ASSIMILATION OF INORGANIC NITROGEN

BY AQUATIC ACTINOMYCETES

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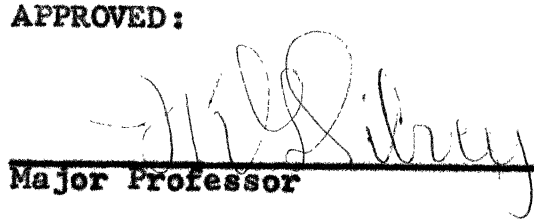

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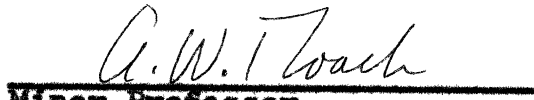

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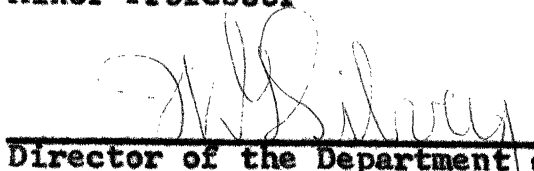

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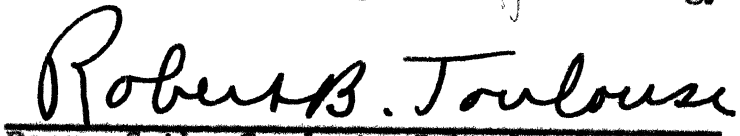

Dean of the Graduate School

ASSIMILATION OF INORGANIC NITROGEN BY AQUATIC ACTINOMYCETES

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF ARTS

By

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CHAPTER I

INTRODUCTION

The actinomycetes, as a group of microorganisms, appear to have metabolic cycles that have not been completely investigated. This is particularly true as regards the aquatic types now under study at North Texas State University. The presence of these organisms in surface water supplies appears to be of considerable importance from a public health standpoint. Certain forms are undesirable since they oftentimes render water unpalatable due to their excreted taste and odor compounds (28, 29, 31). It has been shown that the aquatic forms appear to develop more copiously in alkaline waters than in environments with less mineralization. However, it must be assumed that certain species may grow in almost any aquatic area provided adequate nutrients are available (27). For this reason more knowledge should be acquired regarding the metabolic activity of these organisms.

The term actinomycetes has no taxonomic validity since these organisms are classified as bacteria in a strict sense. The actinomycetes are unicellular microorganisms that produce slender, branched hyphae which may undergo fragmentation and may subdivide to form asexual spores. The hyphae in some genera are aerial and typically exhibit distinct

branching. The individual hyphae or filaments appear morphologically similar to the fungal hyphae but do not compare in size. These hyphae vary from 0.4 to 1.6 microns in diameter. Further taxonomic consideration shows that these organisms are members of the Order Actinomycetales, a category of bacteria that includes thread-like microorganisms forming elongated cells and exhibiting a tendency toward branching. These organisms differ from the fungi in that the cell wall contains no chitin and cellulose (3).

In the Order Actinomycetales there are four families and nine genera at the present time. The Family Mycobacteriaceae contains two Genera, Mycobacterium and Mycococcus. The Family Actinomycetaceae includes the Genera Actinomyces and Nocardia and the Family Streptomycetaceae, the Genera Streptomyces, Micromonospora and Thermactinomyces. Lastly, the Family Actinoplanaceae contains the Genera Actinoplanes and Streptosporangium. Classification of the actinomycetes into separate and distinct species is difficult because of the diverse ideas concerning the characteristics that compose specific and nonvariable characters.

At least one author recognized the similarities between the actinomycetes, the bacteria and the fungi (35, 37). From these studies Waksman has described their similarities. These organisms are similar to bacteria in several respects such as:

> (1) have the same diameter of hyphae and spores; (2) reproduce by fragments or oidia; (3) are acid fast; (4) possess nucleoids (no true mitosis); (5) possess similar chemical properties of cellular structure; (6) have no chitin or cellulose; (7) are procaryotes (not eucaryotes) (8) undergo lysis and phage sensitivity; (9) and are sensitive to antibiotics. (35,37).

On the other hand he indicated that they also exhibit similarities to the fungi such as:

> (1) have aerial mycelia; (2) grow on liquid or solid substrates; (3) exhibit pleomorphism and (4) possess secondary mycelium. (35, 37).

