STUDIES ON THE METABOLISM OF STREPTOMYCES GRISEUS

IN RELATION TO THE PRODUCTION OF STREPTOMYCIN

A Thesis

By

Herman Max Eiser

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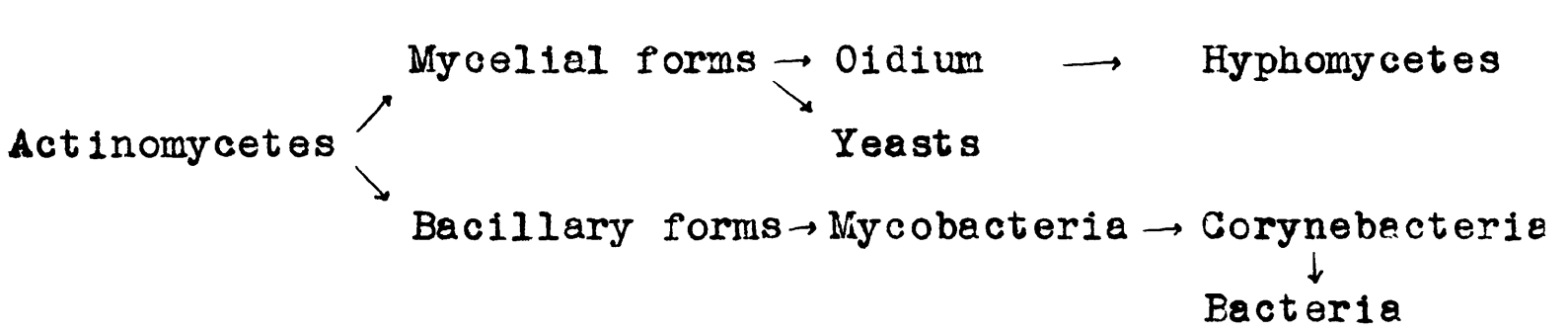
INTRODUCTION

The actinomyces, as a group are present in great abundance in nature, particularly in soil. They are neither true bacteria, although they are often mistaken for such due to the fact that the mycelium is very fine and easily broken, nor are they true fungi because of their great sensitivity to acids. Waksman (1) regards them as an ancestral form of both bacteria and fungi according to the following scheme:



Since the early work of Waksman, some twenty-five years ago, relatively little work has been done on the nutrient requirements of actinomyces. Data on specific requirements for amino acids, growth factors and minor elements are almost entirely lacking. This is all the more surprising since included among the actinomycetes are organisms causing important plant diseases such as potato scab, sugar beet and mangel beet scab and pox of sweet potatoes.

Gratia and Dath (2), in 1924, described a metabolic

product of certain strains of actinomyces which they named "actinomycetin". This substance showed a wide range of antibacterial activity but it was not studied further. Metabolic products showing antimicrobial properties were isolated from other groups of microorganisms during the next fifteen years, but it was Waksman and Woodruff (3), in 1941, who again directed attention to the actinomycetes. A comprehensive survey of the antibiotic producing ability of the group culminating in the discovery of two species Actinomyces lavendulae producing streptothricin (4) and Streptomyces griseus producing streptomycin (5). The latter was particularly interesting because of the low toxicity to animals and wide bacteriostatic spectrum which included gram-positive as well as gram-negative organisms. The possibility that streptomycin might have an effect on Mycobacterium tuberculosis (8) gave rise to widespread research on all phases of the subject.

With a view fo increasing the yield of the antibiotic, research was directed along three main lines-

a. To isolate more active strains, giving higher antibiotic titers.
b. To study the specific nutrient requirements for the synthesis of streptomycin.
c. To develop better methods of isolating the antibiotic from the medium.

The two latter lines of investigation went hand in hand, because the complexity of the media as regards organic constituents made the isolation of streptomycin very difficult; the recovery was poor and the isolated product was colored and often toxic at the dosage used for clinical treatment (7). To quote Hans Molitor (7) writing on "Pharmacology of Streptomycin":

> "It may seem inappropriate to open a discussion of the pharmacology of streptomycin by placing emphasis on impurities, yet, the longer one studies this drug, the less can one escape the conviction that the pharmacology of streptomycin, in the form in which we know it today, is, to a very large degree, due to one of its "standard" impurities. I shall use this term, to designate contaminants which, unless specifically removed, may be expected to be constantly present in preparations of the average type, varying only quantitatively but not qualitatively as long as the parent strains of Streptomyces griseus, the composition of the nutrient media and the process of extraction, concentration and finishing remain the same."

This study was undertaken to develop new media, preferably of known composition; to study the metabolism of the organism when grown in these media; to elucidate the mechanism of streptomycin production, and finally to develop new methods of extraction and purification of streptomycin to obtain a product of higher purity, lighter color, and lower toxicity.

LITERATURE REVIEW

Studies on the Metabolism of Actinomyces up to 1944

The term Actinomyces was coined by Hartz (8) in 1877 to describe the organism Actinomyces bovis causing "lump jaw" of cattle. However, the species belonged to a genus already described under eight different generic terms (9): Streptothrix, Cladothrix, Sphaerotilus, Actinomyces, Discomyces, Actinocladothrix, Norcardia and Oospora. This illustrates the confusion regarding the classification of the genus which arose from the fact that the actinomyces show greater variability in morphological and physiological characteristics, than any other group of organisms. This variability was partly due to the complex nature of the nutrient media.

F.Münter (10) was amongst the first to study the growth of the orgenisms on synthetic and specific media. However, the work was mostly descriptive as to color and type of mycelium. Incubation required as long as three weeks because of the poor growth on some of his media. The study included seven actinomyces isolated from various soils. He concluded that the best nitrogen sources were complex substances such

as casein and albumin, and found no difference between the efficiency of nitrate, ammonia and asparagine-nitrogen. The latter observation is in contradiction with later work and Münter's results may have been influenced by the long incubation period. Using an inorganic source of nitrogen, he obtained good growth with the following carbohydrates: glycerol, levulose, dextrose, galactose, mannitol and starch. Arabinose was only utilized by some actinomycetes while sucrose was well utilized by the quinone producing actinomyces. Interesting results were obtained with some organic acids, e.g., oxalic, acetic and hippuric acid were very sparingly utilized, whereas succinic and citric acid were readily available sources of carbon. The addition of a carbonyl group to succinic acid as a malic acid decreases its availability and with tartaric acid the growth is practically nil. Lactic acid seems to be an exception to this rule. This does not agree with Salzmann (11) findings that succinic, malic, tartaric and citric acids were well utilized, but formic, acetic, propionic, butyric, valerianic, lactic, benzoic and oxalic acids were not readily available. It appears that acids containing one carboxyl group were sparingly utilized, while good growth occurred when a second carboxyl group was introduced.

Lieske (12) employed a solution in distilled water of one per cent urea, traces of basic potassium phosphate and

magnesium sulphate and two per cent of the corresponding carbon source. He found that the aerobic saprophytic strains utilized maltose, lactose, levulose, dextrin, glucose, glycerol, glycogen, inulin, mannitol, asparagine, human blood serum and sucrose with efficiency decreasing in the order named.

Krainsky (13) was the first to name and classify Actinomyces griseus. He recorded its pigment production on various synthetic media. However, the first extensive study of the organism and many other actinomyces was presented by Selman A. Waksman in a survey published in 1919 and 1920. In his first paper in 1919 (14) Waksman used two media, one a milk and the other a blood medium. He found that he could divide the actinomyces into five main groups based on their action on milk. Actinomyces griseus was classed with the group which were highly proteolytic, i.e., they coagulated milk rapidly and then peptonized the coagulum. Apparently two types of enzymes were active in Actinomyces griseus, one rennet-like which was in the medium (exo enzyme) and another in the mycelium (endoenzyme) which was highly proteolytic. Actinomyces griseus was also very active on blood media, producing haemolysis and liquefaction. A rapid increase in α amino-nitrogen, indicated hydrolysis of serum proteins.

Further proof of the high proteolytic activity of Actinomyces griseus was contained in his second paper (15)

in which several media containing egg extract were used to grow the organisms. Actinomyces griseus was again among the most active and hydrolysed the coagulated egg albumin. It also liquefied gelatin and the amino nitrogen, measured after forty days incubation, was as high as with any of the organisms tested. Since acid or gas production by fermentation of carbohydrate is commonly used to differentiate bacterial species, Waksman attempted a classification of actinomyces on this basis but no gas or acid production was observed; visual growth and reduction of nitrate to nitrite was recorded. With Actinomyces griseus, mycelium production was never optimal, probably due to the inorganic nitrogen source used. The order of availability of the carbohydrates as carbon sources for Actinomyces griseus was as follows: starch, glucose, mannitol, sodium malate, arabinose, lactose and glycerol. The average efficiency for all the organisms was calculated and starch and glucose headed the list. However, malate was not readily available and this is the most pronounced difference between Actinomyces griseus and the average for all actinomyces. It must be borne in mind that these results obtain only if nitrate is the sole source of nitrogen, a fact already pointed out by Krainsky (13). Production of diastase by Actinomyces griseus was high, but its action on cellulose was very poor.

In his third paper Waksman (16) dealt with nitrogen

metabolism, particularly as affected by various carbon sources. With glycerol, which was not a very good source of carbon for Actinomyces griseus, the following order of efficiency was established: casein, Witte peptone, fibrin, leucine, asparagine, glycine, egg-albumin, urea, acetamide, sodium nitrate, sodium nitrite, ammonium sulphate, ammonium carbonate. Proteins and amino acids were readily available sources of nitrogen, whereas amides (e.g. acetamide and urea) and the inorganic nitrogenous compounds were a much poorer source of nitrogen.

The availability of nitrate depended upon the carbon source. Waksman showed that forty-five species reduced nitrate when starch was present but only twenty showed the same activity in the presence of sucrose. With urea the media turned alkaline due to the liberation of ammonia, but with acetamide the reverse was true probably because the amino nitrogen was used up and was replaced by a hydroxyl group to give an acid. Of the different amino acids used, leucine tended to give a more acid medium while asparagine and glycine produced a more alkaline reaction. The protein and peptone media all tended to become more acid. This may have been due to the high glycerol concentration.

Results of experiments in which actinomycetes were grown in a medium containing both gelatin and starch, indicated a protective action of the carbohydrates upon the proteins.

However, the effect was not as pronounced with actinomyces as it was with proteolytic bacteria and molds.

The accumulation of ammonia by actinomyces was slow, but increased steadily. From a comparison with bacteria and molds which show rapid accumulation of ammonia, it is concluded that the actinomyces split the proteins chiefly to the amino acid stage and to a limited extent to ammonia. If a long enough period was allowed, however, a considerable amount of ammonia accumulated especially with media containing peptone and asparagine as nitrogen sources.

