# THE NITROGEN METABOLISM OF SELECTED *STREPTOMYCES* SPECIES

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# THE NITROGEN METABOLISM OF SELECTED *STREPTOMYCES* SPECIES

## INTRODUCTION

The genus *Streptomyces* is regarded as a rather unique microbial group. Some investigators look upon species of this genus as transition organisms forming a link between the filamentous fungi and the true bacteria.

A considerable volume of research has been carried out on the morphology of members of the *Streptomyces*. However, the general physiology and more particularly the characteristic chemical activities of these cellular forms has received little attention. Within recent years the latter genus has assumed a position of major importance in view of the production of several antibiotics by certain species of this genus. It has become apparent that more comprehensive metabolic studies are needed.

The present research program was outlined as a part of a general project on the carbon and nitrogen metabolism of representative *Streptomyces* species. This report will deal with a study of methods and results obtained while investigating some aspects of the nitrogen requirements of several *Streptomyces* species.

# HISTORICAL

The nitrogen requirements of Streptomyces cultures have received added attention since the discovery of streptomycin and other useful antibiotics by members of this microbial group. In searching the pertinent literature on nitrogen metabolism, emphasis has been placed on studies dealing with the utilization of inorganic nitrogen, proteins and amino acids.

At an early date Waksman (23, p.9) observed that inorganic nitrogen sources such as ammonium sulfate and ammonium carbonate would not support adequate growth of selected Streptomyces species. This poor growth response was attributed to the acidic conditions of the medium arising from the residual sulfate radical formed after the utilization of the ammonium ion. However, the inclusion of calcium carbonate in the growth medium effectively neutralized the residual sulfate and/or carbonate ion. Then moderate growth was noted.

Some workers have observed that the nitrate ion often provides a better source of nitrogen than the aforementioned ammonium salts (19, p.274; 23, p.11). However, in a recent study, Cochrane (3, p.179) noted that Streptomyces griseus utilized nitrates only if the mycelium was preformed or if growth was stimulated by

yeast extract. The stimulatory effect of yeast extract suggested to Cochrane that a specific growth factor or hydrogen donor might be lacking in the nitrate medium. Dulaney (9, p.309), while studying the yield of streptomycin, also noted the inability of S. griseus to utilize nitrates. However, an ammonium chloride and ammonium sulfate medium served as an adequate source of nitrogen as long as calcium carbonate was present.

Most of the Streptomyces species are able to reduce nitrates to nitrites. However, nitrites can only be utilized at low nontoxic concentrations. Cochrane (6, p.17) studied the inhibitory effects of nitrites with Streptomyces coelicolor. Apparently the toxic nature of nitrites was increased by the organic acids produced in the glucose medium. He suggested that nitrites might possibly block the action of the carboxylase enzymes. On the other hand, when glycerol was used as a carbon source no acid was detected and apparently nitrites were not toxic to growth of the culture. Waksman (24, p.83), however, observed acid production from glycerol by S. coelicolor and by many other Streptomyces species.

Many investigators have found that optimum growth of Streptomyces species occurs in media containing some form of organic nitrogen (2, p.254; 5, p.218; 2, p.11). Proteins, peptide fractions and amino acids have been

tested. Apparently a large number of these organisms are proteolytic in nature (16, p.699; 22, pp.506-522). Waksman (23, p.15) does not consider the amino acids produced during the hydrolysis of protein as waste products but instead as essential metabolites. Apparently those amino acids do not accumulate in the medium but are either deaminated to ammonia or are directly assimilated into cellular substance.

In a related study Waksman and Curtis (25, p.131) stated that considerable variation occurs among the actinomycetes in respect to extent and rate of gelatin liquifaction. In further experiments these workers (24, p.83) noted increases in amino-nitrogen with casein, peptone and asparagine. Ammonia could not be detected except on prolonged incubation.

Apparently a synthetic medium supplemented with amino-nitrogen will support good growth of some Streptomyces cultures. Dulaney (9, p.309) added various amino acids to a synthetic ammonium hydrogen phosphate medium. Good growth of S. griseus was observed with DL $\alpha$ alanine, $\beta$-alanine, glycine, L arginine HCL, L histidine HCL, and L proline when added to the medium singly or in various combinations. Woodruff and Rugers (31, p.317) noted that the growth of S. griseus could be stimulated by proper concentrations of ammonium hydrogen phosphate and

proline. He concluded that the ammonium hydrogen phosphate supplied nitrogen for an unknown synthesis while proline supplied the major portion of the nitrogen needs of the organism.

Eiser and McFarlane (10, pp.166-167) observed that valine and arginine apparently had little effect on the formation of mycelium of S. griseus. Histidine, on the other hand, appeared to have a direct influence on mycelium production. Recently Okami (19, p.265) tested the growth of 37 Streptomyces strains on media containing 17 nitrogen sources. All the strains were able to utilize proline, glycine and alanine. Most of the test cultures also utilized asparagine, arginine, and glutamic acid.

O'Brien, Wagman, and Perlman[1] have described a simple glycine containing medium in which streptomycin yield by Streptomyces griseus equals the yield produced on any other available medium. The omission or replacement of glycine from the medium resulted in poor growth and low streptomycin titer. Isotopic tracer work by Numerof, et al. (18, p.1344) made evident that the function of glycine in the production of high streptomycin titer is concerned with some phase of cellular metabolism and that glycine cannot be considered a precursor

1 O'Brien, E., G. H. Wagman, D. Perlman. Unpublished information, Squibbs, 1954.

of streptomycin.

The observed growth of Streptomyces cultures in the presence of a single amino acid indicates that these organisms do possess mechanisms which enable them to synthesize many of the other essential amino acids. Carbon skeletons of amino acids are apparently synthesized by yeast cells from degradation products of simple carbohydrates. Evidence seems to point to the fact that a glycolytic scheme and a Krebs cycle are present. By isotopic tracer studies the latter pathways have been shown to play important roles in the biosynthesis of amino acids (12, pp.689-698; 27, pp.645-653; 27, pp.663-667; 29, pp.683-688). Alanine, glutamic acid and aspartic acid are believed to be transamination products of the corresponding keto acids occurring in Krebs cycle. The biosynthesis of the other amino acids have also been postulated to be synthesized by side reactions along the TCA cycle (30, pp.462-463; 20, p.191).

However, microorganisms do vary in their ability to synthesize amino acids. Many groups such as the lactic acid bacteria require preformed amino acids. Gale and Stokes (11, p.485) commented on the synthetic abilities of Staphylococcus aureus. Maximum growth was obtained in 10-12 hours if 19 different amino acids were present in the synthetic medium used. The omission of

arginine, lysine, methionine, and hydroxy proline, respectively, had no effect on growth of S. aureus. However, when either histidine, aspartic acid, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophane, or threonine was omitted from the medium, no growth was observed at 128 hours. Growth was delayed in the absence of alanine, glycine, proline, glutamic acid, or serine. The delay in growth may have been due to selection of mutants which are able to synthesize the missing amino acids in question.

Scevala and Valcurone (21, p.624) apparently believe that a Meyerhof glycolytic pattern is active in the cells of Streptomyces cultures. Cochrane and Peck (7, pp.42-43) found that intact cells of S. coelicolor were able to oxidize many of the compounds of the TCA cycle. Cell free preparations were able to oxidize glucose plus ATP, citrate, $\alpha$-ketoglutarate, fumarate, succinate, malate, and decarboxylate oxalacetate. In later studies using S. coelicolor, Cochrane (4, pp.468-469) also noted that succinic acid accumulated from glucose or polyhydric alcohols. Recently Cochrane, Peck, and Harrison (8, pp.22-23) postulated that a typical Meyerhof glycolytic system was absent. Evidence was obtained which apparently indicated that the initial

metabolism of glucose was via the hexose monophosphate shunt reaction. By the use of labeled glucose 1 $C^{14}$ and glucose 3 and 4 $C^{14}$, Cochrane, et al. noted that $C^{14}O_2$ was liberated more rapidly from the former than the latter. Conclusions, however, could not be drawn with precision since the endogenous rate of respiration was high and some of the liberated $CO_2$ was assumed to have come from the complete oxidation of some of the glucose via the TCA cycle.

In studies on a mutant strain of Streptomyces venezuelae, Matsuoka, Yagishita, and Umezawa (15, p.169) reported on the isolation of pyruvic, citric, $\alpha$-ketoglutaric, succinic, malic, and acetic acids from the medium which suggested the utilization of carbohydrates through Krebs cycle. The presence of glutamic acid, they believed, linked carbohydrate and nitrogen metabolism.