Although the taxonomic classification of the aquatic forms is not precise at the present time, they were temporarily placed in the genus Waksmara (33). It is apparent at this time that these organisms under investigation are not members of the Streptomyces and/or Micromonospora because of their bisporulative characteristic.

History

There has been only a meager amount of information contributed by various investigators on the metabolism of these aquatic organisms. Most of this work has been done since 1950, a large portion of which has originated from North Texas State University. Investigations on terrestrial forms have been extensive, and many citations in the literature make reference to specific metabolic activity.

Some investigators have not separated their work on the carbon and nitrogen metabolism of various actinomycetes. They feel that a close relationship exists between the two.

Cochrane (9) in one of his investigations involving several species of Streptomyces showed the formation of various organic acids produced by these forms using glucose as a carbon source. He also showed that whole cells of these organisms oxidized some, but not all, of the compounds of the tricarboxylic acid cycle (10). He found that they failed in particular to metabolize citrate. Harvey (15) studied some qualitative effects of different carbon-nitrogen ratios on soil forms of these organisms. Mohan (22) used a single compound, urethan, as the only source of nitrogen and carbon. Several other investigators have worked with the actinomycetes on determinations of nitrogen metabolism, and many of them selected single organic compounds bearing the nitrogen in some form. Nickerson (24) used glutamate which he found supported growth of several species of Streptomyces. Monosodium glutamate was the organic nitrogen energy source of the synthetic medium developed by Romano (26). Zygmunt (38) found in his analyses for the presence of the metabolic byproduct, oxytetracycline, that an organic nitrogen source was essential and that inorganic sources would yield no oxytetracycline. The carbohydrate to nitrogen ratio was shown to be important in the work of Federov (12). He found that when the ratio fell below 20:1 the ammonifying activity of the species was high, and free ammonia

accumulated in the medium. Hirsch (16) showed that a 1:10 ratio of carbon to nitrogen inhibited growth and nitrite accumulation; whereas, a ratio of 10:1 was best for maximum metabolic activity. Waksman (37) found that if the carbon to nitrogen ratio was above 20:1 the glucose inhibited nitrification. Krasilnikov (20) showed that the metabolic utilization of carbon, and in some cases nitrogen, was a good species differentiation within determined groups of actinomycetes.

Almost all of the isolates at North Texas State University grew well when brown sugar, peptone, and tyrosine were added to the growth medium (28, 30). Zygmunt (38) described a chemically defined medium for analysis of oxytetracycline but maintained that an organic nitrogen source was necessary for its production. Waksman (35, 37) stated that proteins were preferred by some of these organisms as both a nitrogen and carbon source.

Some investigators have used various organic acids in their media. The amino acids appear to be the most used. Federov (13), Gottlieb (14), Nickerson (24) and other investigators (1, 8) have shown that the presence of amino acids caused greater growth to occur. Gottlieb (14) stated that a great deal of variation existed among the organisms as to the mechanism and rate of nitrogen assimilation.

Inorganic sources of nitrogen, it would seem, would not be as complex as organic compounds when used as analytical

tools in a metabolic investigation. However, there is relatively little data on the subject of inorganic nitrogen utilization in comparison to the vast amounts dealing with other phases of actinomycology. Concurrently, there is almost a complete lack of any quantitative data. The literature concerned with the effects of inorganic nitrogen compounds on the actinomycete growth varies from work done on soil actinomycetes (2) to quantitative work carried out under exacting controlled conditions in the laboratory as accomplished by Hirsch (16). It was shown as early as 1903 that either a nitrate or an ammonium salt served as an adequate source of nitrogen for actinomycete metabolism (4). By 1916 Munter (23) believed that nitrates and ammonium salts were a much better source of nitrogen than proteins, peptones, and amino acids. He also showed that 5% sodium chloride, potassium chloride, and potassium or sodium nitrate permitted growth of some of the actinomycetes. Cochrane (5) maintained that Streptomyces griseus was unable to grow in a medium containing nitrate as the only source of nitrogen and glucose as the only source of carbon. In other investigations (8) he showed that another species of Streptomyces could utilize nitrate, but in the presence of organic acids produced from glucose, the nitrite which was produced from the nitrate was toxic. Dietzel (11) cited work concerning nitrate consumption from a nutritive solution which contained sodium

nitrate, mineral salts, and lactose. Waksman (35) specified that, on the whole, the Streptomyces has the ability to reduce nitrate to nitrite.