Waksman's fourth paper (17) dealt with changes in the nutrient media as a result of the growth of actinomyces. He investigated the changes in the hydrogen-ion concentration of the media with different sources of carbon and nitrogen. Since most of the media turned alkaline he concluded that no acids were produced from carbohydrate metabolism. However, he also stated that the presence of an available carbohydrate in a protein medium seemed to favor the change of reaction to the acid side. This, he pointed out, was not because acid was produced in the metabolism of carbohydrates, but rather that the carbohydrate had an effect on nitrogen metabolism. Waksman also studied the effect of different initial hydrogen-ion concentrations upon the growth of actinomyces. He found the hydrogen-ion concentration tended to reach an optimum, the more acid media tending to become less acid and the more

alkaline media (above the optimum), less alkaline.

The above summarizes the available information on the metabolism of actinomyces in general and Actinomyces griseus in particular. In the interim between Waksman's studies in 1920 and the rediscovery of the actinomyces a few years ago, there was a complete absence of publications on the actinomyces.

Studies on the Production of Streptomycin by Streptomyces griseu

It has now been definitely established that a considerable number of the actinomyces isolated from soils, or other natural substances, have the capacity to inhibit the growth of, or even destroy, bacteria and other microorganisms. The nature of the antibiotics produced by the organisms depends upon the composition of the medium, the conditions of cultivation and on the species and frequently on the strain of the organism (18).

Gratia and Deth (2), in 1924, described an antibacterial substance produced from certain actinomyces and Rosenthal (19) in 1925, isolated an actinomycete active against diphtheria bacteria. In 1941, Waksman and Woodruff (3) discovered a new actinomyces, producing a very active antimicrobial agent which was very toxic to animals. The agent was resolved into two crystalline components, named actinomycin A and B. The former was highly bacteriostatic and the latter highly bacteriocidal.

Streptomycin (5) is the most important antibiotic so far obtained from actinomycetes. Its antibacterial spectrum

includes both gram-positive and gram-negative organisms, moreover in pure form its toxicity is low. The original culture of Streptomyces griseus which had been kept in the culture collection of Dr. S.A. Waksman since 1915, does not now possess the property of producing streptomycin, and when recently tested was found to exert only very weak antibiotic effect against Bacillus subtilis and none against Escherichia coli (18).

Schatz, Bugie and Waksman (5), employed a medium very similar to the one recommended, in 1920, for production of the actinomyces (17). However, the amount of peptone was reduced by half and the hydrogen-ion concentration of the medium adjusted to pH 6.8. The new carbohydrate-protein ratio was arrived at in order to counterbalance the tendency toward development, during fermentation of too high an alkalinity. Waksman and coworkers stated that the production of streptomycin by Streptomyces griseus required the presence of a specific growth promoting substance which was supplied by 0.3 per cent meat extract or 1.2 per cent corn steep liquor. The medium finally used was composed of 0.3 per cent meat extract, 0.5 per cent peptone, 1 per cent glucose and 0.5 per cent sodium chloride.

In a later paper, Waksman and coworkers (20)dealt with the metabolism of Streptomyces griseus in surface and submerged culture. From the data given it appeared that the

rate of metabolism was greatly accelerated when submerged culture was used. However, lysis set in quite rapidly and the hydrogen-ion concentration changed to pH 8.9. The data for the fourth day were not given and the other results indicate that on this day the highest production of mycelium and streptomycin was obtained. Glucose had completely disappeared by the fifth day. It was impossible to correlate and compare the metabolic activities in stationary and submerged culture since the same variables were not considered in each experiment. It can only be said that with surface culture the rate of metabolism was slower, as judged by mycelium production; that the amino nitrogen reached its highest level coincident with the beginning of streptomycin production, and that the ammonia concentration increased continuously throughout.

Some investigations have been conducted to determine the role of meat extract in the medium and, as has already been stated, it was found that meat extract was required for streptomycin synthesis (5). Furthermore, dried mycelium of both active and inactive strains of Streptomyces griseus was able to replace meat extract, thus indicating that the mycelium contained a precursor of streptomycin. The organism was able to produce streptomycin in small quantities without meat extract or corn steep liquor showing that it could synthesize the streptomycin factor. It was proposed to designate the specific substance required for maximum

production of an antibiotic agent as "the activity factor" in contradistinction to the accepted term "growth factor" to indicate substances essential for maximum cell aynthesis.

Streptomyces griseus was reported to produce another antibiotic substance which was present mainly in the mycelium. The second substance was distinct from streptomycin since it was soluble in organic solvents and not active against gram negative organisms. There appeared to be no correlation between the production of streptomycin and the second antibiotic factor.

An antifungal substance has also been reported to be produced by Streptomyces griseus (21). This substance had a different antibacterial spectrum from streptomycin and moreover was soluble in chloroform. Apparently it was present in the medium and evidently its action differs from Waksman's "second antibiotic".

Rake and Donovick (22) studied various other materials as nutrients for Streptomyces griseus and devised a media giving high yields of streptomycin, which contains neither beef extract nor corn steep liquor. The problem with a meat extract-peptone medium lies in the fact that the extraction of streptomycin is rendered difficult because certain of the basic components of beef extract are concentrated along with streptomycin and are found as impurities in the final product. Rake and Donovick used soybean meal as the nitrogen

source along with glucose and sodium chloride. It was pointed out that the volume of medium used in shake-flask culture was an important factor in the concentration of streptomycin in the broth. A medium composed of soybean meal and glucose was distinctly inadequate as it yielded less than 10 units of streptomycin per ml. The addition of meat extract greatly improved the yield but not as much as the addition of sodium chloride. substituting sulphate-ion for chloride-ion had no significant effect on streptomycin production, but substituting magnesium for sodium gave lower streptomycin concentrations.

Indian workers (23), demonstrated that an enzymic hydrolysate of ground nut cake compared favorably with Waksman's medium in inducing streptomycin production by surface cultures.

Two other media have been reported as being satisfactory for streptomycin production, one by Le Page and Campbell (24), contains Bacto-yeast extract, glucose, sodium chloride, and small amounts of iron and magnesium sulphate and othe other by VanderBrook and coworkers (25), is composed of dextrose, Curbay B-G (soluble products from butanol fermentation, United States Industrial Chemicals, Inc.) sodium chloride, basic potassium phosphate, ammonium sulphate, magnesium sulphate and calcium carbonate .

It is evident that the media used industrially for streptomycin production tend to be less and less complex for the advantages in a defined synthetic medium are manifold. It has already been stated (9) that the variability of the actinomyces on complex organic media is pronounced and even slight changes in the carbon-nitrogen ratio will affect their cultural and biochemical characteristics. The composition of corn steep-liquor has been reported to vary from batch to batch, causing a lack of uniformity in the response (26). Furthermore, the isolation and purification of the antibiotic is made easier when a simple medium is used and the final antibiotic product is more potent, less toxic and altogether more suitable for clinical use.

METHODS AND PROCEDURES

Organism

In the early stages of this investigation the strain Actinomyces griseus 19 (Waksman) was employed as the experimental organism, however, it was soon replaced by a more active strain Actinomyces griseus 10 (Waksman)#. It was observed that, on continued transfer from slant to slant, the organism lost its ability to synthesize streptomycin. The original strain was therefore grown in soil culture and the following scheme employed to obtain slants for experimental work.

soil culture → master slant → inoculation slant
inoculation slant
inoculation slant

Difco nutrient agar was used as substrate in the preparation of the slant. The inoculated slant was incubated at 26.6°C for 72 hours and stored in the refrigerator for a period not exceeding two weeks.

Inoculation

The slant was flooded with 10 ml. of sterile distilled

Kindly supplied by Dr.G.A.Grant, Ayerst, McKenna and Harrison Ltd., Montreal, P.Q.

water. The practice of adding a drop of aerosol to loosen the spores was discontinued because it appeared to affect the production of streptomycin (27). Consequently the spores were loosened with a sterile needle and the spore suspension thoroughly mixed. 1 ml. of inoculum was used for 50 ml. of medium and that ratio was maintained throughout the work.

Culturing the Organism

Submerged culture was used throughout this investigation. During the earlier part of this research, 250 ml. Erlenmeyer flasks were employed, each containing 50 ml. of the medium, but later test tubes (25 x 200 mm) were used, and these were agitated in a slanting position. This method offered several advantages, e.g., the small volume gave greater economy, especially when working with expensive amino acids, easier handling, and a more uniform inoculum (one inoculation slant served 40 test tubes). The test tubes, each containing 10 ml. of medium, were plugged with cotton and autoclaved at 15 pounds pressure for 15 minutes. They were inoculated with 0.2 ml. of spore suspension and placed in a slanting position on the shaker. A slant of 23° proved best, giving a yield of streptomycin equivalent to that obtained with 50 ml. of medium in 250 ml. Erlenmeyer flasks on the same shaker and within the same period of time.

The shaker, which had a backward and forward motion (84 strokes per minute) was in a constant temperature room (26.6°C), constant humidity room (75% relative humidity). When streptomycin production reached a maximum, the tubes were removed from the shaker, and the original volume of the medium was restored to compensate for loss due to evaporation. The mycelium was separated by centrifuging or filtering, the medium placed in the refrigerator and aliquots withdrawn for analysis.

Microbiological Assay of Streptomycin

To estimate the streptomycin potency of culture, two methods were employed which were based upon the agar plate diffusion technique, using a streptomycin sensitive strain of Bacillus subtilis, The first method utilized the technique developed by Wilkins and Harris (28).

> 0.1 ml. of a spore suspension of Bacillus subtilis prepared as outlined belowwas distributed in each petri plate. This was covered with 20 ml. melted nutrient agar and the plate was well agitated to distribute the inoculum evenly. The petri plate was then placed in the refrigerator to harden the agar. When thoroughly chilled, holes (11 mm. diameter) were cut into the agar base with a cork borer. 0.1 ml. of the solution to be assayed was placed in each cup and the plate incubated at 32°C for at least 16 hours. The diameter of the zone of inhibition gave an indication of the potency of the test solution.

A second more refined technique replaced the above method. It was based upon the procedure developed for the assay of penicillin by Foster and Woodruff (29), and took

into consideration the special precautions observed in the assay of streptomycin as outlined by Loo and coworkers (30). Neither of the methods was followed in exact detail and modifications were introduced in the course of this investigation. The details of the method of assay actually used are as follows:

A spore suspension of a streptomycin sensitive strain of Bacillus subtilis was prepared by inoculating 100 ml. of Difco nutrient broth with the organism and cultivating the broth on a rotary shaker at 26.6°C. for seven days. The vegetative cells were killed by heating the broth to 80°C and holding it at that temperature for ten minutes. The spore suspension was stored in the refrigerator and used as required. The inoculum was held at a temperature as close to 0°C as possible, to prevent the reappearance of the vegetative cells. A further heat treatment of five minutes duration eliminated this trouble.

To petri dishes, selected for uniform flat bottoms, 10 ml. of melted nutrient agar were added and allowed to harden preferably in the refrigerator. For each plate, 0.1 ml. of spore suspension of Bacillus subtilis was added to 5 ml. of melted nutrient agar (55-60°C) and the seeded agar was agitated to distribute the spores uniformly throughout. Using an open mouthed pipette for quick delivery, 5 ml. of the seeded agar was pipetted onto the agar base and allowed to harden. In the meantime, a petri dish containing standard size penicylinders was heated over a water bath. This warmed the cylinders sufficiently so that when placed quickly on the agar, a uniform firm seal was effected between cup and agar. Four or five drops of the solution to be assayed were placed in each cup, the plate incubated at 32°C. and the zone of inhibition measured after sixteen hours.