# EXPERIMENTAL METHODS

Preliminary studies provided evidence indicating that more than one approach should be used in studying the nitrogen metabolism of the test organisms. Therefore, three approaches were investigated. These included pour plate, shake flask and turbidimetric studies. Each method supplied data relative to growth response in a defined medium.

## Treatment of Stock Cultures

### Cultures

Seven *Streptomyces* cultures (A.T.C.C.) were used in the present investigation. Stocks were maintained in sterile soil and successive transfers on laboratory media were limited to insure physiological constancy of the test species.

### Media

The medium used for the cultivation of spores contained 0.5 per cent glucose, 0.5 per cent peptone, 0.3 per cent beef extract, a trace of calcium chloride and yeast extract, 1.5 per cent agar, and water. The *Streptomyces* species which would not sporulate on the glucose nutrient agar were grown on a glycerol asparagine

medium. This medium contained 1.0 per cent glycerol, 0.1 per cent asparagine, 0.3 per cent dibasic potassium phosphate, 0.25 per cent sodium chloride, a trace of ferric chloride and 1.5 per cent agar and water. These media were dispensed in 50 ml aliquots into 200 ml prescription bottles and slanted. After the pH (6.8 to 7.0) was standardized, all media were autoclaved at 15 lbs. pressure for 20 minutes.

Treatment of Spores

The bottle slants were inoculated with spores of the test species and incubated at 28°C. Maximum sporulation was obtained in 4 days. At the end of the incubation period, the spores were carefully teased off the slants with a sterile wire loop into 25 ml of sterile distilled water. The spore suspensions were then transferred to sterile bottles and kept in the refrigerator at 4°C. Spores for the turbidimetric study received special attention. The bottle slants were streaked with spores from sterile soil. After maximum sporulation, the spores were harvested into approximately 10 ml of sterile 0.85 per cent saline. The resulting spore suspension was then filtered through 30 layers of 50 mesh gauze held in a Seitz filter to remove all spore clumps and soil. Finally the spores were centrifuged and

washed three times in physiological saline. A fresh spore suspension was prepared for each experiment to eliminate the possibility of any variation in the culture caused by prolonged storage.

## Pour Plate Studies

### Media

The basal medium contained the following salts: 0.1 per cent dibasic potassium phosphate, 0.25 per cent magnesium sulfate, 0.05 per cent sodium chloride, a trace of ferrous sulfate and distilled water. Washed agar prepared according to the method of Ayers, et al. (1, p.571) was added at a concentration of 2.0 per cent. Then the medium was divided equally into two portions and autoclaved at 15 lbs. pressure for 20 minutes.

Stock solutions of glucose (10 per cent) and various amino acids (5.0 per cent) were sterilized by filtration and stored in the refrigerator at 4°C. Glucose was added aseptically to one lot of the basal medium at a concentration of 1.0 per cent. The other half of the basal medium received no glucose. Then the following amino acids were added singly to both media at a concentration of 0.1 per cent: arginine, serine tryptophane, valine, glutamine, aspartic acid, cysteine,

glutamic acid, glycine, histidine, tyrosine, asparagine, isoleucine, leucine, lysine, methionine, proline, sodium nitrate, ammonium chloride, and peptone. All test amino acids were either the DL or L forms.

Growth Conditions

Pour plates were prepared in the usual manner. However, each pour plate was divided into four sections. Spore suspensions of the test species were streaked in each section with a sterile wire loop. The streaks were made from the center of the plate to the periphery. Then the plates were incubated at 28°C and observed daily for one week.

## Shake Flask Studies

Media

The basal salt medium with and without added glucose already described for the pour plate procedure was used. However, in this instance, no agar was required. Various nitrogen sources were added singly to the basal medium containing glucose and also to the medium with no added carbohydrate. Difco isoelectric casein, gelatin, and peptone were tested at a concentration of 1.0 per cent; alanine, glutamic acid and asparagine at 0.5 per cent and proline at 0.1 per cent concentration. The

respective media were dispensed into Erlenmeyer flasks in 50 ml aliquots and autoclaved at 15 lbs. pressure for 20 minutes.

## Cultural Conditions

Duplicate flasks were inoculated with 2 drops of a heavy spore suspension from a one ml pipette. Uninoculated duplicate flasks served as controls. These flasks were incubated at 30°C on a rotary type shaker set at 230 revolutions per minute for 4 days. At the end of the incubation period extent of cell growth was observed in the media with the amino acids. However, in the media containing isoelectric casein, gelatin, and peptone the cells were removed by filtration. The filtrates were then tested for the degree of utilization of the test nitrogen source by one or more of the following chemical determinations.

## Chemical Determinations

Amino-nitrogen determination. A modified formaldehyde titration method was used. Ten ml of the filtrate was dispensed into a 100 ml beaker. By means of the Fisher automatic titrimeter the filtrate was brought to a pH of 8.2 with 0.01N sodium hydroxide. Five ml of a 37 per cent formaldehyde solution and 5 ml of distilled water were added and allowed to stand one minute with

thorough mixing. The mixture was brought to a pH of 9.3. The amino-nitrogen present was then determined by using the proper conversion factor.

Non-Protein Nitrogen. Twenty ml of a 20 per cent trichloroacetic acid solution was added to 50 ml of the filtrate. The mixture was allowed to stand for 10 minutes to allow sufficient time for the complete precipitation of protein. Then the precipitate was removed by filtration. A micro-Kjeldahl determination was carried out on the non-protein fraction as described by Ma and Zuazaga (14, pp.280-282).

Total Nitrogen. The total nitrogen was determined by the aforementioned micro-Kjeldahl method.

Ammonia Nitrogen. Twenty-eight ml of distilled water and 2.8 ml of a phosphate buffer were added to 5 ml of the culture filtrate. The mixture was poured into a micro-Kjeldahl distillation apparatus. The mixture was distilled for 10 minutes or until 15 ml passed into 5 ml of a saturated boric acid solution. A methyl red-brom cresol green mixture was used as an indicator. The distillate was titrated with standardized sulfuric acid (17, pp.311-313).

## Turbidimetric Studies

Rate of growth as revealed by net increases in cell mass may be followed by noting changes in the optical density of the test culture. The value of this approach in attempting to study the influence of a specific microbial nutrient is apparent.

### Media

A non-synthetic medium and a strictly synthetic medium were used. The non-synthetic medium contained the basal salt mixture plus 1.0 per cent glucose already outlined for the pour plate and shake flask studies. One portion of the glucose basal salt medium received 0.5 per cent peptone and 0.3 per cent beef extract, a second lot 0.5 per cent peptone, a third 0.3 per cent beef extract and a fourth portion 0.5 per cent neutralized casein hydrolysate. In the case of the casein hydrolysate set, a further test was conducted in which several growth factors and amino acids were included with the hydrolyzed casein. These included: DL tryptophane 100 gammas, L cystine 100 gammas, pyridoxal 40 gammas, biotin .08 gamma, calcium pantothenate 40 gammas, folic acid 4 gammas and nicotinic acid 40 gammas per 200 ml of the medium.

A synthetic medium similar to that used by Henderson

and Snell (13, pp.15-29) was also tested. The composition of this medium is outlined in Table 1. Filtered stock solutions of the salts, purines, pyrimidines, vitamins and amino acids were prepared. The concentrations of the stocks were such that one ml was equivalent to the amount needed for 200 ml of the synthetic medium. Each chemical used was of C.P. or equivalent grade. The amino acids, vitamins, purines, and pyrimidines were of Nutritional Biochemical "Purity" grade. All glassware received special attention. Usually a concentrated sulfuric acid and potassium dichromate solution was used for washings with adequate rinsing in distilled water. Each filter used to sterilize the various solutions was washed and neutralized. The sintered glass filters were washed in hot concentrated nitric acid and neutralized with 1N sodium hydroxide. Fresh Seitz filter pads were used for each filtration process. These pads were neutralized with 1N sodium hydroxide and 1N hydrochloric acid.

Growth Conditions

Variations in the growth of Streptomyces albus in the synthetic medium were observed in the presence and absence of various components of the complete medium. The purines and pyrimidine bases and vitamins were omitted as separate groups. Amino acids were omitted

singly and in various groups.

Media were made up in 50 ml aliquots and the pH was adjusted to 6.7 to 7.0. Ten ml aliquots of each medium were dispensed into four sterilized cuvettes. As a further precaution against contamination, the cuvettes were autoclaved at 15 lbs. pressure for 10 minutes. Duplicate cuvettes were inoculated with two drops of a spore suspension of S. albus from a one ml pipette. Duplicate uninoculated tubes were set up as controls. The cuvettes were incubated at 30°C on a rotary type shaker set at 320 revolutions per minute. Turbidity was followed for 15 hours by a Beckman Model B spectrophotometer. The spectrophotometer was standardized with control tubes for each medium. All values were read as optical density (2 - Log G) giving a direct indication of cell mass.

