies with fungi and actinomyces. J. Agr. Res. 49:1115-1123.

Bach, E. 1956. The agaric Pholiota aurea: Physiology and ecology. Dansk Bot. Ark. 16(2):1-120.

Beneke, E. S. 1963. Calvatia, calvacin and cancer. Mycologia 55:257-270.

Blumer, S. and W. H. Schopfer. 1940. Beiträge zur Biologie und Wirkstoffphysiologie von Ustilago scabiosae (Sowerby) Winter. Ber. Schweiz. Bot. Ges. 50: 248-272.

Cancer Chemotherapy Reports. 1962. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. No. 25 Cancer Chemotherapy National Service Center. Bethesda 14, Maryland.

Chibata, I., T. Tosa and R. Sano. 1964. A new mould D-amino acid oxidase. Nature 204:684-685.

Christie, T. 1958. Nutritional studies of Phytophthora cactorum (Leb. and Cohn) Schroet. Part 1: Utilization of nitrogen and carbon compounds. New Zeal. J. Sci. 1:83-90.

Christie, T. 1959. Nutritional studies of Phytophthora cactorum (Leb. and Cohn) Schroet. Part 2: Influence of pH of a culture medium on utilization of some nitrogen and carbon compounds. New Zeal. J. Sci. 2:320-322.

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

Cook, W. L. 1962. Physiological studies of spore strains of Calvatia gigantea. M.S. Thesis, Michigan State University.

Czapek, F. 1902, 1903. Untersuchungen über die Stickstoffgewinnung und Eiweissbildung der Schimmelpilze. Beitr. Chem. Physiol. Pathol. 1:538-560, 2:557-590, 3:47-66.

Demain, A. L. 1963. On the utilization of D-alpha aminoadipic acid by lysineless mutants of ascomycetous fungi. Can. J. Microbiol. 9:111-115.

Dimond, A. E. and G. L. Peltier. 1945. Controlling the pH of cultures of Penicillium notatum through its carbon and nitrogen nutrition. Amer. J. Bot. 32:46-50.

Duggar, B. M. and A. R. Davis. 1916. Studies in the physiology of the fungi. I. Nitrogen fixation. Ann. Missouri Bot. Gard. 3:413-437.

Emerson, R. L., M. Puziss and S. G. Knight. 1950. The D-amino acid oxidase of molds. Arch. Biochem. 25:299-308.

Falanghe, H. 1962. Production of mushroom mycelium as a protein and fat source in submerged culture in medium of vinasse. Appl. Microbiol. 10:572-576.

Falanghe, H., A. K. Smith and J. J. Rackis. 1964. Production of fungal mycelial protein in submerged culture of soybean whey. Appl. Microbiol. 12:330-334.

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

Fraser, I. M. and B. S. Fujikawa. 1958. The growth-promoting effect of several amino acids on the common cultivated mushroom, Agaricus bisporus. Mycologia 50: 538-549.

Fries, L. 1955. Studies in the physiology of Coprinus. I. Growth substance, nitrogen and carbon requirements. Svensk Bot. Tidskr. 49:475-535.

Fries, N. 1950. Growth factor requirements of some higher fungi. Svensk Bot. Tidskr. 44:379-386.

Fries, N. 1965. The chemical environment for fungal growth 3. Vitamins and other organic growth factors. p 491 to p 523. In G. C. Ainsworth and A. S. Sussman. The Fungi. I. The fungal cell. Academic Press, Inc., New York.

Gottlieb, D. 1946. The utilization of amino acids as a source of carbon by fungi. Arch. Biochem. 9:341-351.

Gregory, F. J., E. M. Healy, H. P. K. Agersborg and G. H. Warren. 1966. Studies on antitumor substances produced by Basidiomycetes. Mycologia 58:80-90.

Hacskaylo, J., V. G. Lilly and H. L. Barnett. 1954. Growth of fungi on three sources of nitrogen. Mycologia 46: 691-701.

Hagem, O. 1910. Untersuchungen über norwegische Mucorineen. Vidensk. Selsk. Skrift. I. Math-Naturw. Klasse 4:1-152. Ann. Myc. 8:265-286.