In order to increase the accuracy in measuring the zone of inhibition, a specially constructed box was used. All samples to be assayed were diluted with 0.1 M phosphate buffer (pH 7.8) in the proportion of 1:10, so as to minimize the effect on the assay of the hydrogen-ion and salt concentration of the media (30). For a time the cups were cleaned in cleaning solution, but thorough rinsing failed to remove all the acid and so instead they were suspended in the phosphate buffer, rinsed with water and dried and sterilized with dry heat.

Most of the results are expressed as zones of inhibition in millimeters, however, when "S" units were introduced (31) a standard curve# was prepared for that particular experiment.

Extraction and Purification of Streptomycin

A modification of the method of Brook and coworkers (25) was employed to prepare streptomycin concentrates. As outlined later, media were developed which changed the distribution of streptomycin between mycelium and medium. The greater part of the streptomycin was now in the mycelium and the modification of the extraction procedure described below is concerned with the recovery of streptomycin from the mycelium.

The mycelium was separated by centrifuging and washing thoroughly with distilled water. When the washings were water clear, the washed mycelium was freeze-dried in order to break up the mycelium. When dry, the mycelium was powdered in a mortar and suspended in 20 per cent acetone made acid with sulphuric acid to pH 2.5. The mycelium was then filtered-off and washed twice with the acidified acetone. The washings were all combined and more acetone added to increase the concentration to 75-80 per cent. The solution turned milky and was placed in the refrigerator overnight. The supernatant was decanted, the residue taken up in a minimum amount of water and the solution neutralized with sodium hydroxide. The solution was freeze-dried and the streptomycin concentrate, a very light colored non-hygroscopic powder, was stored in the cold.

A sample of streptomycin concentrate of known potency was kindly supplied by Dr. G.A. Grant, Ayerst, McKenna and Harrison Ltd., Montreal.

Analytical Methods

Glucose was determined by Hanes (32) modification of the Hagedorn Jensen method. pH hydrogen-ion concentration was with a Hellige pH-meter. The media were all adjusted to pH 6.8 - 7.0 before each run. Histidine was determined according to Macpherson's (33) modification of the Hanke-Koessler reaction. Ammonia was measured by the method of Van Slyke and Cullen (34) using 20 per cent sodium carbonate instead of a saturated solution of potassium carbonate and aerating for at least four hours and usually overnight, to insure complete distillation. Amino nitrogen was determined by the method of Van Slyke as outlined in the "A.O.A.C. Book of Methods" (35). The weight of mycelium was determined by filtering or centrifuging, weshing thoroughly and weighing after drying at 80°C.

EXPERIMENTAL

I. Fractionation of Two Industrial By-products which Stimulate Streptomycin Production

Introduction

Several industrial and agricultural products when used as substrates for growth of Streptomyces griseus were shown to promote a very high production of the antibiotic. Since it was desirable to simplify the medium as much as possible in order to facilitate the isolation of the antibiotic, fractionation studies were carried out to:

a. Devise a simple and effective medium for streptomycin production.

b. Identify the factor or factors which stimulate the production of streptomycin.

A. Fractionation of Fish Solubles.

This preparation is a concentrate of fish-press juice, containing 50 per cent solids. When used as a medium in concentrations of 2 per cent (1 per cent solids) it promoted streptomycin production equal to that of Waksman's medium (5) (0.3 per cent meat extract, 0.5 per cent peptone, 1 per cent glucose, 0.5 per cent NaCl). The following fractionation of fish solubles was carried out:

The fish syrup was diluted with water and then adjusted to pH 3 with 2 N H_2SO_4

insoluble — soluble

evaporated under CO_2, residue suspended in 50% ethanol

soluble — insoluble

70% ethanol

insoluble — soluble

80% ethanol

soluble — insoluble

over 90% ethanol

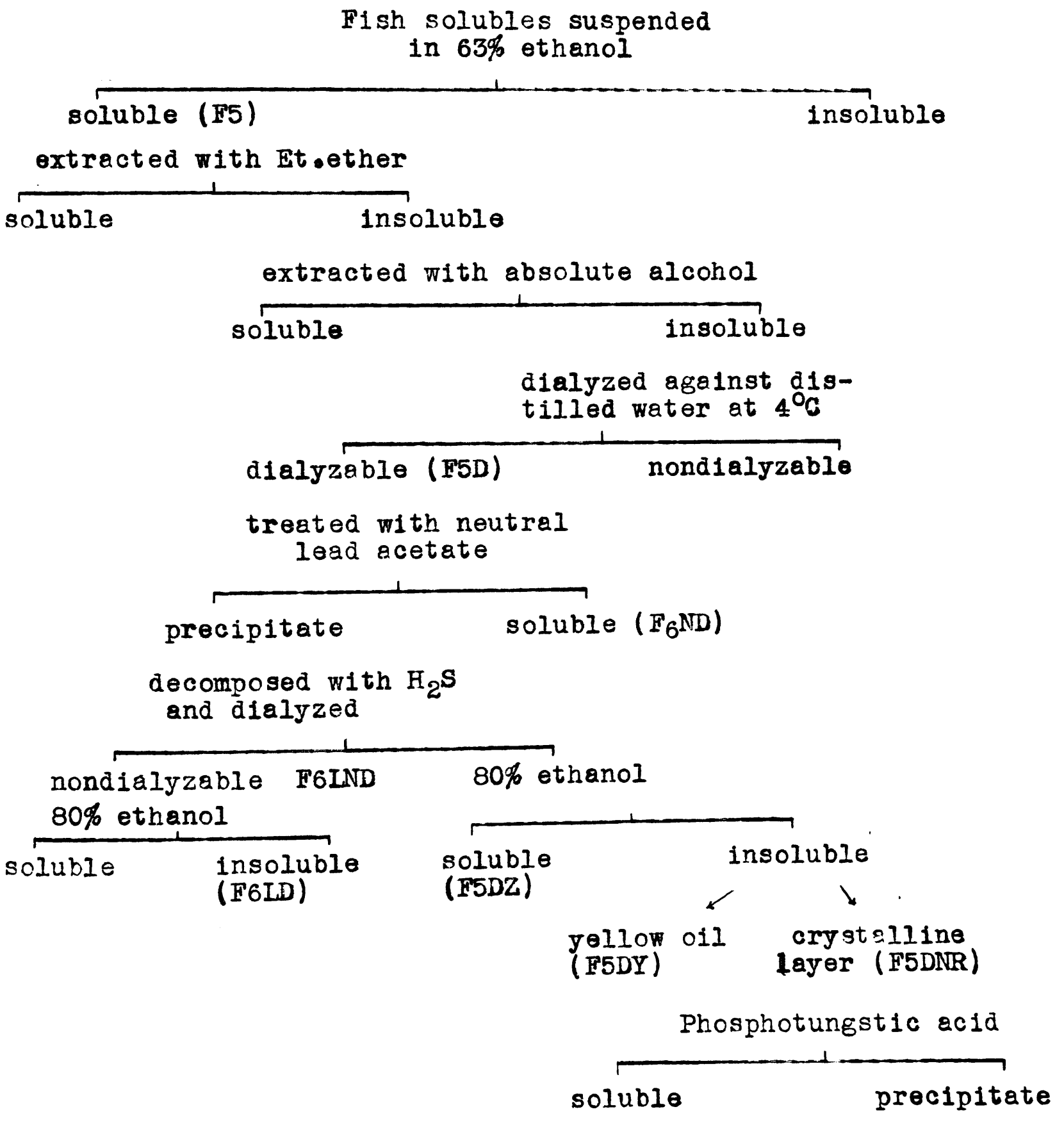
soluble — insoluble

acetone

soluble — insoluble

Waksman's medium without meat extract showed practically no streptomycin production and was therefore adopted as the basal medium. The addition of 1 per cent fish solubles to this medium stimulates streptomycin production to a level equivalent to 0.3 per cent meat extract. Consequently the fractions obtained in the above scheme were added to the basal medium in amounts equivalent to 1 per cent of the original fish syrup.

The fractions soluble in 50 per cent alcohol and insoluble in 70 and 80 per cent alcohol induced streptomycin synthesis, thus indicating that ethanol could be used to concentrate the active fraction(s). A second more elaborate fractionation was carried out according to the following scheme, based upon the preceding results.



Each fraction was tested at different levels but always at concentration equivalent to a 1 per cent solution of the original fish solubles. The active fractions are indicated in the above schematic outline by initials. It was possible to effect a ten-fold concentration of the active factor, indicating the possibility of preparing concentrates which would stimulate streptomycin production (Table I). Phosphotungstic acid appeared to be inhibitory to the organism, since the fractionation of the active F5DNR resulted in two inactive components which even when assayed together showed no further stimulation of streptomycin production.