TABLE 1

Complete Synthetic Medium*

| | |
|---|---|
| Glucose | 1.00 g. |
| Ammonium chloride | 0.30 g. |
| Dibasic potassium phosphate | 0.50 g. |
| Salt Mixture | 1.0 ml |
| Magnesium sulfate | 10.00 g. |
| Ferrous sulfate | 0.50 g. |
| Sodium chloride | 0.50 g. |
| Manganese sulfate | 2.00 g. |
| Distilled water | 250.00 ml |
| Adenine sulfate | 1.0 mg. |
| Guanine hydrochloride | 1.0 mg. |
| Uracil | 1.0 mg. |
| Xanthine | 1.0 mg. |
| Thiamin | 100 gammas |
| Riboflavin | 100 gammas |
| Pyridoxal | 20 gammas |
| Calcium pantothenate | 100 gammas |
| Niacin | 100 gammas |
| PABA | 20 gammas |
| Biotin | 1 gamma |
| Folic acid | 1 gamma |
| Amino acids Group A | |
| DL Alanine | 100.0 mg. |
| DL Aspartic acid | 100.0 mg. |
| L Glutamic acid | 100.0 mg. |
| Amino acids Group B | |
| L Arginine | 20.0 mg. |
| L Lysine | 20.0 mg. |
| Other L forms 10 mg. each, DL forms 20 mg. each | |
| L Histidine | L Valine |
| L Leucine | DL Trypto-phane |
| DL Isoleucine | L Cysteine |
| L Methionine | L Cystine |
| DL Phenyl-alanine | DL Serine |
| L Proline | Glycine |
| L Threonine | L Asparagine |
| DL Tyrosin | L Glutamine |

* The above concentrations are based on 200 ml of the medium.

# EXPERIMENTAL RESULTS

## Pour Plate Studies

A study of the data shown in Table 2 calls attention to culture variation in respect to utilization of the test nitrogen sources. In the absence of glucose each culture is dependent upon the included nitrogen compound for both carbon and nitrogen. In most instances only peptone, proline, glutamic acid, histidine and glutamine served as excellent sources of both carbon and nitrogen. Only slight growth responses were obtained with the remaining amino acids. The poor growth noted for each species with ammonium chloride and sodium nitrate is not surprising because of the absence of available carbon in the medium.

The inclusion of glucose brought about an increase in growth of the test Streptomyces species. This observation applies particularly to the stimulation of growth noted in media containing nitrogen compounds previously considered to be poor sources of carbon. This point is well illustrated in the poor growth response obtained with asparagine alone and the growth stimulation observed when glucose was added to the asparagine medium. On the other hand, it is interesting to note that the inclusion of glucose better illustrated the

TABLE 2

Observations on Growth of *Streptomyces* Species on Pour Plates*

| | Glucose Absent | | | | Glucose Present | | | |
|---|---|---|---|---|---|---|---|---|
| | S. griseus | S. albus | S. coeli-color | S. fla-veolus | S. griseus | S. albus | S. coeli-color | S. fla-veolus |
| Control | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Peptone | 2 | 3 | 3 | 2 | 3 | 4 | 4 | 3 |
| $NH_4Cl$ | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
| $NaNO_3$ | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 |
| Alanine | 2 | 1 | 3 | 2 | 2 | 1 | 3 | 3 |
| Arginine | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 |
| Glycine | 1 | 1 | 1 | 1 | 1 | 2 | 3 | 2 |
| Isoleucine | 1 | 2 | 1 | 1 | 2 | 2 | 3 | 2 |
| Leucine | 1 | 1 | 1 | 1 | 4 | 2 | 3 | 1 |
| Serine | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| Aspartic Acid | ND | 1 | 1 | ND | ND | 2 | 2 | ND |
| Methionine | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| Proline | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Lysine | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| Glutamic Acid | 4 | 4 | 4 | 4 | 4 | 3 | 4 | 4 |
| Tryptophane | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 |
| Valine | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 |
| Asparagine | 1 | 1 | 1 | 2 | 4 | 4 | 4 | 4 |
| Tyrosine | 2 | 1 | 2 | 1 | 4 | 2 | 4 | 1 |
| Histidine | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Glutamine | 1 | 2 | 4 | 4 | 1 | 4 | 4 | 4 |

* 1. Slight growth
2. Moderate growth
3. Good growth
4. Excellent growth
ND No determination

differences in utilization of the nitrogen sources by the individual test cultures. For example, Streptomyces flaveolus showed slight growth with leucine, Streptomyces albus moderate growth, Streptomyces coelicolor good growth and Streptomyces griseus excellent growth. Similar growth variations may be observed with other nitrogen compounds in the data recorded in Table 2. Apparently these observations reflect species differences.

## Shake Flask Studies

### Utilization of Gelatin and Casein

The free ammonia and more directly the non-protein nitrogen determinations given in Table 3 provide information on the proteolytic activity of the listed cultures. It is apparent that considerable variation exists among these cultures. In the absence of glucose, S. griseus, Streptomyces willmorei, and Streptomyces lipmanii effected very extensive proteolysis of isoelectric casein; S. albus and S. flaveolus showed moderate activity; Streptomyces viridochromogenus and Streptomyces olivaceus only slight proteolytic action. The aforementioned groups may also be detected by a study of the non-protein nitrogen fractions given for gelatin minus glucose. The higher free ammonia

TABLE 3

Proteolysis of Casein and Gelatin by Various *Streptomyces* Species

| Organisms | Isoelectric Casein | | | | Gelatin | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose Absent | | Glucose Present | | Glucose Absent | | Glucose Present | |
| | mg $NH_3$-N / 100 ml | mg N-P-N / 100 ml | mg $NH_3$-N / 100 ml | mg N-P-N / 100 ml | mg $NH_3$-N / 100 ml | mg N-P-N / 100 ml | mg $NH_3$-N / 100 ml | mg N-P-N / 100 ml |
| Control | 1.39 | 6.18 | 1.19 | 5.78 | 1.20 | 2.59 | 2.39 | 3.39 |
| *S. albus* | 8.07 | 30.10 | 1.69 | 32.79 | 17.34 | 18.54 | 4.59 | 10.26 |
| *S. flaveolus* | 5.58 | 32.19 | .60 | 26.03 | 14.75 | 21.22 | 1.19 | 6.18 |
| *S. griseus* | 8.77 | 86.12 | 1.79 | 52.43 | 31.10 | 56.41 | 3.69 | 15.15 |
| *S. viridochromogenus* | 7.28 | 19.93 | .70 | 16.04 | 20.50 | 1.10 | 1.10 | 6.28 |
| *S. olivaceus* | 6.08 | 24.22 | .90 | 49.11 | 3.39 | 4.39 | 1.00 | 3.49 |
| *S. willmorei* | 9.07 | 78.03 | 1.00 | 44.24 | 67.78 | 52.63 | 1.10 | 6.67 |
| *S. lipmanii* | 14.75 | 89.11 | 3.79 | 45.05 | 67.38 | 51.63 | 1.59 | 7.57 |

determinations observed with gelatin gives indication that this protein has undergone a more rapid and complete hydrolysis than the isoelectric casein.

In the presence of glucose, the strongly proteolytic species effected far less hydrolysis of both isoelectric casein and gelatin. Apparently, these cultures preferred glucose as the source of carbon and thereby spared the protein. This sparing action is also reflected in the free ammonia determinations. Significantly enough, far less sparing of these proteins occurred with the moderately and weakly proteolytic cultures.

Growth in Peptone

In respect to amino nitrogen and free ammonia determinations, the data presented in Table 4 attests to the ability of the Streptomyces cultures to utilize peptone. Again it is evident that variations exist among these cultures. In the absence of glucose, S. flaveolus, S. griseus, and S. lipmanii effectively used peptone; moderate activity was noted with S. albus and S. willmorei. S. viridochromogenus and S. olivaceus were weakly active. In most cases, the aforementioned groups are detected also by reference to the free ammonia determinations. S. lipmanii which appears to be only weakly active in regard to the release of free amino-nitrogen can be considered highly active because

TABLE 4

Utilization of Peptone by Various *Streptomyces* Species

| Organisms | Glucose Absent | | Glucose Present | |
|---|---|---|---|---|
| | Mg $NH_3$-N / 100 ml | Mg $NH_2$-N / 100 ml | Mg $NH_3$-N / 100 ml | Mg $NH_2$-N / 100 ml |
| Control | 1.40 | 25.80 | 1.19 | 27.73 |
| *S. albus* | 30.70 | 59.34 | 3.98 | 25.80 |
| *S. flaveolus* | 52.28 | 74.43 | 3.78 | 25.80 |
| *S. griseus* | 68.85 | 66.43 | 8.97 | 31.50 |
| *S. viridochromogenus* | 5.18 | 36.38 | .80 | 25.80 |
| *S. olivaceus* | 1.39 | 48.37 | 1.00 | 26.44 |
| *S. willmorei* | 33.29 | 59.98 | 10.76 | 30.21 |
| *S. lipmanii* | 61.67 | 48.68 | 6.38 | 29.03 |

of the extensive change of peptone nitrogen to free ammonia.