Waksman showed in a later work (36) that the availability of different nitrogen compounds for growth was possibly good criteria for diagnostic evaluation. He further found that nitrate made a better source of nitrogen. The residual effect of the basic ion left from nitrate compared with the acid ion left from the ammonium salts made the medium less favorable for growth. Nitrate was never reduced to atmospheric nitrogen or ammonia and, whenever it was reported, it was due to secondary reactions rather than direct reduction of nitrate. Gaseous nitrogen is formed when nitrate and amino acids are placed in an acid medium, that is, $2NO_2^- + 4NH_2 \longrightarrow 3N_2 + 4H_2O$. This combination was very unlikely to appear in actinomycete cultures. Waksman (36) further showed that a buildup in ammonia content in a culture medium which had nitrate as a nitrogen source was due to the autolysis of synthesized cell materials.

In a later work Waksman (37) found that in a culture medium containing nitrate as the nitrogen source, if the amounts of nitrite and NH_2OH were lower than the amount of nitrate that was assimilated then this was due to a reduction to molecular nitrogen.

In other investigations, initially the same results were confirmed. Okami (25) claimed that all strains of Streptomyces grew better on sodium nitrate than on ammonium sulfate. He attributed this to the nitrate ion being more diffusible in agar because of its negative charge, whereas ammonium was less diffusible with its positive charge. Harvey (15) was by no means the first to show that alkaline conditions must be met before optimum growth can be obtained. Silvey (27) showed this as early as 1950 regarding the actinomycete growth in its natural aquatic environment and later (28) described a medium on which actinomycetes obtained optimum growth. This medium consisted of a base of dark brown sugar, mineral salts, peptone, and tyrosine or phenyl alanine. This also showed a reduction in growth with removal of sodium nitrate and corresponding 30% loss of nitrogen from the medium in six weeks using ammonium nitrate as the inorganic source. Gottlieb (14) studied the utilization of nitrate by these organisms. His methods were semi-quantitative, and he only reported that various amounts either were or were not utilized. He showed that a great deal of variation existed in nitrate reduction among the different Streptomyces. Hotchkiss (17) claimed that growth of Nocardia was dependent on nitrogen in the form of sodium and potassium nitrate and various ammonium salts in the

concentration of 0.2 milligram nitrogen/ml. Her work was relatively quantitative in that she showed a decrease in the nitrogen content as the mycelium increased and also showed that sodium nitrate produced more mycelium than ammonium chloride. Federov (12) showed, in his work on the actinomycetes, a rapid reduction of sodium nitrate and sodium nitrite to ammonia derivatives which were utilized by actinomycetes in synthesizing nitrogen compounds. He claimed that ammonia and NH_2OH were intermediates in the reaction. Krasilnikov (19) claimed that ammonia and nitrate salts act differently in the metabolism of actinomycetes for pigment production. Tendler (34) in his work with the Thermoactinomyces showed that only nine of 805 strains had the capacity to reduce nitrate. In contrast, 113 of 121 strains of thermophilic Streptomyces reduced nitrate. Lechevalier (21) worked with a defined medium but offered no explanation as to the method of sterilization. He supported the idea that in the presence of glucose, sucrose, or glycerol, ammonium nitrate and ammonium sulfate would not support the growth of the actinomycetes with which he worked. By 1958 Cochrane (7) showed that nitrate reduction was common but not universal in actinomycetes but concluded that some strains could not grow in a medium which contained nitrate as the only nitrogen source of nutrition.

It was the opinion of some investigators that nitrite was toxic to some forms of actinomycetes. Hotchkiss (17) showed that a concentration of 0.2 mg nitrite nitrogen per milliliter was toxic and prevented growth. She was able to detect, in some instances, nitrite concentrations up to 50 mg/L as nitrogen. Hirsch (16) showed that nitrite could be formed in a medium of ammonium hydrogen phosphate by members of the Mycobacterium, Nocardia, Streptomyces, Micromonospora, and Streptosporangium. All of these mentioned except a few Streptomyces and Nocardia metabolized nitrite further so that the concentration diminished with time. A number of Streptomyces released nitrite only after the maximum cell concentration had been attained. He never found over 2.39 mg/L nitrite. Alexander (3) reported that Streptomyces cultures rarely produce more than 1-2 mg/L nitrite: the source for nitrite production being the ammonium ion. Also he maintained that if the concentrations of sugar to ammonium ion was as low as 10:1, nitrite could be detected.