TABLE I - ASSAY RESULTS OBTAINED WITH FRACTIONS FROM FISH SOLUBLES

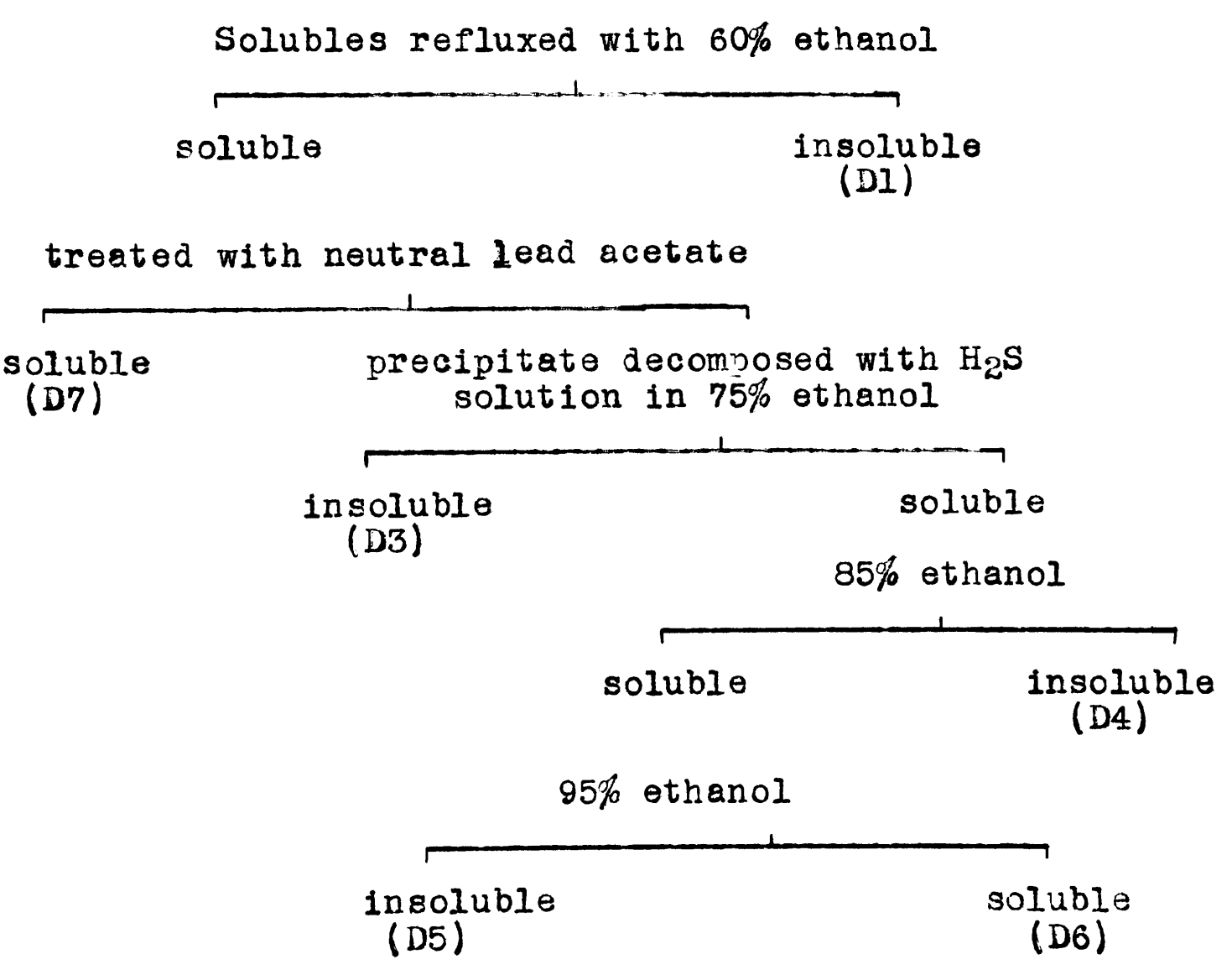
Medium	Zone of inhibition (mm)
Basal medium alone	17.0
Basal medium + 1.0% fish solubles	33.0
Basal medium + 0.2% F5	32.0
Basal medium + 0.19% F5D	33.5
Basal medium + 0.025% F6ND	20.0
Basal medium + 0.03% F6LND	26.0
Basal medium + 0.05% F6LD	28.0
Basal medium + 0.05% F5DY	32.0
Basal medium + 0.5% F5DZ	32.0
Basal medium + 0.025% F5DNR	30.0

Basal medium: Waksman's medium without meat extract.

The active components were dialyzable, precipitated by lead acetate and insoluble in 80 per cent ethanol.

B. Fractionation of Distillers Solubles.

Distillers solubles is a concentrate of spend mash and yeast cells obtained as a by-product of the fermentation alcohol industry. As a basal medium, it did not promote a high streptomycin production. This was thought to be due to the presence of inhibiting substances so a fractionation with alcohol was carried out. In this case ethanol would be a most suitable fractionating solvent since the process could be carried out in the distillery.



The active components of distillers solubles were apparently split by the treatment with lead acetate. This was in contrast to the fractionation of fish solubles where all the active components were precipitated. The presence of inhibiting substances in the original solubles was indicated by the fact that the component fractions induced a higher streptomycin production than the original solubles (Table II).

TABLE II - ASSAY RESULTS OBTAINED WITH FRACTIONS OF DISTILLERS SOLUBLES

Medium	Zone of inhibition (mm)
Basal medium alone	17
Basal medium + 1.0% solubles	27
Basal medium + 0.2% D1	17
Basal medium + 0.1% D3	33
Basal medium + 0.1% D4	30
Basal medium + 0.1% D5	28
Basal medium + 0.1% D6	21
Basal medium + 0.2% D7	34

Basal medium: Waksman's medium without meat extract.

Active fractions were precipitated in a range of ethanol concentration from 65-95 per cent whereas with fish solubles all the activity was removed by treatment with 80 per cent ethanol. It is possible that the factors which

stimulated streptomycin synthesis may be linked with different type molecules, thus explaining the different results in the two fractionations.

Fraction D7 was studied by means of electrodialysis, carried out at room temperature, with the initial pH 5.4, a current of 220 volts D.C. adjusted to 0.06 amps by a resistance of 3000 ohms. The membrames were sheets of parchment, and the area of the electrodes was 3.2 square centimeters.

The growth promoting substances appeared to dialyze quite rapidly and concentrated in the anode compartment of the dialysis apparatus. The process was discontinued after twelve hours, the results indicating that an active concentrate could be prepared. A more complete experiment was therefore attempted using a slurry of distillers solubles. The condition of dialysis was the same as before. After 48 hours the apparatus was emptied and the fractions assayed (Table III). The nondiffusible fraction and the fraction in the anode compartment contained precipitates which were centrifuged-off and analyzed separately.

TABLE III - ELECTRODIALYSIS OF DISTILLERS SOLUBLES

Medium	Zone of inhibition (mm)
Basal medium alone	17.0
Basal medium + 0.1% anode fraction	24.0
Basal medium + 0.1% anode insoluble fraction	17.0
Basal medium + 0.1% cathode fraction	0
Basal medium + 0.1% nondiffusible fraction	19.0
Basal medium + 0.1% nondiffusible insoluble fraction	17.0

Basal medium: Waksman's medium without meat extract.

The low activity of the anode components was surprising, particularly in view of the results obtained with the dialysate of fraction D7. It was questionable whether the deep color of the fraction was associated with activity in view of its slow accumulation during the experiment. The components of the anode fraction were subjected to another dialysis and samples were taken during the experiment. It was found that the active components accumulated rapidly while the colored substance, with inhibitor properties, diffused slowly. The active fraction could also be precipitated with 90 per cent acetone and it had an alkaline reaction, whereas the acetone soluble part was inactive and neutral in reaction.

II. Development of a Chemically Defined Medium Promoting Maximum Streptomycin Production

Introduction

As already mentioned in the literature review, it had been known for some time that actinomyces will thrive best in nutrient media containing complex organic nitrogen sources, such as proteins or their breakdown products, i.e., peptides and amino acids. It has also been reported that streptothricin was a by-product of the nitrogen metabolism of Actinomyces lavendulae (36), certain amino acids stimulated the production of the antibiotic, others did not.

It was thought that a study of the exact nutrient requirements of Streptomyces griseus for streptomycin synthesis, could best be accomplished by developing a synthetic medium and conducting a study of the metabolism of the organism in that medium.

A. Inorganic Requirements

From the results obtained with the fractions of fish and particularly distillers solubles, it was indicated that the active products contained inorganic ions probably combined with certain organic substances of low molecular weight; the availability of the inorganic component

apparently depended on the type of organic complex it was bound to. With fish and distillers solubles, and fraction D7, the addition of inorganic salts did not stimulate streptomycin production. A pronounced increase in streptomycin yield was obtained when a salt mixture was added to media containing wheat germ extract# and peptone (Table IV).

Meat extract, distillers solubles and fish solubles were ashed at 550°C and the ash added to the basal medium. Both strains of Streptomyces griseus, Waksman 10 and 19 were tested and were found to vary considerably in their requirements for streptomycin synthesis. The more active strain showed no variation in streptomycin production when the ash was substituted for the complex. However, the maximum production of streptomycin occurred on the fourth day whereas with fish solubles, fraction D7 and meat extract the fermentation took only three days. The active strain was unaffected by considerable variation in the amount of inorganic salts. Results with the less active slant indicated that factors other than inorganic salts were required for maximum streptomycin production. It is also possible that precursors of streptomycin containing inorganic-ions are only slowly synthesized by this strain, when these inorganic-ions are supplied in the medium.

The sample was kindly prepared by V.K.Collins, Ogilvie Flour Mills Co., Montreal, by extracting defatted wheat germ with 0.1% NaOH and acidifying the extract to pH 4.8. The precipitated protein was removed, the extract adjusted to pH 6.9 and concentrated. The solids contained 51% sugars, 3.5% nitrogen and 21.1% ash.

TABLE IV - THE EFFECT OF ADDING THE ASH PREPARED FROM THREE CONCENTRATES TO A BASAL MEDIUM OF PEPTONE-GLUCOSE-NaCl

Medium	Zone of inhibition (mm) Strain Waksman 19	Strain Waksman 10
Basal medium alone	16.0	17.0
Basal medium + 0.3% D7	28.0	31.0
Basal medium + 0.3% D7 + 0.12% ash of dist. solubles	26.0	31.0
Basal medium + 0.12% distillers solubles ash	25.0	31.0
Basal medium + 1.0% fish solubles	28.5	30.0
Basal medium + 1.0% fish solubles + 0.12% ash of fish solubles	26.0	31.0
Basal medium + 0.12% ash of fish solubles	24.0	30.5
Basal medium + 0.3% meat extract	28.5	31.0
Basal medium + 0.3% meat extract + 0.12% ash of meat extract	27.0	30.0
Basal medium + 0.12% ash of meat extract	25.0	31.0
Basal medium + 0.4% wheat germ extract		23.0
Basal medium + 0.4% wheat germ extract + 0.12% ash of distillers solubles		30.0

Because of its greater adaptibility and higher streptomycin production, strain Streptomyces griseus 10 Waksman was selected for future study. The concentration of the ash in the medium could vary over a wide range before it affected the

yield of streptomycin. Maximum streptomycin production was maintained within the range of 0.036 - 0.32 per cent ash.

By analysis of the inorganic salts in distillers solubles (36) the amount of each ion equivalent to the original concentration in a medium containing one per cent distillers solubles was added to a basal medium (Table V).

TABLE V - THE EFFECT OF INORGANIC-IONS ON THE PRODUCTION OF STREPTOMYCIN

Medium	Zone of inhibition (mm)	Medium	Zone of inhibition (mm)
Basal medium alone	16.0	Basal medium + ash but minus Ca	31.5
Basal medium + complete ash	31.5	Basal medium + ash but minus Si	31.5
Basal medium + ash but minus K	20.0	Basal medium + ash but minus Co	31.0
Basal medium + ash but minus P	18.0	Basal medium + ash but minus Mn	31.5
Basal medium + ash but minus Mg	26.5	Basal medium + ash but minus S	31.0
Basal medium + ash but minus Fe	26.0	Basal medium + ash but minus Cu	29.5

Basal medium: Waksman's medium without meat extract

In a further experiment each ion was added in normal and double concentration to the basal medium, but no increased streptomycin production resulted. The combination of ions which produced stimulation equivalent to the complete ash, was K,P, Mg, Fe, Cu and Na. The effect of various other ions was studied

but none stimulated streptomycin synthesis. The minimum amount of phosphorus which sustained maximum streptomycin production with all other ions present, was found to be 3.2 mg P. per 50 ml of medium. The minimum amount of ash required for maximum production of streptomycin was 18 mg per 50 ml of medium and contained 2.8 - 3.0 mg phosphorus. It would appear, therefore, that the phosphorus content of the ash was the limiting factor determining the amount of ash required to induce maximum streptomycin production.

The following salt mixture, containing each of these essential elements, was formulated by calculation of the actual content of each element to a medium of 1 per cent distillers solubles which in turn was based on the analysis of the ash of distillers solubles.