An apparent sparing action on peptone by the cultures is noted in the presence of glucose. This is striking in both the amino-nitrogen and free ammonia determinations.

Observed Variations

In reference to Tables 3 and 4, it is interesting to note that several variations occur with individual cultures in the ability to hydrolyze the different types of organic nitrogen sources. S. flaveolus which is only moderately active on isoelectric casein and gelatin is highly active on peptone. On the other hand, S. willmorei which is highly active on isoelectric casein and gelatin is only moderately active on peptone.

Typical growth data obtained by the pour plate and shake flask methods are shown in Table 5. It is evident that compatible results are not obtained when testing single amino acids. All three organisms grew well with proline in pour plates but showed little activity with this amino acid under shake flask conditions. However, the test cultures grew equally well under both conditions whenever peptone was used.

TABLE 5

Comparison of Results Obtained With the Pour Plate and Shake Flask Studies

| Nitrogen Sources | Pour Plate (0.1%) | | Shake Flasks (0.5%) | |
|---|---|---|---|---|
| | Glucose Absent | Glucose Present | Glucose Absent | Glucose Present |
| *S. albus* | | | | |
| Proline | 4 | 4 | 1 (0.1%) | 1 (0.1%) |
| Alanine | 1 | 1 | 1 | 1 |
| Asparagine | 1 | 4 | 1 | 1 |
| Peptone | 2 | 4 | 4 | 4 |
| *S. flaveolus* | | | | |
| Proline | 4 | 4 | 1 (0.1%) | 1 (0.1%) |
| Alanine | 2 | 3 | 1 | 1 |
| Asparagine | 2 | 4 | 1 | 2 |
| Peptone | 2 | 3 | 4 | 4 |
| *S. griseus* | | | | |
| Proline | 4 | 4 | 1 (0.1%) | 1 (0.1%) |
| Alanine | 2 | 2 | 1 | 1 |
| Asparagine | 1 | 4 | 1 | 1 |
| Peptone | 2 | 3 | 4 | 4 |

* 1. Slight growth
2. Moderate growth
3. Good growth
4. Excellent growth

## Turbidimetric Studies

### Growth in Non-Synthetic Media

Variation in growth lag and the various growth rates illustrated in subsequent figures attest to the influence of specific nutrients on the growth of *Streptomyces albus*. A study of Figure 1 shows that the individual components of glucose nutrient broth are necessary for optimum growth of *S. albus*. However, the omission of beef extract from the complete medium caused only a slight decrease in total growth. On the other hand, the absence of peptone lengthened the lag phase and decreased the rate and subsequent growth of the culture.

It is interesting that the medium containing neutralized casein hydrolysate supported only poor growth of *S. albus*. For this reason, a medium was prepared containing casein hydrolysate and the missing vitamins and amino acids. This enriched medium also failed to support growth of the test culture. The latter observation was substantiated by other shake flask experiments. Apparently the vitamins can not be considered as essential for growth.

### Growth in Synthetic Media

The concentration of the salt mixture prescribed

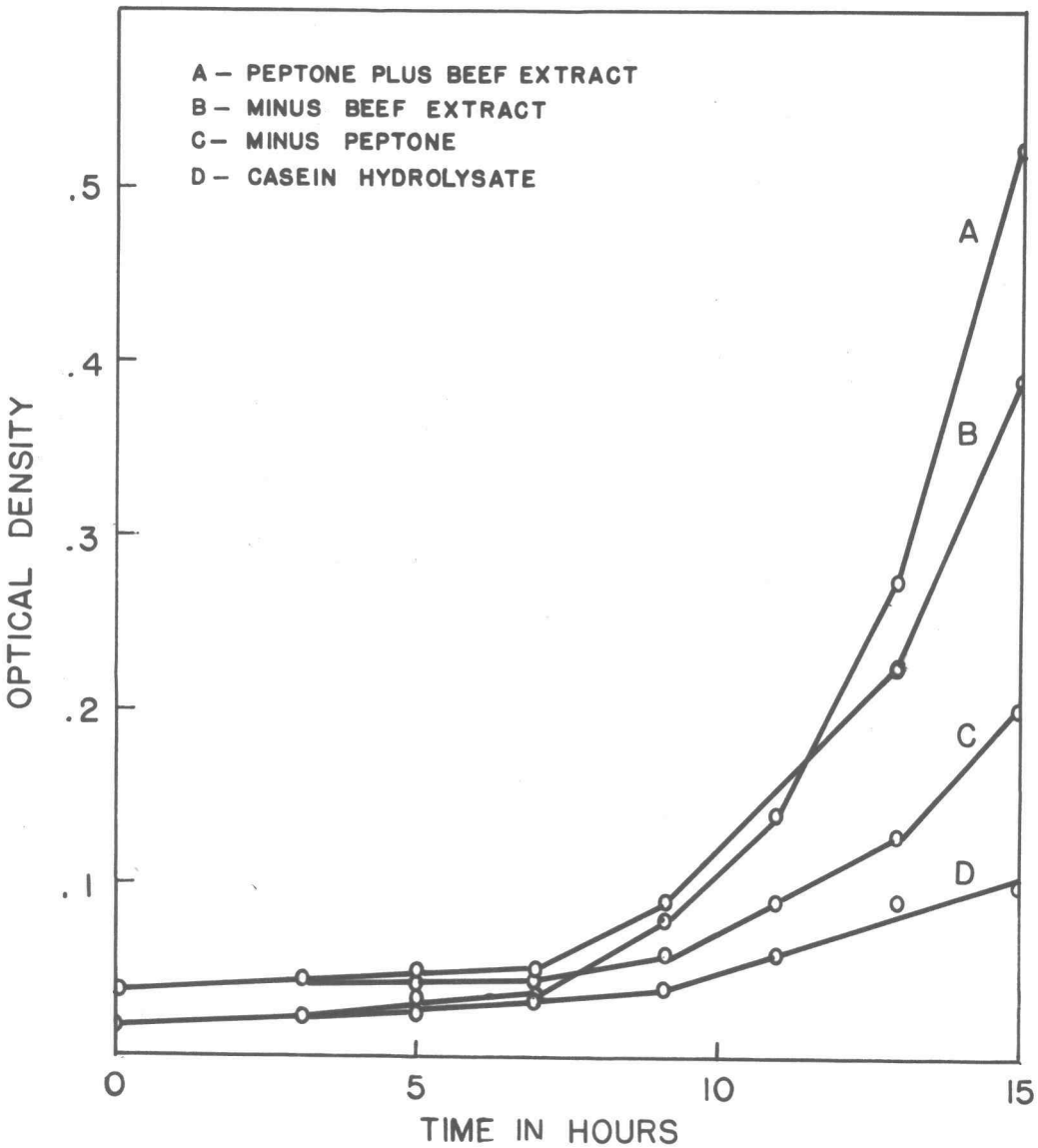


FIG. 1 OBSERVED GROWTH OF S. ALBUS IN MODIFIED GLUCOSE NUTRIENT BROTH AND CASEIN HYDROLYSATE

by Snell caused considerable precipitation in the cuvettes. For this reason, the turbidimetric readings could not be properly standardized. A clear solution was obtained when only half the concentration of the original salt mixture was used. Reference to Figures 1 and 2, justifies the use of the reduced salt concentration. Growth of S. albus obtained in the minimal synthetic medium (glucose, salts, and amino acid groups A and B) was comparable to the growth observed in the non-synthetic medium containing peptone. The growth rate of S. albus was also increased in the synthetic medium. However, the omission of amino acid group A decreased the rate and therefore the total growth. A longer lag phase with a decrease in total growth was observed in the absence of Group B. The growth of S. albus was poor when all the amino acids were omitted and ammonium chloride served as the sole nitrogen source.