Dietzel (11) was one among the few investigators who showed the presence of ammonia-nitrogen production from a nutritive solution containing sodium nitrate. Waksman (35) claimed that ammonium salts could be used as a source for growth. Cochrane (7) brought out other pertinent points

in ammonia metabolism. He stated that ammonia utilization increased with the pH and had no pH optimum. He also reported that there was no optimum concentration of nitrogen for best growth and development of a culture. He maintained that the demand for nitrogen depended on the carbon supply. By 1961 (6) he showed that young cells (colonies) of actinomycetes lost ammonia to the medium, but he could not establish a point of separation between the ability to reduce nitrate from the capacity to grow on it as the only source of nitrogen. Cochrane (6) found one enzyme which was stabilized by the presence of calcium but irreversibly inactivated by the presence of ethylenediaminetetraacetic acid in the medium. Simon (32) carried his work to the point where the available carbohydrates were exhausted. At this point the ammonia content increased. He attributed the increase in organic nitrogen to autolysis after the first three days of growth. Alexander (3) showed that application of ammonium ion to soil actinomycetes resulted in the oxidation of aniric acid by microbial action and the resultant fall in pH was detrimental to the metabolic activity of these organisms. Hirsch (16) did work using ammonium hydrogen phosphate as the only nitrogen source. He claimed that a pH of over 7.5 allowed ammonia volatilization to take place.

Very little recognition has been given to the possible influence of free gaseous (molecular) nitrogen on the metabolism of the actinomycetes. Federov (13) showed that certain actinomycetes were capable of accumulating nitrogen in their bodies in a medium free of added nitrogen sources. He ascribed this to the ability of the organisms to fix atmospheric nitrogen. Howell (18) grew his cultures in a 95% atmosphere of nitrogen at a pH of 6.5. In this environment he claimed that they grew better at that pH than at the neutral to alkaline pH of 7.5. Waksman (36) did say, however, that actinomycetes were unable to fix atmospheric nitrogen.

Statement of Problem

It was the purpose of this investigation to present laboratory data concerning the assimilation of inorganic nitrogen by the aquatic actinomycetes. The strains of aquatic actinomycetes under consideration represented a cross section of those currently under culture at North Texas State University. One factor considered in this study was the development of a chemically defined synthetic medium which could be subjected to quantitative chemical analyses and from which adequate nutrition could be derived for growth and development of the organisms. Various containers in which the different strains of actinomycetes could

assimilate nitrogen under optimum laboratory conditions were investigated.

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CHAPTER II

METHODS AND MATERIALS

Microorganisms such as the actinomycetes are capable of carrying on normal metabolic activity under a variety of ecological conditions. Thus, it must be assumed that adequate sources of nutrition that are assimilated in their energy cycles are available in natural environments. Their growth on different types of artificial media in a laboratory presents a rather complex problem if quantitative metabolic data are to be accumulated. Of the many and varied synthetic media commercially available, it would seem that cultivation of these organisms should be easily obtained. This is true for most of the isolates, but there are some strains that do not develop properly under such conditions. Several isolates in the collection at North Texas State University require an organically rich medium, while others respond to nutrient sources containing only glucose as a carbon source. Roach (7) developed several methods and media for both isolation and cultivation of most strains. Silvey (9) reported a medium on which maximum growth could be obtained for production of odor compounds produced by these organisms. He also reported a synthetic medium for optimum growth of most strains of the actinomycetes (10).

In order to do accurate quantitative studies on inorganic nitrogen assimilation by certain strains of aquatic actinomycetes, it was necessary to employ a synthetic medium capable of rearing the microorganisms and, at the same time, properly compounded so as to yield accurate analytical results. Several such media have been described in the literature from time to time. Lechevalier (3) developed one which showed promise, but his medium was a solid one and eliminated the possibility of any quantitative chemical analyses.

Raymond (6) utilized a defined medium for his work on Nocardia. This contained n-alkane as an energy source, as well as ashed yeast extract, which would not be amenable to rapid quantitative chemical analyses. Sikyta (8) developed a reasonably defined medium in which he employed ammonium sulfate as the sole nitrogen source. He sterilized the medium by autoclaving. Small amounts of magnesium phosphate precipitated as a result so that quantitative results could not be obtained. The principle in developing a chemically defined medium was to produce one in which an optimum carbon source could be added along with essential quantities of required cations and anions. The ingredient in such a medium that would be a variable one would be the inorganic nitrogen source. It was realized before the work of developing this medium was begun that a source of calcium

and phosphate would be essential for growth. These two ions, along with magnesium, would precipitate as calcium and magnesium phosphate unless properly stabilized. Attempts were made to stabilize all ingredients continuously in solution.