KNO_3	0.05%
$Na2HPO_4\ 12H_2O$	0.18%
$MgCl_2 \cdot 6H_2O$	0.05%
$FeCl_3 \cdot 6H_2O$	0.003%
$CuSO_4$	0.001%

The basal medium containing this salt mixture plus 0.5 per cent NaCl, induced maximum production of streptomycin.

B. Growth Factors

Before studying in detail the nitrogen requirements of

Streptomyces griseus, it was thought advisable to examine the growth factors in regard to their effect on streptomycin production. The composition of the basal medium was as follows:-

Glycine 0.4% (equal in nitrogen content to .5% peptone)

Glycose 0.8%

NaCl 0.5%

Salt mixture (as described above).

The growth factors were added individually and streptomycin and mycelium production were measured. The results are given in Table VI.

TABLE VI - THE EFFECT OF GROWTH FACTORS ON THE PRODUCTION OF STREPTOMYCIN AND MYCELIUM

Medium	Zone of inhibition (mm)	Weight of mycelium mg/50 ml of culture
Basal medium alone	22	64
50 ml Basal medium + 10 mg thiamine hydrochloride	22.5	68
50 ml Basal medium + 10 mg riboflavin	23	66
50 ml Basal medium + 10 mg nicotinic acid	24	62
50 ml Basal medium + 10 mg folic acid	21	48
50 ml Basal medium + 10 mg pyridoxine hydrochloride	19	47
50 ml Basal medium + 10 mg para amino-benzoic acid	20	52
50 ml Basal medium + 10 mg Ca pantothenate	21	53
50 ml Basal medium + 10 mg choline chloride	22	67
50 ml Basal medium + 25 mg/50 cc. inositol	25.5	80

Inositol increased the yield of both streptomycin and mycelium, riboflavin and nicotinic acid seemed to have some effect on stimulating streptomycin production but none of mycelium formation (Table VI). The results, even with inositol present, were significantly lower than those obtained with Waksman's medium thus indicating the need for a detailed study of the nitrogen source.

C. Nitrogen Requirements

As pointed out by Waksman (14), actinomyces develop most readily if complex nitrogenous substances such as proteins are included in the medium. Chemically defined media have been used in the study of the organisms and the nitrogen source was either an amino acid, urea, creatine, nitrate or ammonium salt. However growth was slow (10) and streptomycin production was poor.

All naturally occurring amino acids were incorporated individually into basal medium in amounts so that the total nitrogen content was equal to that of a 0.5 per cent solution of peptone. An exception was made in the case of histidine and tryptophane since the utilization of the ring nitrogen is in doubt. The basal medium was composed of 1.0 per cent glucose, salt mixture and 0.0005 per cent inositol. In all subsequent experiments, penicylinders were employed in the assay, hence, the smaller zones of inhibition. The results are given in Table VII.

TABLE VII - THE EFFECT OF SPECIFIC AMINO ACIDS ON STREPTOMYCIN PRODUCTION

Addition to Basal medium#	Zone of inhibition (mm)	Addition to Basal medium#	Zone of inhibition (mm)
0.4% glycine	17.0	0.52% alanine	17.0
0.3% arginnine	18.0	1.0% histidine	25.0
0.94% glutamic	17.0	0.37% asparagine	19.0
0.69% cystine	0	0.74% norlencine	19.0
0.52% lysine	17.0	0.85% methionine	0

#Basal medium: 1.0% glucose, inorganic salt and 0.0005% inositol.

The efficiency of the individual acids in inducing mycelium and strepomycin production was variable and stimulation was not confined to any particular group. In general, highest antibiotic titers were obtained on the fifth day. Histidine at a concentration of 1 per cent, gave the highest streptomycin production, whereas the sulphur containing amino acids gave no streptomycin. This can be explained by the inactivation of streptomycin by sulphydryl groups. The optimum ratio of histidine to glucose was next studied. Mycelium production was estimated visually, a growth of 5 representing the optimum for that experiment (Table VIII).

Apparently, 0.7 per cent histidine and 1.3 per cent glucose is the best ratio for optimum yields. Histidine could not be replaced by the other two basic amino acids, lysine and arginine. However, if half of the histidine was

TABLE VIII - THE EFFECT OF THE CARBON-NITROGEN RATIO (GLUCOSE:HISTIDINE) ON THE PRODUCTION OF STREPTOMYCIN AND MYCELIUM

Medium#		Zone of inhibition (mm)	Mycelium production
% Histidine (monohydrochloride)	% Glucose		
0.1	1.0	19.0	1
0.2	1.0	20.5	3
0.5	1.0	23.0	4
1.0	1.0	24.5	5
2.5	1.0	22.0	4
0.7	1.5	24.5	5
0.7	1.3	25.5	5
0.7	1.0	23.5	5
0.7	0.7	22.0	4

In addition the medium contained inorganic salts and 0.0005% inositol.

replaced by an equivalent amount of histamine, the yield of streptomycin was equal to or greater than with histidine alone. Inorganic nitrogen source such as nitrate or ammonium salts could not be substituted for histidine, even in part.

With a view to obtaining a higher streptomycin production, one half of the histidine was replaced by the other amino acids in amounts to give a total nitrogen content equal

to that of 0.25 per cent peptone which is half of the usual peptone concentration. Mycelium production was estimated visually (Table IX).

TABLE IX - THE EFFECT OF COMBINATIONS OF AMINO ACID ON STREPTOMYCIN AND MYCELIUM PRODUCTION

Amino acid combinations favoring high streptomycin production	Zone of inhibition (mm)	Amino acid combinations favoring high mycelium production	Zone of inhibition (mm)
Addition to Basal Medium#		Addition to Basal Medium#	
0.35% histidine-0.35% valine	27	0.35% histidine-0.47% glutamic	18
0.35% histidine-0.15% arginine	26	0.35% histidine-0.38% aspartic	20
0.35% histidine-0.26% alanine	25	0.35% histidine-0.34% cystine	20
0.35% histidine-0.33% proline	25	0.35% histidine-0.48% phenyl alanine	22
0.35% histidine-0.34% threonine	26	0.35% histidine-0.50% tyrosine	23

Basal Medium contained 1.3% glucose, inorganic salts and 0.0005% inositol.

It was possible to distinguish between the mechanism of mycelium and streptomycin production. To obtain still higher yields of the antibiotic, combinations of amino acids were tested in which each of the amino acids contributed one third of the total nitrogen in a medium containing 0.5 per cent peptone (Table X).

All combinations containing valine proved superior. The histidine-valine-arginine medium produced less mycelium and, therefore, a higher yield of streptomycin per milligram of mycelium, than did the histidine-valine-threonine combination.

TABLE X - THE EFFECT OF COMBINATIONS OF AMINO ACIDS ON STREPTOMYCIN PRODUCTION

Addition to Basal medium#	Zone of inhibition (mm)
0.23% histidine, 0.23% valine, 0.22% proline	24.0
0.23% histidine, 0.1% arginine, 0.33% tyrosine	20.0
0.23% histidine, 0.23% threonine, 0.18% lysine	22.0
0.23% histidine, 0.1% arginine, 0.23% valine	26.0
0.23% histidine, 0.23% proline, 0.33% tyrosine	20.5
0.23% histidine, 0.23% valine, 0.23% threonine	25.5
0.23% histidine, 0.23% proline, 0.18% lysine	23.0

#Basal medium: 1.3% glucose, inorganic salts, inositol.

The effect of varying the concentration of histidine, valine and arginine was next studied (Table XI).

TABLE XI - THE EFFECT OF VARYING PROPORTIONS OF HISTIDINE, VALINE AND ARGININE ON STREPTOMYCIN AND MYCELIUM PRODUCTION

Addition to Basal medium#	Zone of inhibition (mm)	Growth
0.23% histidine, 0.23% valine, 0.1% arginine	24.5	4
0.23% histidine, 0.46% valine, 0.1% arginine	25.5	4
0.23% histidine, 0.12% valine, 0.1% arginine	22.5	4
0.1% histidine, 0.23% valine, 0.2% arginine	23.5	3
0.05% histidine, 0.46% valine, 0.2% arginine	22.5	2
0.05% histidine, 0.23% valine, 0.3% arginine	22.5	2

#Basal medium: 1.3% glucose, inorganic salts, inositol.

If the amount of histidine was kept constant and the valine concentration varied there was a direct correlation between valine concentration and streptomycin production but not with mycelium formation. The concentration of histidine must be above a certain level if this was to be observed. If the amount of valine was constant and the amount of histidine varied, there was a direct correlation between histidine concentration and streptomycin and mycelium production. Arginine seemed to have little effect on the production of streptomycin or mycelium.

The medium containing 0.23 per cent histidine, 0.23 per cent valine and 0.1 per cent arginine was compared with Waksman's medium and the zone of inhibition expressed in "S" units of streptomycin. Waksman's medium produced 180 "S" units, whereas the synthetic medium gave up to 250 "S" units per ml of medium.

A study was undertaken to see if histidine or valine should be replaced by compounds containing the imidazole nucleus. It was found that histamine could replace up to half of the histidine without affecting the yield of streptomycin. Carnosine replaced histidine effectively. Imidazole, guanido-glyoxaline hydrochloride, nucleic acid, barbitol and uric acid failed to substitute for histidine. Particularly interesting was the complete ineffectiveness of imidazole to replace histidine. The imidazole sample was not pure, however, and may have contained inhibiting substances.

Isovaleric acid did not replace valine although tested at many levels. Apparently the nutrient requirements of the organism for streptomycin synthesis is quite specific.

III. Studies on the Metabolism of Streptomyces griseus Grown on Chemically Defined Media

In this investigation it was observed that replacing valine with aspartic acid greatly enhanced the mycelium production but lowered significantly the yield of the antibiotic. When the metabolism of Streptomyces griseus was studied, using a complex medium as substrate, it was found (20) that maximum strepomycin production coincided with maximum mycelium formation. It was hoped that a detailed study of two synthetic media, one supporting streptomycin production and the other mycelium production, would show which metabolic changes were associated with growth and which with the formation of the antibiotic.

The two media had the following composition:

Medium I	Medium II
0.23% valine	0.23% aspartic acid
0.23% histidine	0.23% histidine
0.10% arginine	0.10% arginine
1.30% glucose	1.30% glucose
inorganic salts	inorganic salts
0.0005% inositol	0.0005% inositol

The media were prepared in batches and then dispensed into the tubes. All results are for duplicate experiments and all the analyses were performed on the same day the sample was withdrawn from the tube at the shaker. The results are presented in tabular form and in graphs, the percentage is expressed relative to the highest value obtained for any one variable with both media which is rated as 100 per cent (Tables XII and XIII; Graphs I and II).

The metabolism in the aspartic acid medium was characterized by a high production of mycelium, rapid uptake of glucose and histidine, ammonia production, increase in alkalinity and a low yield of streptomycin. The lag phase was only about twenty-four hours and then changes occur which are characteristic of rapid growth. The results obtained resemble those reported by Waksman and coworkers (20) using complex media. It can be concluded that the changes are all characteristic of growth and can not be correlated with the streptomycin producing mechanism. Histidine and glucose are consumed at approximately the same rate and practically disappear at the time mycelium production is maximal. The alkalinity increases steadily and is directly related to the corresponding rise in ammonia. Streptomycin production is low and bears no relation to mycelium production.