The significant growth response of S. albus with the synthetic medium containing only glucose, salts and group B amino acids warranted further consideration. The data presented in Figures 3, 4 and 5 make it apparent that the omission of different amino acids from group B caused variations in growth. No adverse effects were seen when glutamine, asparagine, histidine, and tryptophane were absent. However, a marked decrease in the growth of S. albus was noted when the following

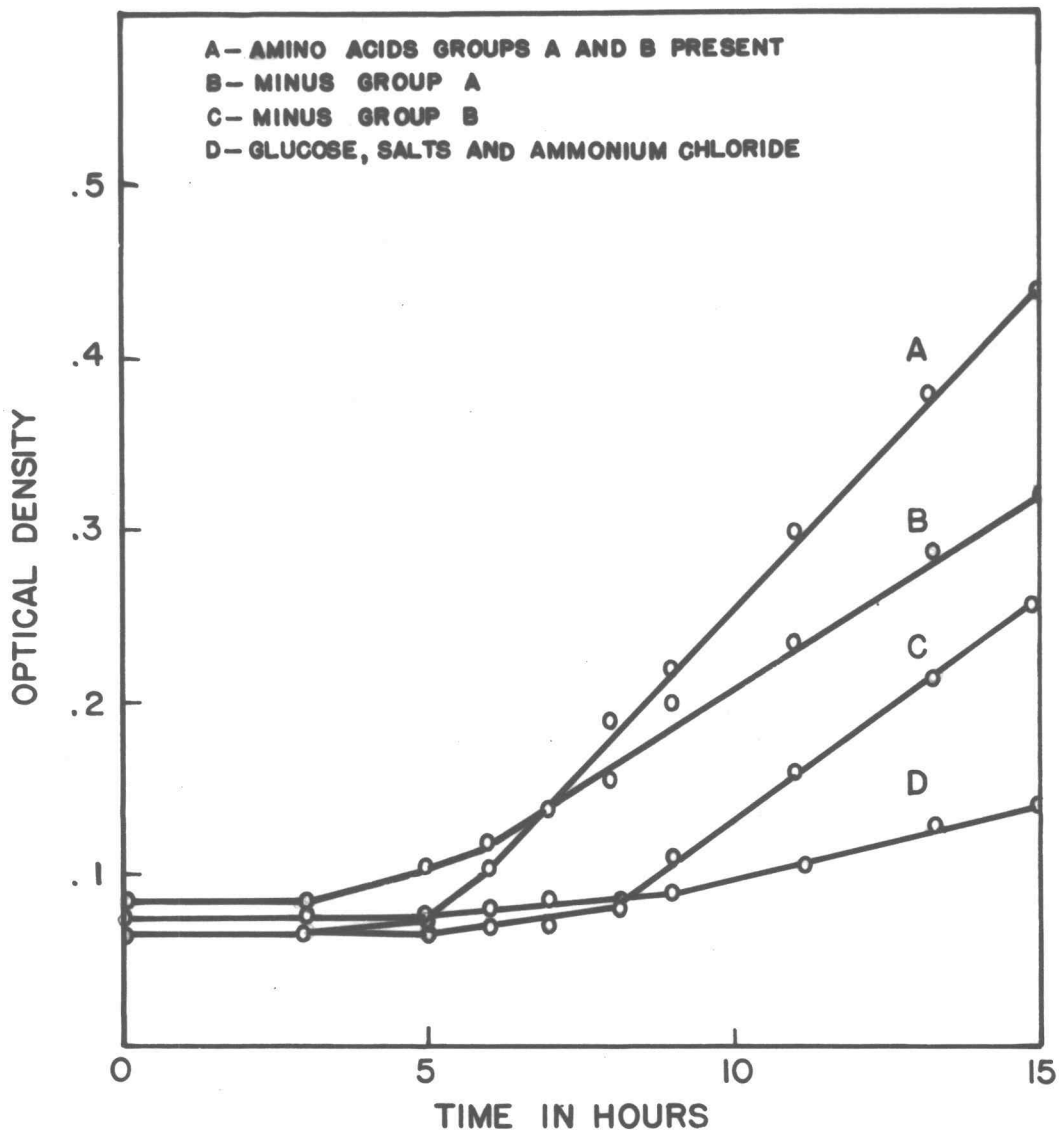


FIG. 2 OBSERVED GROWTH OF S. ALBUS IN PRESENCE OF AMINO ACID GROUPS A AND B

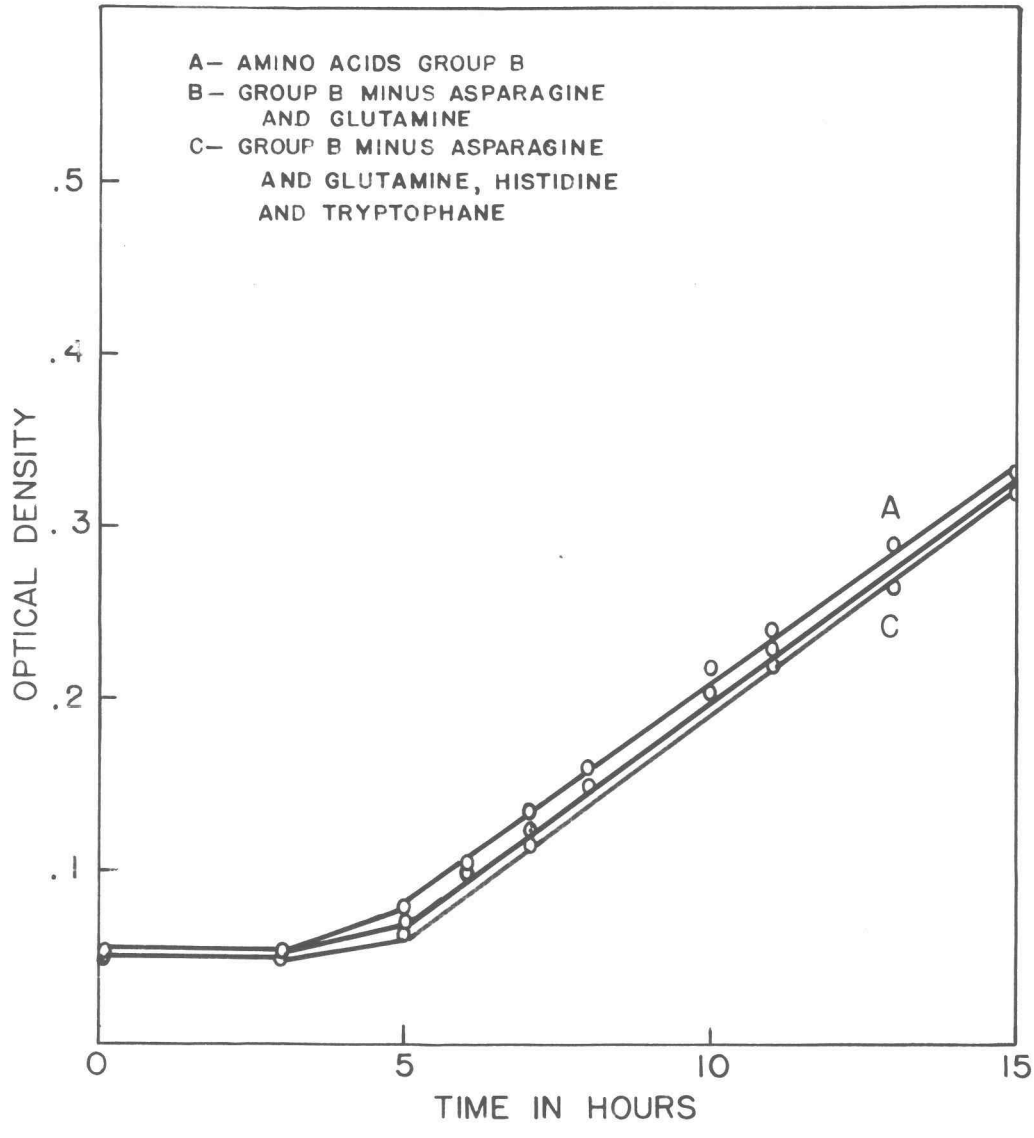


FIG. 3 INFLUENCE OF VARIOUS GROUPS OF AMINO ACIDS ON GROWTH OF S. ALBUS

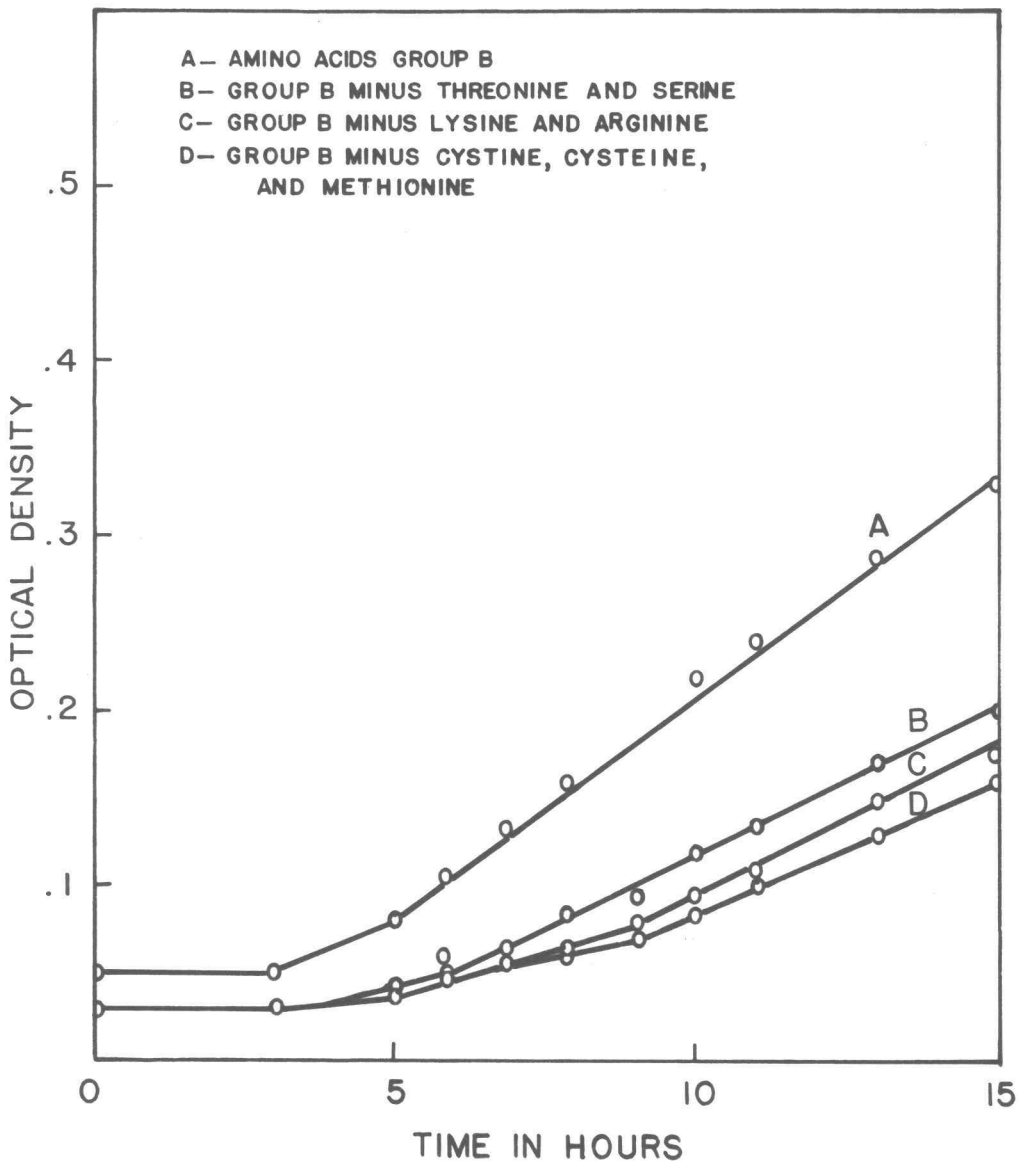


FIG. 4 INFLUENCE OF VARIOUS GROUPS OF AMINO ACIDS ON GROWTH OF S. ALBUS

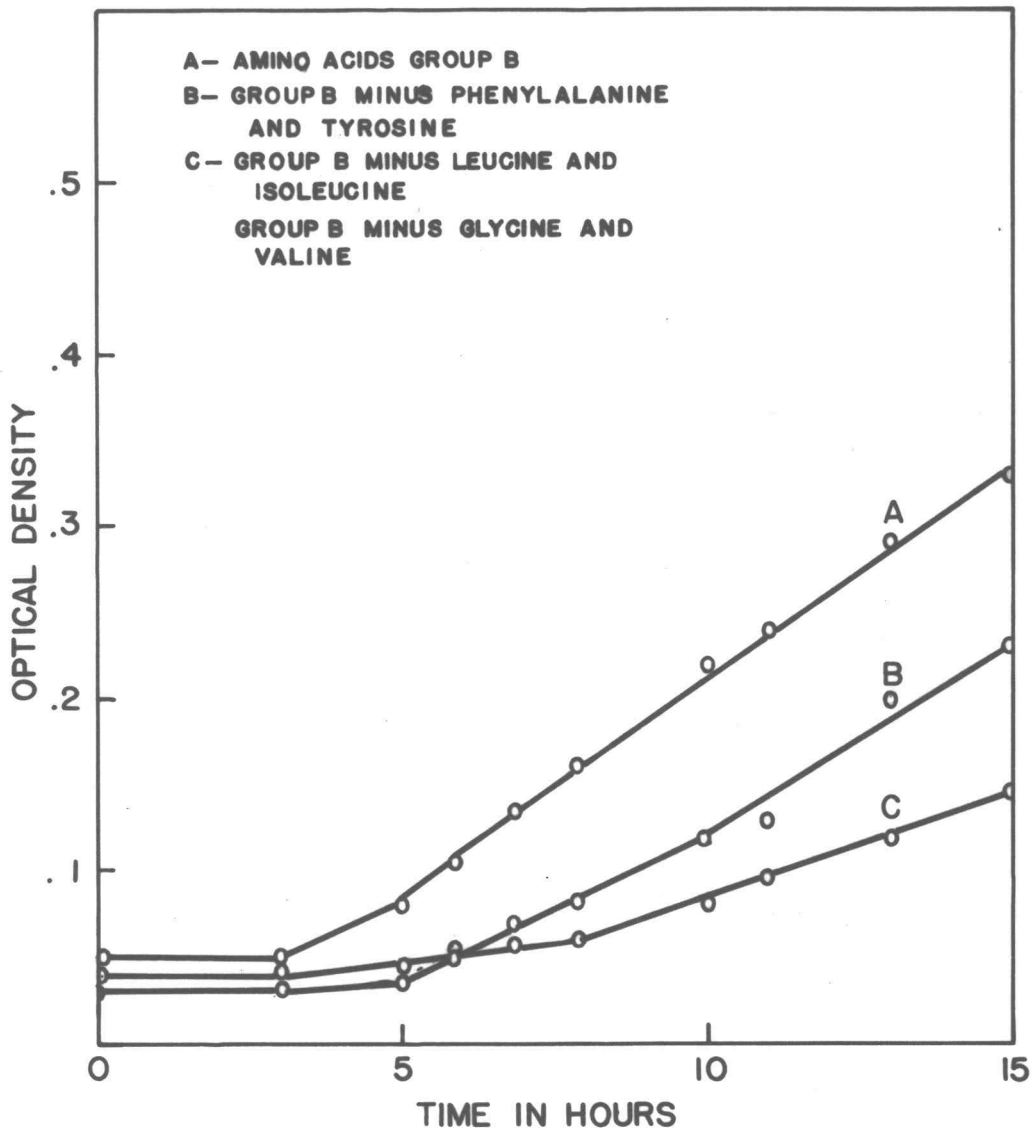


FIG. 5 INFLUENCE OF VARIOUS GROUPS OF AMINO ACIDS ON GROWTH OF S. ALBUS

groups were omitted: threonine and serine; lysine and arginine; cystine, cysteine and methionine; phenylalanine and tyrosine; leucine and isoleucine; and glycine and valine. The absence of the latter two groups also caused a longer lag phase.

Most of the amino acid groups eliminated showed decreases in the growth of S. albus. For this reason, subsequent work was designed to test the effect of removing single amino acids from group B. As shown in Figure 6, glycine had no effect on growth. Similar results were obtained with leucine, serine, asparagine, glutamine, proline, histidine, tryptophane, tyrosine, cysteine, and cystine. Slight effects were noted when the following amino acids were absent: methionine, phenylalanine, arginine, lysine, and threonine. Isoleucine and valine did significantly influence the growth of S. albus. The longer lag phase is again evident. The slight growth response from the single omission of isoleucine and valine was checked to eliminate the possibility that the amino acids present were contaminated by isoleucine and valine. A 20 fold dilution gave similar results.