Herrick, J. A. 1940. The carbon and nitrogen metabolism of Stereum gausapatum Fries. Ohio J. Sci. 40(3):123-129. (Abstr.).

Herrick, J. A. and C. J. Alexopoulos. 1942. A further note on the nitrogen metabolism of Stereum gausapatum Fries. Ohio J. Sci. 42(3):109-111. (Abstr.).

How, J. E. 1940. The mycorrhizal relations of larch. I. A study of Boletus elegans in pure culture. Ann. Bot. N. S. 4:135-150.

Humfeld, H. 1948. The production of mushroom mycelium ( Agaricus campestris) in submerged culture. Science 107:373.

Humfeld, H. and T. F. Sugihara. 1949. Mushroom mycelium production by submerged propagation. Food Technol. 3:355-356.

Humfeld, H. and T. F. Sugihara. 1952. The nutrient requirements of Agaricus campestris grown in submerged culture. Mycologia 44:605-620.

Jennison, M. W., M. D. Newcomb and H. Henderson. 1955. Physiology of the wood-rotting Basidiomycetes. I. Growth and nutrition in submerged culture in synthetic media. Mycologia 47:275-304.

Jennison, M. W. and A. M. Perritt. 1960. Physiology of wood-rotting Basidiomycetes. III. Studies on the utilization of optical isomers of amino acids. Mycologia 52:628-635.

Klotz, L. J. 1923. Studies in the physiology of the fungi. XVI. Some aspects of nitrogen metabolism in fungi. Ann. Missouri Bot. Gard. 10:299-368.

Kluyver, A. J. and L. H. C. Perquin. 1933. Zur Methodik der Schimmelstoffwechseluntersuchung. Biochem. Z. 266:68-81.

Lamprecht, L. 1958. Beiträge zur Physiologie des Echten Hausschwamms. Über die Verwertung organisch gebundenen Stickstoffs und Kohlenstoffs. Arch. Mikrobiol. 30:190-210.

Leonian, L. H. and V. G. Lilly. 1940. Studies on the nutrition of fungi. IV. Factors influencing the growth of some thiamine-requiring fungi. Amer. J. Bot. 27: 18-26.

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

Lindeberg, G. 1944. Über die Physiologie Ligninabbauender Boden Hymenomyzeten. Symbolae Bot. Upsaliensis 8 (2):1-183.

Lucas, E. H. 1960. Folklore and plant drugs. Mich. Acad. Sci., Arts, and Letters. 45:127-136.

Lucas, E. H., R. U. Byerrum, D. A. Clarke, H. C. Reilly, J. A. Stevens and C. C. Stock. 1958-59. Production of oncostatic principles in vivo and in vitro by

species of the genus Calvatia. Antibiotics Annual. p 493-496. (Medical Encyclopedia, Inc., New York).

Lucas, E. H., R. L. Ringler, R. U. Byerrum, J. A. Stevens, D. A. Clarke and C. C. Stock. 1957. Tumor inhibitors in Boletus edulis and other Holobasidiomycetes. Antibio. and Chemo. 7:1-4.

Majid, F. Z. 1965. Carbohydrate utilization and its relation to the production of antitumor substance in Suillus luteus. Ph.D. Thesis, Michigan State University.

Mandels, G. R. 1965. Kinetics of fungal growth. p 599 to 612. In G. C. Ainsworth and A. S. Sussman. The Fungi. I. The fungal cell. Academic Press, Inc., New York.

Meister, A. 1965. Biochemistry of the Amino Acids I. 2nd ed. Academic Press, Inc., New York.

Melin, E. 1922. Untersuchungen über die Larix-Mykorrhiza. I. Synthese der Mykorrhiza in Reinkulture. Svensk Bot. Tidskr. 16:161-196.

Melin, E. 1923a. Experimentelle Untersuchungen über die Birken- und Espenmykorrhizen und ihre Pilzsymbioten. Svensk Bot. Tidskr. 17:479-520.