Up to the fifth day, the metabolism of the valine medium was characterized by less mycelium, slower uptake of glucose

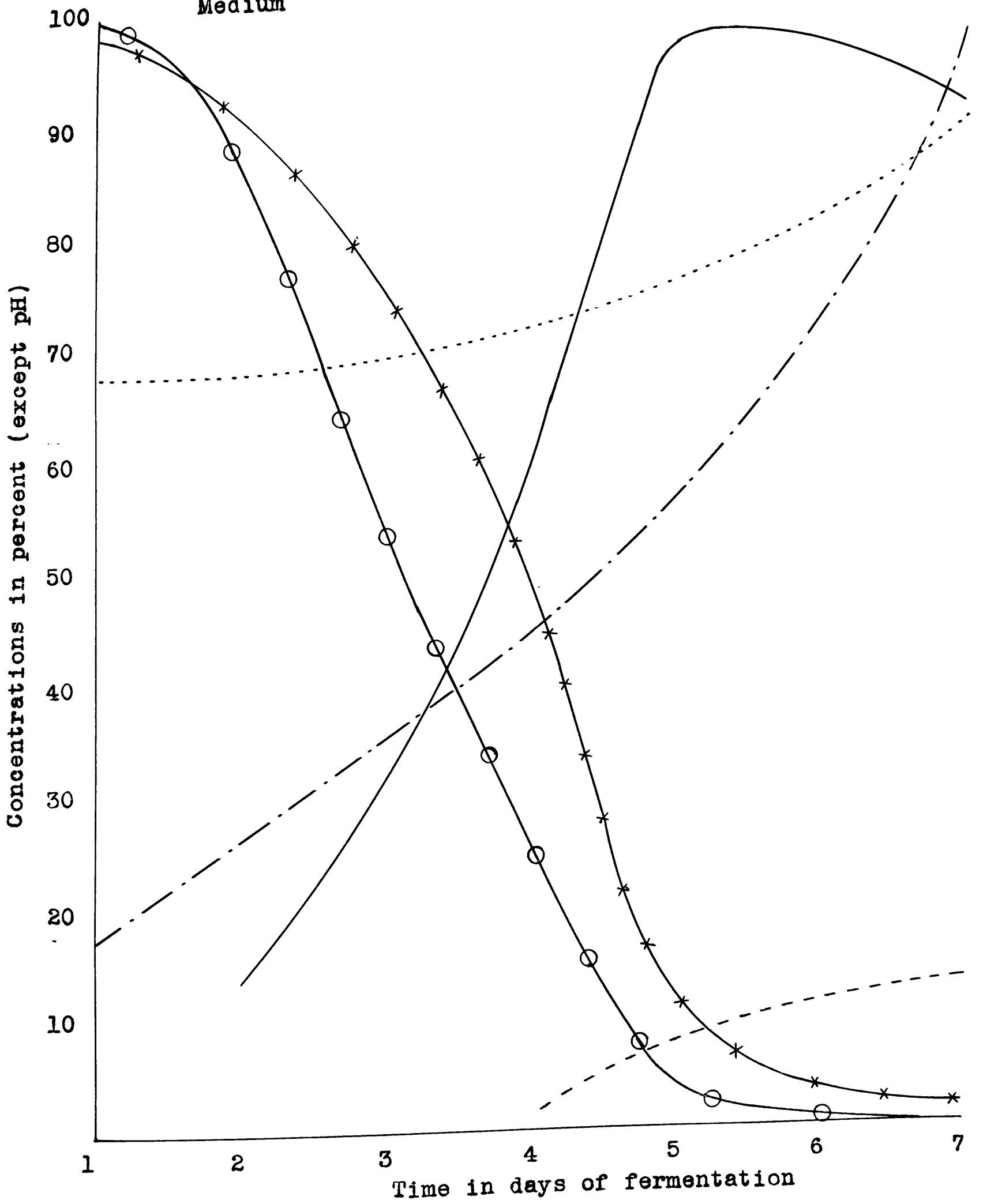
TABLE XII - METABOLIC CHANGES DURING GROWTH IN A HISTIDINE-VALINE-ARGININE MEDIUM

Analysis of Medium	Before sterilization	After sterilization	Duration of Fermentation 1 day	2 day	3 day	4 day	5 day	7 day
pH	6.90	6.90	6.90	6.80	6.75	6.70	6.85	8.50
Residual glucose (mg per ml)	12.2	12.4	12.4	11.3	10.6	8.8	6.25	0.60
Mycelium production (mg per 20 ml)	-	-	-	5	7	20	48	65
Ammonia (mg per 10 ml)	0.68	0.88	0.89	0.88	0.90	0.90	0.91	5.32
Residual histidine (mg per ml)	2.15	2.15	2.15	2.15	1.95	1.90	1.68	0.523
Streptomycin production ("S" units per ml)	-	-	-	-	-	40	230	80

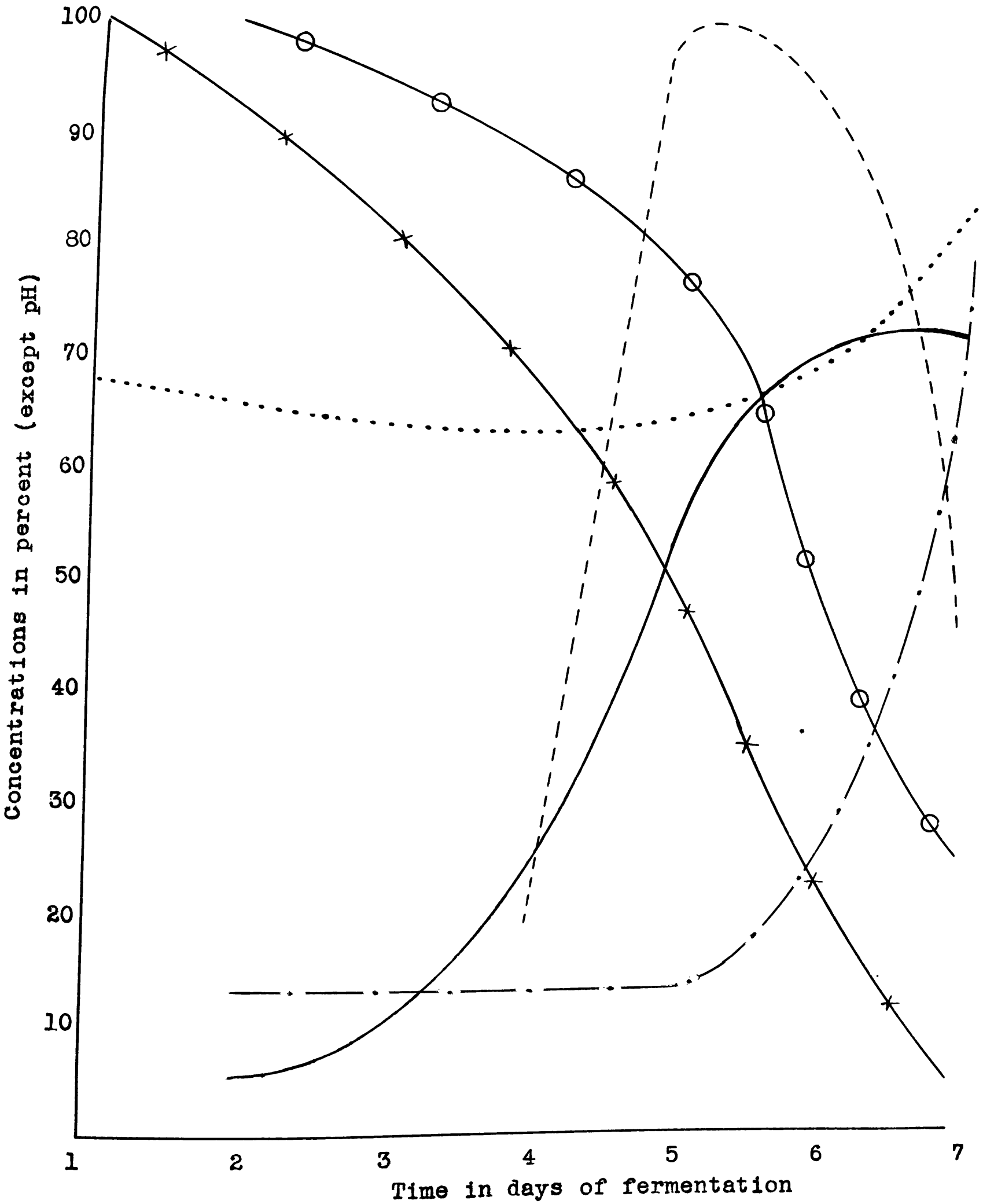
TABLE XIII - METABOLIC CHANGES IN THE HISTIDINE-ASPARTIS ACID-ARGININE MEDIUM

Analysis of Medium	Before sterilization	After sterilization	Duration of Fermentation 1-day	2-day	3-day	4-day	5-day	7-day
pH	6.90	6.80	6.80	6.80	7.10	7.30	7.70	9.10
Residual glucose (mg per ml)	12.2	12.4	12.2	11.3	9.4	6.0	1.25	0.25
Mycelium production (mg per 20 ml)	-	-	-	11.5	29.1	55.5	85.0	80.0
Ammonia (mg per 10 ml)	0.886	1.06	1.06	1.86	2.40	2.94	4.17	6.60
Residual histidine (mg per ml)	2.15	2.15	2.15	1.88	1.10	0.55	0.06	0.02
Streptomycin production ("S" units per ml medium)	-	-	-	-	-	10	18	30

GRAPH I. Metabolic Changes in the Histidine-Aspartic acid Medium



GRAPH II. Metabolic Changes in the Histidine-Valine Medium.



and histidine, practically no formation of ammonia, no change in hydrogen-ion concentration but a high yield of streptomycin. The lag phase was of longer duration and although mycelium production was rapid from the third day on, the concentration of ammonia and the hydrogen-ion concentration remained constant. It was not until the fifth day that the overall changes resembled those in complex media. It is doubtful whether any of these factors are associated with the streptomycin producing mechanism of stimulate production. It can be tentatively concluded that the development of an alkaline reaction in complex media has no effect on the production of streptomycin.

It was possible to replace aspartic acid with glutamic or even with succinic acid plus nitrate and still obtain the same results as with aspartic acid, namely, high mycelium but low streptomycin production. From these data, it is suggested that the metabolism of the aspartic acid, involving the aspartase-transaminase reaction, increases mycelium production at the cost of streptomycin, whereas another, as yet unexplained, mechanism operates in the case of valine. It appears as if the mycelium production does not parallel streptomycin production if the aminopherase-aspartase systems are predominantly involved in the synthesis of mycelial protein.