The influence of isoleucine and valine on the growth of S. albus was studied further in the presence of amino acids groups A and B. As shown in Figure 7, the lag

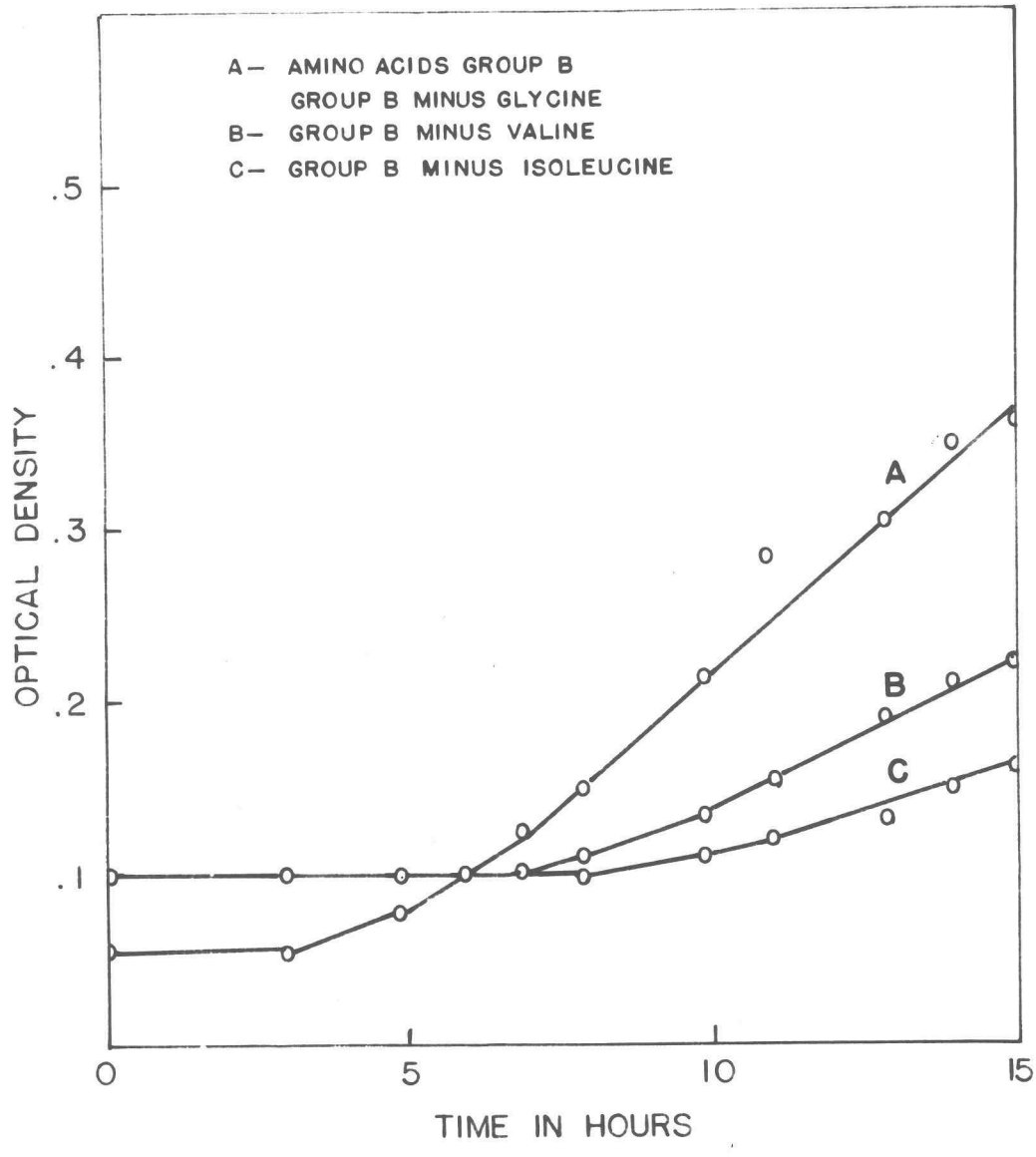


FIG. 6 OBSERVED GROWTH OF S. ALBUS WHEN SINGLE AMINO ACIDS ARE OMITTED

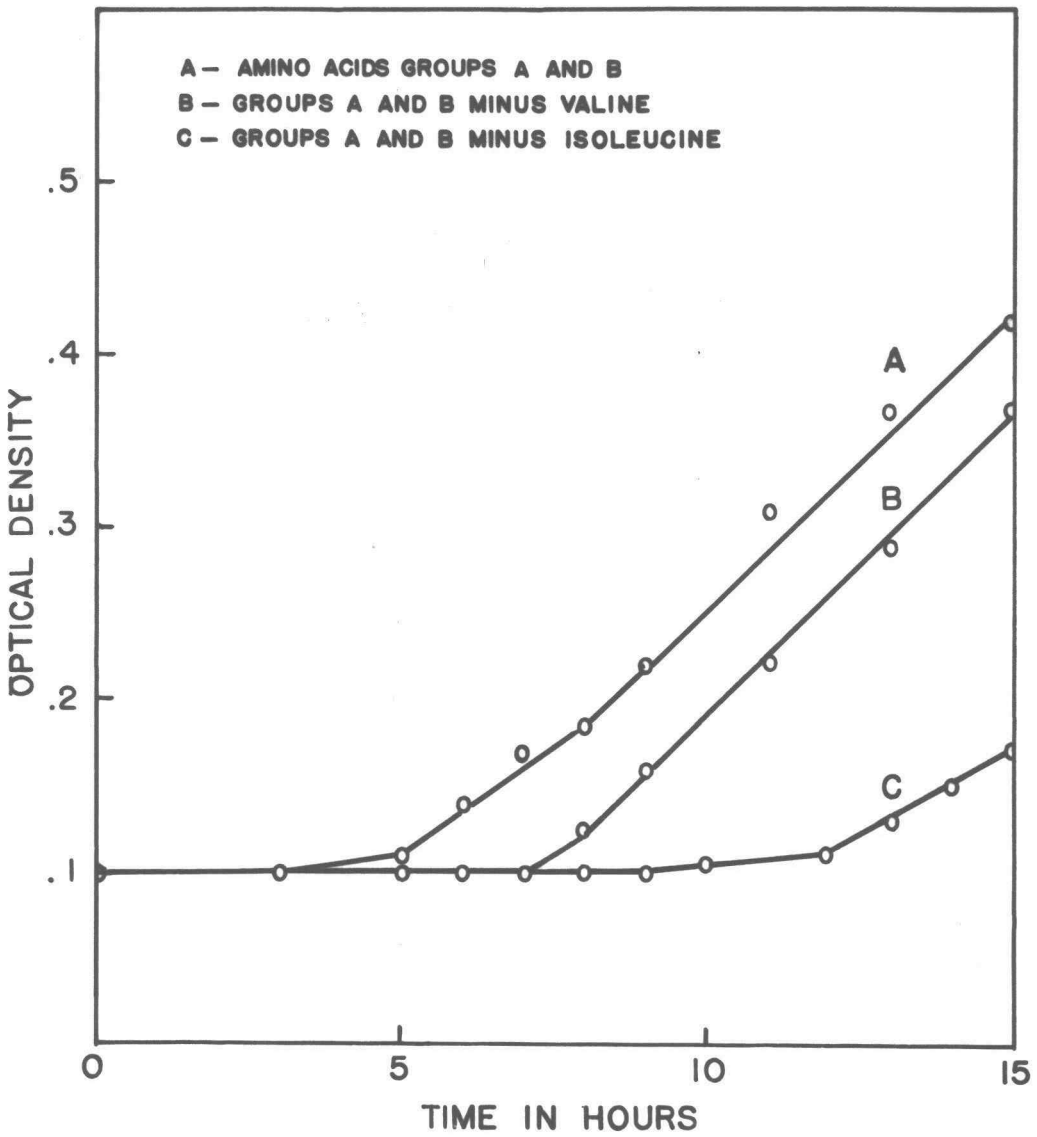


FIG. 7 INFLUENCE OF VALINE AND ISOLEUCINE ON THE GROWTH OF S. ALBUS

phase was still prolonged in the absence of valine. However, after two hours the rate of growth was similar to that noted when the amino acids were all present. Isoleucine, on the other hand, produced a longer lag and the growth rate never equaled that obtained in the medium containing all the amino acids. This observation indicates that valine may be synthesized.

Reference to Figure 8 shows that of the three amino acids present in group A, glutamic acid is the most stimulatory. In a final experiment, a medium was prepared including all the amino acids which seemed to have an effect on growth. These amino acids included isoleucine, valine, lysine, arginine, phenylalanine, methionine, threonine, and glutamic acid. In all concentrations tested, these amino acids could not support growth comparable to the growth obtained when all the amino acids were present.