Melin, E. 1923b. Experimentelle Untersuchungen über die Konstitution und Ökologie der Mykorrhizen von Pinus silvestris L. und Picea abies (L.) Karst. Mykol. Untersuch. und Ber. von R. Falck. 2:73-331.

Melin, E. and P. Mikola. 1948. Effect of some amino acids on the growth of Cenococcum graniforme. Physiol. Plantarum 1:109-112.

Melin, E. and B. Norkrans. 1948. Amino acids and the growth of Lactarius deliciosis (L.) Fr. Physiol. Plantarum 1:176-184.

Melin, E. and B. Nyman. 1940. Weitere Untersuchungen über die Wirkung von Aneurin und Biotin auf das Wachstum von Wurzelpilzen. Arch. Mikrobiol. 11:318-328.

Modess, O. 1941. Zur Kenntnis der Mykorrhizabildner von Kiefer und Fichte. Symbolae Bot. Upsaliensis 5(1): 1-146.

Morton, A. G. and A. MacMillan. 1953. The assimilation of nitrogen from ammonium salts and nitrate by fungi. J. Exp. Bot. 5:232-252.

Nicholas, D. J. D. 1965. Utilization of inorganic nitrogen compounds and amino acids by fungi. p 349 to 376 In G. C. Ainsworth and A. S. Sussman. The Fungi. I. The fungal cell. Academic Press, Inc., New York.

Nicolas, G. and J. R. Villanueva. 1965. Physiological studies on the rust hyperparasite Darluca filum. I. Carbon and nitrogen nutrition. Mycologia 57: 782-788.

Norkrans, B. 1950. Studies in growth and cellulolytic enzymes of Tricholoma. Symbolae Bot. Upsaliensis 11 (1):1-126.

Norkrans, B. 1953. The effect of glutamic acid, aspartic acid, and related compounds on the growth of certain Tricholoma species. Physiol. Plantarum 6:584-593.

Regnery, D. C. 1944. A leucineless mutant strain of Neurospora crassa. J. Biol. Chem. 154:151-160.

Reusser, F., J. F. T. Spencer and H. R. Sallans. 1958. Protein and fat content of some mushrooms grown in submerged culture. Appl. Microbiol. 6:1-4.

Sedlmayr, M. 1960. Physiological studies on Calvatia species. Ph.D. Thesis, Michigan State University.

Smith, A. H. and H. D. Thiers. 1964. A contribution toward a monograph of North American species of Suillus. Ann Arbor, Mich.

Srb, A. M. and N. H. Horowitz. 1944. The orinthine cycle in Neurospora and its genetic control. J. Biol. Chem. 154:129-139.

Steinberg, R. A. 1937. Role of molybdenum in the utilization of ammonium and nitrate nitrogen by Aspergillus niger. J. Agr. Res. 55:891-902.

Steinberg, R. A. 1939a. Effects of nitrogen compounds and trace elements on growth of Aspergillus niger. J. Agr. Res. 59:731-748.

Steinberg, R. A. 1939b. Growth of fungi in synthetic nutrient solutions. Bot. Rev. 5:327-350.

Steinberg, R. A. 1942. Effect of trace elements on growth of Aspergillus niger with amino acids. J. Agr. Res. 64:455-475.

Steinberg, R. A. 1950. Growth of fungi in synthetic nutrient solutions. Bot. Rev. 16:208-227.

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

Treschow, C. 1944. Nutrition of the cultivated mushroom. Dansk Bot. Ark. 11(6):1-180.

Ward, E. W. B. 1964. The utilization of nitrogen compounds, especially ammonia, by a low-temperature Basidiomycete. Can. J. Bot. 42:1071-1086.

Yusef, H. M. 1953. The requirements of some Hymenomycetes for essential metabolites. Bull. Torrey Bot. Club 80:43-64.

Yu-Sun, C. C. C. 1964. Nutritional studies of Ascobolus immersus. Amer. J. Bot. 51:231-237.