At the time of maximum streptomycin production with

valine, that is on the fifth day, about 80 per cent of the histidine and 50 per cent of the glucose had not been metabolized. Perhaps, therefore, the medium could yield another crop of streptomycin when additional nutrients were added. On the fifth day of the fermentation the mycelium was filtered off and one per cent norit A was added. After shaking for fifteen minutes, the carbon was filtered-off and the medium enriched with the inorganic salt mixture and glucose in amounts equivalent to 50 per cent of the original concentration. The medium was re-sterilized and inoculated, and on the fifth day a second yield of streptomycin was obtained amounting to 160 "S" units per ml of medium.

A third run was attempted, but the yield of streptomycin did not exceed 80 "S" units per ml, and the medium turned acid (ph 5.5) presumably because of an unfavorable carbon-nitrogen balance.

IV. Studies on the Distribution of Streptomycin Between Mycelium and Medium

In the study of the nitrogen requirements of Streptomyces griseus, with respect to streptomycin production, the replacement culture technique was introduced in order to speed up the investigation. The organism was cultured in Waksman's medium. When maximum streptomycin production was achieved,

the mycelium was filtered, washed thoroughly, suspended in 0.1M phosphate buffer, pH 7, and stored in the refrigerator. Aliquots of the mycelium, with amino acids added, were placed on the reciprocal shaker for twenty-four hours. Assays showed a high streptomycin yield which was the same with the control (no amino acids added) and the media enriched with amino acids. Moreover, the inoculum taken directly from the refrigerator showed the same assay as when shaken at 26.5°C. for twenty-four or forty-eight hours. It was, therefore, concluded that streptomycin had been produced while the inoculum was in storage at 4°C. and in all probability had dialyzed out from the mycelium into the medium.

To prove this further the mycelium, at the logarithmic phase of growth, was suspended in phosphate buffers of different molarities. One aliquot of the mycelium was ground with sand in a mortar and the macerated mixture extracted with phosphate buffer. All were stored in the cold and none showed any significant production of streptomycin. This proved that if the mycelium does not contain any streptomycin before being suspended in the buffer, none will diffuse into the medium even although the organism is at its most active stage of growth.

It was shown that without sodium chloride Waksman's medium gave a low yield of streptomycin (5). Furthermore, all media so far developed, which gave high streptomycin production,

contained sodium chloride (24) (25). One author reported that the chloride-ion could be replaced by the sulphate-ion, but the sodium-ion could not be replaced by magnesium (22).

Two media were prepared, i.e., Waksman's medium with and without sodium chloride, and tested for streptomycin and mycelium production. The mycelium was separated by centrifuging and a difference in appearance was observed. Mycelium from the sodium chloride medium was dense in appearance, occupying only a small volume in the centrifuge tube and had a dark color. The mycelium grown on the sodium chloride deficient medium was light colored, fluffy in appearance and occupied a volume about two and one half times that of the mycelium from the sodium chloride. When washed, dried and weighed, however, the amount of mycelium produced in both cases was about the same. However, with the sodium chloride media the streptomycin content of the medium was higher.

A study of the metabolism of Streptomyces griseus in both media was carried out to elucidate the function of the high concentration of sodium chloride in Waksman's medium. The results with both media are given in one table, Table XIV, since there was no significant difference attributable to the influence of sodium chloride. However, as reported previously, the yield of streptomycin and the physical consistency of the mycelium was not the same in the two media. The overall destruction of streptomycin was rapid, either it was

TABLE XIV - METABOLIC CHANGES IN WAKSMAN'S MEDIUM WITH AND WITHOUT SODIUM CHLORIDE

Period of fermentation	pH#	Streptomycin ("S" units per ml) With NaCl	Streptomycin ("S" units per ml) Without NaCl	Glucose (mg per ml)#	Ammonia (mg per 10 ml)#	Mycelium (mg per 10 ml)#	Amino nitrogen (mg per 10 ml)#
0	6.9	-	-	9.5	0.15	-	2.4
1 day	7.5	-	-	9.0	0.29	28	2.0
2 days	7.5	130	25	5.0	0.27	104	1.0
3 days	7.9	180	30	1.7	0.30	82	1.1
4 days	8.5	180	70	0.8	0.47	64	0.9
5 days	8.7	130	130	0.8	0.50	49	1.0
6 days	8.8	80	80	0.7	0.52	42	1.0

Values represent the average of both media.

metabolized or inactivated at the alkaline reaction. A close relationship between the hydrogen-ion concentration and the concentration of ammonia was again evident, apparently they are directly related. The alpha-amino nitrogen dropped to a minimum, apparently as proteolysis proceeded the free amino acids were immediately utilized as shown by the accumulation of ammonia. In the sodium chloride-deficient medium, lysis of the mycelium was accompanied by an increase in streptomycin until equal to the sodium chloride medium.

This observation provided an explanation of the action of sodium chloride. As the concentration of salt increases in the medium, the permeability of the membrane is affected in such a way as to cause an accumulation of streptomycin within the mycelium which is only liberated by lysis of the cell. In order to prove this hypothesis, mycelium was produced on Waksman's medium with and without sodium chloride and ten ml aliquots were transferred to centrifuge tubes. The medium was separated from the mycelium by centrifugation and thoroughly washed by dispersing in water and recentrifuging. The mycelium of both media was treated as follows (Table XV).

All volumes were kept constant and the results are comparable. Excellent agreement in the amount of streptomycin produced, was obtained by mechanical rupture of the mycelium, as compared to shaking in saline or to slow diffusion at 4°C. for seven days. From these results it seems evident that

TABLE XV - THE EFFECT OF SODIUM CHLORIDE IN WAKSMAN'S MEDIUM IN THE DISTRIBUTION OF STREPTOMYCIN BETWEEN MYCELIUM AND MEDIUM

Treatment of mycelium	Mycelium from medium without NaCl	Mycelium from medium with NaCl
	(Zone of inhibition in mm	
Tap water added, stored in refrig. (7 days)	27.0	21.0
0.5% NaCl added, stored in refrig. (7 days)	25.5	22.0
1% NaCl added, stored in refrig. (7 days)	26.0	22.0
0.1M phosphate pH 7 added, stored in refrig. (7 days)	25.5	22.5
0.2M phosphate pH 7 added, stored in refrig. (7 days)	26.5	22.0
Shaken with tap water 24 hrs.	23.5	21.0
Shaken with 0.5% NaCl 24 hrs.	26.0	23.5
Ground in mortar	26.5	22.5
Ground in mortar and mixture suspended in 0.1M phosphate buffer pH 7; stored in cold for 7 days.	25.5	22.0

streptomycin synthesis occurs when the metabolic activity is at a minimum.

The distribution of streptomycin between mycelium and medium depends upon the sodium chloride concentration. When

Streptomyces griseus is grown in Waksman's medium without sodium chloride, 75 per cent of the streptomycin is in the mycelium and 25 per cent remains in the medium. This distribution depends on the sodium chloride concentration (Table XVI).

TABLE XVI - THE EFFECT OF SODIUM CHLORIDE IN THE DISTRIBUTION OF STREPTOMYCIN BETWEEN MYCELIUM AND MEDIUM AFTER THREE DAYS GROWTH

Per cent sodium chloride added to Waksman medium	Zone of inhibition	
	Mycelium	Medium
Nil	26.5	21.0
0.1	25.5	22.0
0.2	24.0	23.5
0.3	23.0	25.0
0.4	23.0	26.0
0.5	22.5	26.5

The effect of sodium chloride was the same, whether the culture vessels were test tubes placed on the slant or, 250 cc. or 2 liter Erlenmeyer flasks and using a reciprocal or rotary shaker. However, the type of container influenced the time at which the greater part of the streptomycin was contained in the mycelium. With the slanted test tubes, the time was three days and with the two liter Erlenmeyer flasks, four days.

An exact study of the daily variations in the distribution of streptomycin between mycelium and media was next carried out (Table XVII).

TABLE XVII - STREPTOMYCIN DISTRIBUTION BETWEEN MYCELIUM AND MEDIA - WAKSMAN'S MEDIUM WITH AND WITHOUT SODIUM CHLORIDE

Days	Medium with NaCl Zone of inhibition (mm)		Medium without NaCl Zone of inhibition (mm)	
	Mycelium	Medium	Mycelium	Medium
1	11.0	23.0	21.0	23.5
2	23.0	25.5	26.0	21.0
3	22.0	26.5	25.0	22.5
4	20.0	25.5	20.0	25.0
5	19.0	24.5	19.0	24.5

There appears to be a limit to the amount of strepomycin synthesized because the total production is constant, however, the distribution varies with salt concentration and duration of fermentation. As lysis proceeds, the streptomycin is liberated from the mycelium and is destroyed at the same rate in both media. The antibiotic does not seem to be metabolized but is most probably inactivated by the accumulation of alkali.

Since it has been reported (22) that some ions can replace sodium chloride, whereas others can not, an experiment was carried out in which the sodium or chloride ion was

replaced by other cations or anions. If the sodium chloride affects the permeability of the cell membrane, then the Hofmeister ion series should be reproducible as follows: Li› K› Na› NH_4› Mg for cations; and citrate › tartrate› SO_4 › acetate › Cl › NO_3 › Br › ClO_3 › J › CNS for anions (Table XVIII).

TABLE XVIII - THE REPLACEMENT OF THE SODIUM AND CHLORIDE ION BY IONS IN THE LYOTROPIC SERIES

Concentration of salts replacing sodium chloride in Waksman's medium	Zone of inhibition (mm)	
	Mycelium	Medium
0.36% LiCl	18.0	22.0
0.64% KCl	24.0	26.5
0.5% NaCl	23.0	26.0
1.97% Na_2 tartrate	19.0	25.5
1.2% Na_2SO_4	18.0	25.5
0.87% NaBr	23.0	23.0
0.69% NaCNS	24.5	22.0

It was apparent that the ions had a selective action upon the permeability of the membrane. Little or no difference was observed in the cation series. Magnesium was also used to supplement sodium, however, the high concentration of the ion inhibited the growth of the organism. With the anions a more selective action was observed. This was apparent, not only in the distribution of streptomycin but also in the type of

mycelium produced. With tartrate the mycelium was dense and dark in color, while with the thiocyanate it appeared fluffy, swollen and light in color. This was proof that the action of the sodium or chloride ion was not specific but that the anion and cation could be replaced by others, provided they were close to sodium or chloride ion in the Hofmeister lyotropic series.

The results obtained in the study of Waksman's medium also held true for other media which have been used for streptomycin production (22) (24) (Table XIX).

TABLE XIX - THE EFFECT OF SODIUM CHLORIDE ON THE DISTRIBUTION OF STREPTOMYCIN IN VARIOUS MEDIA

Media	Zone of inhibition (mm) Mycelium	Medium
1% soybean flour, 1% glucose	24.0	21.0
1% soybean flour, 1% glucose, 0.5% NaCl	19.0	24.0
Waksman's medium, no 0.5% NaCl	25.5	22.0
Waksman's medium	22.0	26.0
1% glucose, 1% Bacto yeast extract, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.025% $MgSO_4 \cdot 7H_2O$	23.0	19.0
1% glucose, 1% Bacto yeast extract, 0.5% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.025% $MgSO_4 \cdot 7H_2O$	18.0	23.0

It was evident that the conclusion drawn by previous workers that sodium chloride promoted the production of streptomycin is erroneous. Its absence merely altered the distribution between

mycelium and media and this possibility had not been considered by other workers.