Over twenty-two different media were tested before the optimum chemical composition was finally determined. Required quantities of each component were ultimately found by using varying mixtures in a series of 14, 250 milliliter Ehrlenmeyer flasks as shown in Table I.

TABLE I

FLASK SERIES DETERMINATION OF SYNTHETIC CHEMICAL MEDIUM OPTIMUM FOR GROWTH OF ACTINOMYCETES

		Flask													
Compound	g/L	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Glucose	10	X	X	..	..	X	X	X	X	X	X	..	..	..	..
Sodium citrate	10	X	X	X	X	..	..	X	X	..	..	.X	X	..	..
Glycerol	10	..	..	X	X	X	X	X	X	..	..	..	..	X	X
Sodium asparaginate	0.1	X	..	X	..	X	..	X	..	..	X	..	X	..	X
Ammonium nitrate	6	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Calcium chloride	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Magnesium sulfate	0.5	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Dipotassium phosphate	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Sodium chloride	2.5	..	..	..	..	X	X	..	..	X	..	..	..	X	..

X = Component added to the medium

Of the fourteen different media indicated in Table I the variation between the amounts of growth of one strain of actinomycete was quite evident. The amounts of growth are indicated in Table II below. Strain "S" was used as the test organism.

TABLE II

RESULTS OF GROWTH OF STRAIN "S" IN SYNTHETIC CHEMICAL MEDIA SHOWN BY TABLE I

Flask	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth	0	0	0	XX	pt	pt	XX	0	pt	pt	0	X	pt	pt

pt = medium precipitated
0 = no increase in growth
X = sparse growth
XX = good growth

As a result of these investigations a synthetic medium was compounded that contained sodium citrate-- 10.0 g., glucose-- 10.0 g., ammonium nitrate-- 6.0 g., calcium chloride-- 0.1 g., magnesium sulfate-- 0.05 g., dipotassium phosphate-- 2.0 g., and distilled water-- 1000 ml. It was termed medium $M1B_1$, and the growth of at least one strain of actinomycete was satisfactory. A change in the nitrogen source to ammonium chloride (2.5 g/L) reduced the growth and development to less than that which was produced in the original M1B. This medium which was devoid of nitrate was termed $M1B_1$. A second variation using 2.0 g/L each of

sodium nitrate and potassium nitrate (4.0 g/L total) produced better growth than M1B. This latter medium was termed $M1B_2$, and the final pH of the medium was 7.49.

A series of experiments were undertaken next to determine the most desirable conditions under which the actinomycetes utilized the inorganic nitrogen sources supplied them in the media. Since autoclaving appeared to alter the ingredients, these media were passed through a Seitz filter which had a pore tolerance of 0.1 micron. The media were always filtered into previously autoclaved, two-liter Ehrlenmeyer flasks (Bellco Glass Corporation). These incorporated an outlet near the top and near the base. To the lower outlet, a 10 milliliter automatic syringe was attached by the use of gum tubing. The liquid medium was filtered into the flask under 12 inches of vacuum. It was then possible to dispense that medium, by way of the automatic syringe, into the desired type of sterile receptacle. Generally either 50 or 100 milliliters of medium were autopipetted into each culture flask and the meniscus carefully marked. A known volume of a spore suspension of a specific actinomycete was added to each flask. The quantity of innoculum was similar in each instance so that growth weights would not be affected in any manner. It should be observed that the cultures used to prepare the innoculum were of about the same age which minimized the chances of introducing extraneous nitrogen sources from other media.

A series of 18-250 ml Ehrlenmeyer flasks containing actinomycetes and medium was placed in a rotary shaker which agitated the flasks at 240 revolutions per minute. The purpose of this experimental design was to provide adequate aeration to the medium and the actinomycetes, and to allow for maximum exposure of medium to the actinomycetes so that optimum growth would be obtained. A second series of 32-250 ml Ehrlenmeyer flasks was prepared as previously described except it was not agitated during the period of growth.