# 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 | 88.00 |
| Copper | 19.00 |

| | PER CENT |
|---|---|
| Phosphorus | 0.89 |
| Iron | 0.028 |

Typical analysis of Bacto-Yeast Extract--continued

| PER CENT | |
|---|---|
| $SiO_2$ | 0.052 |
| Potassium | 0.042 |
| Sodium | 0.32 |
| Magnesium | 0.03 |
| Calcium | 0.0406 |
| Arginine | 0.78 |
| Aspartic acid | 5.1 |
| Glutamic acid | 6.5 |
| 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 | 0.88 |
| Tyrosine | 0.60 |
| Valine | 3.4 |

Typical Analysis of Bacto-Yeast Extract--continued

| MICROGRAMS PER GRAM | |
|---|---|
| Pyridoxine | 20.00 |
| Biotin | 1.40 |
| Thiamine | 3.20 |
| Nicotinic acid | 279.00 |
| Riboflavine | 19.00 |
| Folic acid | 0.30 |

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

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

Typical analysis of Bacto-Peptone--continued

| | |
|---|---|
| Manganese | Nil |
| Iron | 0.0033% |
| Ash | 3.53% |
| Lead | 15.00 ppm |
| Arsenic | 0.09 ppm |
| Zinc | 18.00 ppm |
| Copper | 17.00 ppm |
| $SiO_2$ | 0.042% |
| Arginine | 8.00% |
| Aspartic acid | 5.90% |
| Glutamic acid | 11.00% |
| Glycine | 23.00% |
| Histidine | 0.96% |
| Isoleucine | 2.00% |
| Leucine | 3.50% |
| Lysine | 4.30% |
| Methionine | 0.83% |
| Phenylalanine | 2.30% |
| Threonine | 1.60% |
| Valine | 3.20% |
| Pyridoxine | 2.50 gamma/gm |
| Biotin | 0.32 gamma/gm |

| | |
|---|---|
| Thiamine | 0.50 gamma/gm |
| Nicotinic acid | 35.00 gamma/gm |
| Riboflavine | 4.00 gamma/gm |

Formulas of the Amino Acids Employed (From Lilly and Barnett, 1951)

Monoamino dicarboxylic acids:

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

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

Amides of monoamino dicarboxylic acids:

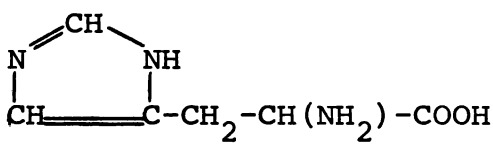
Asparagine: $NH_2OC-CH_2-CH(NH_2)-COOH$

Basic amino acids:

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

Lysine: $NH_2-CH_2-CH_2-CH_2-CH_2-CH(NH_2)-COOH$

Histidine:



Monoamino monocarboxylic acids:

Glycine: $CH_2(NH_2)-COOH$

Alanine: $CH_3-CH(NH_2)-COOH$

Valine: $(CH_3)_2-CH-CH(NH_2)-COOH$

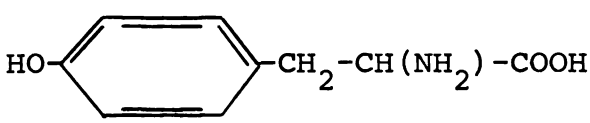
Leucine: $(CH_3)_2-CH-CH_2-CH(NH_2)-COOH$

Isoleucine: $CH_3-CH_2-CH(CH_3)-CH(NH_2)-COOH$

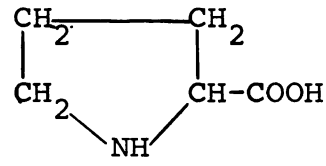
Phenylalanine: $C_6H_5-CH_2-CH(NH_2)-COOH$

Serine: $CH_2(OH)-CH(NH_2)-COOH$

Tryosine:



Formulas of the Amino Acids Employed--continued

Proline: 

Sulfur-containing amino acids:

Cysteine: $CH_2(SH)-CH(NH_2)-COOH$

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

Methionine: $CH_2(SCH_3)CH_2-CH(NH_2)-COOH$