These observations suggest the possibility of developing new methods for the isolation of streptomycin. Rather than recovering the antibiotic from a deeply colored, highly complex medium, it might be easier to isolate it from the mycelium. As Molitor (7) pointed out, "fundamental changes will have to be brought about in the present isolation and purification technique in order to obtain streptomycin concentrates not contaminated with biologically active substances."

In this investigation a method has been developed for the concentration of streptomycin from the mycelium. The method was outlined previously (p.20). It has yielded a very light colored product of high antibiotic activity. Because the medium is unchanged, no chemicals being added, it may be possible to obtain a second yield of mycelium containing streptomycin thereby reducing the present high cost of the drug.

However, a much more intensive study would have to be undertaken in order to perfect a new procedure for the isolation of pure streptomycin from the mycelium and to obtain a concentrate with desirable characteristics in color, consistency, stability and above all a low toxicity.

DISCUSSION OF RESULTS

Schatz, Bugie and Waksman (5) found that the production of streptomycin by Streptomyces griseus requires a specific organic nutrient supplied by beef extract or corn steep-liquor. They called it "an activity factor", and in its absence only small amounts of streptomycin were obtained (20). However in a later paper, Schatz, Waksman and Reynolds (18) do not claim that this activity factor is necessary for maximum streptomycin formation, but now consider that it merely speeds up the rate of production of the antibiotic. This belief was substantiated in this investigation. It was possible to replace meat extract by its inorganic ash, however, the time of maximum production of streptomycin was thereby delayed.

It is possible then, that fish solubles, distillers solubles, corn steep-liquor and meat extract contain substances which accelerate the synthesis of the antibiotic. It also appears from observations recorded in this thesis that some strains of Streptomyces griseus can synthesize these intermediates more readily than others. It is difficult to speculate on the chemical nature of these intermediates of their function, since their absence merely delays but does not prevent the synthesis of the antibiotic.

Amongst the amino acids, histidine alone serves as ready available nitrogen source which promotes both treptomycin and mycelium production. Valine, however, plays more specific role, it promotes streptomycin synthesis nly, provided other factors are present which will promote he formation of mycelium. The addition of aspartic acid efinitely inhibits synthesis of the antibiotic but ccelerates production of mycelium and induces similar etabolic changes in the medium as does a complex organic edia. Since aspartic, glutamic or succinic acid exhibit similar effect upon the organism, it may be concluded that he synthesis of mycelium proceeds by the aspartase trans-minase system but this inhibits streptomycin formation. owever, another mechanism, as yet unexplained, will also ring about the synthesis of mycelial protein and apparently here is some connection between this alternate path of ycelial synthesis, the role of valine, the action of nositol and the production of streptomycin.

The effect of high concentrations of sodium chloride n the nutrient media, on the distribution of streptomycin etween mycelium and medium suggests an entirely new pproach to the production and isolation of the antibiotic. ; has been shown that streptomycin can be concentrated n the mycelium of Streptomyces griseus by proper mani-ılation of the salt concentration in the medium.

Preliminary work indicates that a relatively simple method for recovering streptomycin from the mycelium can be perfected. The streptomycin concentrate obtained by the new method will most probably contain other types of contaminants than the streptomycin concentrates now on the market. It is hoped these impurities will have little or no biological activity and so permit the administration of higher dosage of the drug, and ensure complete inhibition of the particular pathogens.

The influence of high concentrations of sodium chloride can not be ascribed to osmotic pressure effects, aside from considerations of hyper or hypotonicity. It appears more likely, in view of the observations with the ions of the lyotropic series, that the permeability of the membrane is affected by the concentration as well as the nature of the ion. These observations also suggest that the antibiotic is a product of intracellular synthesis and diffuses into the medium when the salt concentration is high. Unexplained, however, is the observation, that on the second day of fermentation in a medium without added sodium chloride, more streptomycin is found in the medium than in the mycelium.

SUMMARY

1. By the fractionation of natural organic complexes, several fractions were obtained which, when added to a basal medium of peptone, glucose and sodium chloride, induce a rapid accumulation of streptomycin equal to the amount obtained with Waksman's medium.

2. With some strains of Streptomyces griseus meat extract, fish solubles and distillers solubles can be replaced by their ash constituents, and obtain as high production of the antibiotic as with the complex medium.

3. Inositol, among the B vitamins tested, promotes streptomycin and mycelium production, in a basal medium of glycine, glucose and inorganic salts.

4. The amino acid histidine promotes both mycelium production and streptomycin synthesis.

5. Valine increases the production of streptomycin in a basal medium containing histidine and arginine as nitrogen sources.

6. Aspartic acid increases the production of mycelium but depresses streptomycin synthesis, when added to a medium containing histidine and arginine as nitrogen sources.

7. Streptomycin can be recovered from the mycelium instead of the medium if the salt concentration is adjusted accordingly.

8. The antibiotic recovered from the mycelium is streptomycin since it is insolubile in organic solvents and is extracted from the mycelium by phosphate buffer. It is not identical with the "second" antibiotic principle produced by the same organism and discovered by Waksman.

9. A method of concentrating streptomycin from the mycelium has been described.

BIBLIOGRAPHY

1. *Waksman*, S.A.

Principles of Soil Microbiology.
Bailliere, Tindall and Cox,
London, 1931. pp.273-297.

2. Gratia, A. and Dath, S.

Proprietee bacteriolytiques de certaines moississures.
Compt.rend.Soc.Biol. 91:1442, 1923.

3. Waksman, S.A. and Woodruff, H.B.

Actinomyces antibioticus, a new soil organism antagonistic to pathogenic and nonpathogenic bacteria.
J.Bact. 42: 231-249, 1941.

4. Woodruff, H.B. and Foster, J.W.

Microbiological aspects of streptothricin.
Arch. Bioch. 2: 301-315, 1943.

5. Schatz, A., Bugie, S. and Waksman, S.

Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria.
Proc.Soc.Exptl.Biol.and Med. 55: 66-69, 1944.

6. Schatz, A. and Waksman, S.A.

Effect of streptomycin upon Mycobacterium tuberculosis and related organisms.
Proc.Soc.Exptl.Biol.and Med. 57: 244-248, 1944.

7. Molitor, H.

The pharmacology of streptomycin and streptothricin.
Ann.of New York Acd. of Sc. 68: 101-111, 1946.

8. Hartz, C.O.

Actinomyces bovis, ein neuer Schimmel in den Geweben des Rindes.
Jahresbencht Tierarzneischule München, p. 125-140, 1877.

9. Breed, R.S. and Conn, H.J.

The nomenclature of the actinomycetaceae.
J. Bact. 4: 585-602, 1919.

10. Münter, F.

Über Actinomyceten des Bodens.
Centralblatt Bact. II, 36: 365-381, 1913.

11. Salzmann, P.

Diss. Königsberg 1902 (ref. in Lafar's Tech. Myk. 3: 212).
Cited from Waksman, S.A. Principles of Soil Microbiology, London, 1931.

12. Lieske, R.

"Morphologie und Biologie der Strahlenpilze".
Leipzig, 1921, p. 275.

13. Krainsky, A.

Die Aktinomyceten und ihre Bedentung in der Natur.
Centralblatt für Bact. II. 41: 649-688, 1914.

14. Waksman, S.A.

Studies in the metabolism of actinomyces.
J.Bact. 4: 189-216, 1919.

15. Waksman, S.A.

Studies in the metabolism of actinomyces.
J.Bact. 4: 307-330, 1919.

16. Waksman, S.A.

Studies in the metabolism of actinomyces.
J.Bact. 5: 1-30, 1920.

17. Waksman, S.A. and Joffe, J.S.

Studies in the metabolism of actinomyces.
J.Bact. 5: 31-47, 1920.

18. Waksman, S. A., Schatz, A. and Reynolds, D.M.

Production of antibiotic substances by actinomyces.
Ann.of New York Acad.of Sc. 68: 73-85, 1946.

19. Rosenthal, L.

Les lyses des Bacilles diphtheriques effectuees par un Streptothrix.
Compt.rend.Soc.Biol. 93: 77-78, 1925.

20. Waksman, S.A., Schatz, A. and Reilly, H.C.

Metabolism and the chemical nature of Streptomyces griseus.
J.Bact. 51: 753-759, 1946.

21. Whiffen, A.J., Bohonos, W. and Emerson, R.L.

The production of an antifungal antibiotic by Streptomyces griseus.
J.Bact. 52: 610-611, 1946.

22. Rake, G. and Donovick, R.

Studies on the nutritional requirements of Streptomyces griseus for the formation of streptomycin.
J.Bact. 52: 223-226, 1946.

23. Rao, R.R., Rao, S.S. and Venkataraman, P.R.

Utilization of ground nut-cake hydrolysate as medium for streptomycin production. Nature 158: 23, 1946.

24. LePage, G.A. and Campbell, E.

Preparation of streptomycin. J.Biol. Chem. 162: 163-171, 1946.

25. Vander Brook, M.J., Wick, A.N., DeVries, W.H., Harris, R., and Cartland, G.F.

Extraction and purification of streptomycin with a note on streptothricin. J.Biol. Chem. 165: 463-468, 1946.

26. Foster, J.W., Woodruff, H.B., Perlman, D., McDaniel, L.E., Wilker, B.L., and Hendlin, D.

Microbiological aspects of penicillin. J.Bact. 51: 695-698, 1946.

27. Trussel, P.C.

Ayerst, McKenna and Harrison Ltd., Montreal. Private communication, Dec. 1945.

28. Wilkins, W.H. and Harris, G.C.M.

Investigation into the production of bacteriostatic substances by fungi. Annals app.Biol. 30: 227-229, 1943.

29. Foster, J.W. and Woodruff, H.B.

Procedures for the cup assay for penicillin. J.Bact. 47: 43-49, 1944.

30. Loo, Y.H., Shell, P.S., Thornberry, H.J., Ehrlich, J., McGuire, J.M., Savage, G.M., and Sylvester, J.C.

31. Waksman, S.A.

Standardization of streptomycin.
Science 102: 40, 1945.

32. Hanes, C.S.

An application of the method of Hagedorn and Jensen to the determination of larger quantities of reducing sugars.
Biochem. J. 23: 99-106, 1929.

33. Macpherson, H.T.

Modified procedures for the colorimetric estimation of arginine and histidine.
Biochem. J. 36: 59-63, 1942.

34. Van Slyke, D.D. and Cullen, G.E.

A permanent preparation of urease and its use in the determination of urea.
J.Biol. Chem. 19: 211-228, 1914.

35. Van Slyke, D.D.

Amino nitrogen in meat products.
Methods of analysis of the A.O.A.C. 1930, pp.297-298.

36. Waksman, S.A.

Production and activity of streptothricin.
J.Bact. 46: 299-310, 1943.

37. Bauernfeind, J.C., Gacey, J.C., Baumgarten, W., Stone, L. and Bornff, C.S.

Nutrient content of alcohol fermentation byproducts from corn.
Ind. and Eng. Chem. 36: 76-78, 1944.