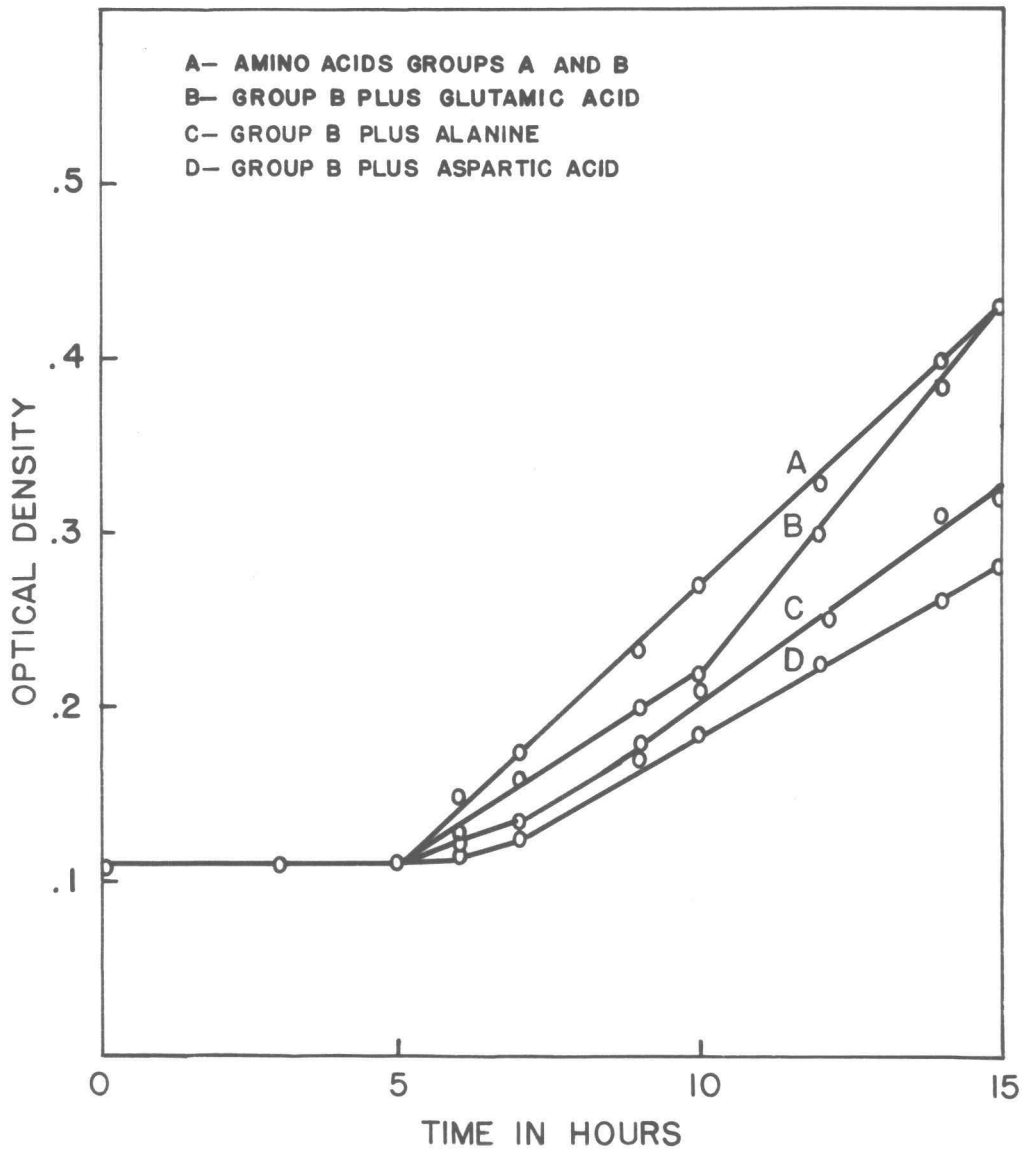


FIG. 8 OBSERVED GROWTH OF S. ALBUS IN THE PRESENCE AND ABSENCE OF AMINO ACIDS IN GROUP A

# DISCUSSION

The results of the present investigation attest to the complex nature of many Streptomyces species. The conflicting data obtained under the shake flask and pour plate conditions may be explained by a consideration of several factors. The nitrogen impurities in the agar may have promoted growth. The heavy spore suspensions were streaked on the pour plates in a small restricted area. Mutant cells which are able to utilize the test amino acids may emerge. Thus the resulting colony growth would not be representative of the original spore population. On the other hand, growth of such mutant cells in shake flask cultures would not be readily detected. Thus a positive growth response observed on solid agar might not be confirmed when liquid media is used.

Early observations by Waksman (23, p.9) on the inability of some Streptomyces cultures to readily utilize inorganic nitrogen have been confirmed. However, considerable growth does take place when either the ammonium or nitrate ion constitutes the sole source of nitrogen. We may conclude, therefore, that these organisms are capable of synthesizing all nitrogen compounds essential for the growth processes of the cell. Yet, since growth with inorganic nitrogen salts cannot be

regarded as optimum, we must regard the aforementioned biosynthetic reactions as sluggish in nature. Obviously other factors are required if maximum growth is desired.

As a group, the Streptomyces reach maximum growth in media containing the more complex organic sources of nitrogen. This result might have been expected since soils rich in organic matter invariably show higher Streptomyces counts than those deficient in organic materials. However, the extent of utilization of proteins as revealed by non-protein nitrogen determinations does vary with the species. Interestingly enough, the inclusion of glucose with either casein or gelatin often leads to a sparing of these proteins. Presumably the strong proteolytic activity noted for several species in the absence of glucose is initiated to satisfy both the nitrogen and carbon requirements of the cells. Inasmuch as the inclusion of glucose did not significantly increase the degree of proteolysis effected by the moderate to weak proteolytic types, we cannot regard, carbon as the limiting factor. However, the further observation that some degree of protein degradation takes place implies that at least the initial protease enzymes are present. Thus species variation in proteolytic ability can be regarded in terms of extent rather than reflecting basic differences in metabolism.

However, basic differences in the mode of utilization of added carbon cannot be excluded.

In the absence of glucose, few of the single amino acids tested served as an adequate source of carbon and nitrogen for the Streptomyces under study. Evidently glutamic acid and alanine which may originate from known reactions of the citric acid cycle can support growth whereas, with the exception of proline and histidine, all other amino acids used proved to be poor sources of carbon and nitrogen. Since the inclusion of glucose stimulated growth in the presence of most of these amino acids, we may assume that the test Streptomyces cultures cannot readily utilize such amino compounds as the sole source of carbon.

Growth of S. albus in Snell's synthetic medium disclosed new sources of information. At this point, attention should be directed to the observed growth of S. albus in the complete and various incomplete media used. Although marked differencesin growth were encountered yet some growth was observed even in minimal media. Thus, all questions must by inference refer to stimulation of growth up to a maximum level.

Apparently S. albus can synthesize any required accessory growth factors since the omission of the latter in the complete synthetic medium did not cause a decrease

in growth. The high level of growth noted with glutamic acid, alanine and aspartic acid as the sole amino acids present, attests again to the ability of S. albus to synthesize other essential amino acids. Thus, it is probable that a system of interrelated enzymic reactions characteristic of Krebs citric acid cycle may play an important role in the biosynthesis of these amino acids. Certainly, the aforementioned observations relating to the utilization of glutamate, aspartate and alanine support such a suggestion.

With the omission of valine, isoleucine, and glutamic acid, respectively, from the synthetic medium, suboptimal growth was observed. Yet we must assume that these amino acids are formed during the steady state of cell metabolism. It may be that the net synthesis of these amino acids cannot meet the growth needs of the organism. In this respect, Gale (30, p.465) has stated that when cells are actively growing, the free amino acid pool within the cells acts as a reservoir. The concentration of amino acids in the metabolic pool is determined by the rate of entry and by the rate of utilization. Consequently, any amino acid increase in the medium may stimulate cell metabolism and growth. It is probable that the higher levels of growth observed with isoleucine and other amino acids may be explained on

the latter basis.

# SUMMARY AND CONCLUSIONS

The growth of several Streptomyces species was observed in non-synthetic and synthetic media. It was found that inorganic nitrogen salts do not supply an adequate source of nitrogen for these cultures. Maximum growth responses were observed with the more complex nitrogen substances such as peptone, gelatin and casein. Variation in the degree of proteolysis of gelatin and casein was observed among the test species. In some instances the inclusion of glucose brought about a very significant decrease in the hydrolysis of casein, gelatin and peptone.

With the exception of glutamic acid, glutamine, histidine, and proline, single amino acids did not support good growth of the Streptomyces cultures unless glucose was added. Optimal growth of Streptomyces albus was obtained in a modified synthetic medium. Apparently S. albus is able to synthesize some of the known accessory growth factors and various amino acids. The participation of Krebs citric acid cycle has been suggested in this synthesis since a high level of growth was obtained when only alanine, aspartic acid and glutamic acid were present. Of all the amino tested, isoleucine, valine and glutamic acid appeared to be the most

essential for optimum growth of *S. albus*.

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