As the actinomycete growth proceeded, complete data were gathered by using one flask per 24 hours to obtain nitrogen uptake and total mat weight. The growth medium was filtered through a pre-weighed Millipore filter having a pore size of 0.45 microns. If any loss in medium volume had occurred during incubation it was replaced with an identical volume of demineralized water. After recovery of the liquid medium, successive washings of the sample flask with demineralized water insured that actinomycetes attached on the flask wall would be transferred to the Millipore filter. The filter, with the mycelial mat, was dried in a desiccator for 48 hours and weighed so as to obtain the quantity of total growth in a particular time over the weight of the original innoculum. The actinomycete-free medium was then analyzed for its nitrogen

content as will be described later. Data presented in Chapter III illustrate the results obtained from these two series.

In the next series of experiments two liter flasks were used to evaluate the effects of larger quantities of medium in greater volumes on growth rates and nitrogen uptake. Approximately one liter of the $M1B_2$ medium was Seitz filtered into the flask, and a strain of actinomycete was innoculated into the medium. Two types of growth conditions were provided by these studies. In one flask, a teflon-covered, magnetic bar was placed for stirring of the medium. The other flask remained static. Comparison of the rates of metabolism of the actinomycetes present in each flask were observed.

It soon became apparent in these investigations that small volumes of sample in restricted containers were inadequate for complete studies. Greater parameters were essential if the desired information was to be obtained. For example, the studies should include data on pH, dissolved oxygen, cation or anion concentrations in addition to nitrogen assimilation. These requirements could be more adequately satisfied by employing large culture chambers currently used in metabolic studies of the actinomycetes. Originally the chambers, or columns as they are often

referred to in the laboratory, were constructed with glass and asbestos cores to provide a substance for colony development. In these investigations such solid substrates appeared to be undesirable so that the construction was modified somewhat from that described by Silvey (11). Plate I illustrates one such column. In addition, the heating elements appeared to serve no useful purpose in these studies so they were not included in construction of the columns. Other general features such as sterilization of the columns, flow of sterile air, inlets and outlets and general handling was not modified from the techniques generally employed in using these chambers for rearing the actinomycetes. Since the synthetic medium used in these studies was sterilized by filtration, special techniques were developed to insure its flow into the columns under aseptic conditions. A sterile Seitz filter was attached to a 3/16-inch tygon tubing attached to each outlet on the column. A vacuum of 10 inches was drawn inside the column and the medium slowly filtered into the column. Generally, three liters of medium were added to each column so that adequate volumes would be available for all types of analytical procedures required in the investigation.

Innoculation of these columns with spore suspensions followed the same methods previously described for the flask

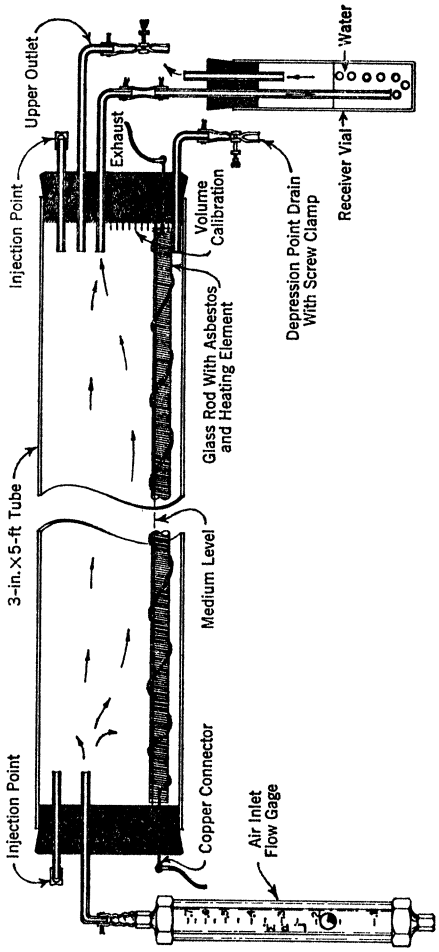

Plate I - Cultural Column. Columns similar to this (but lacking the core) were used for quantitative studies on actinomycetes.

studies. Effluent air was passed out of the column by means of an outlet above the surface of the medium and at the opposite end of the chamber from which it entered. This effluent air was then passed through a tygon tube into a buffered chlorine solution so as to prevent bacterial contamination of the medium in the column. The quantities of exhausted air were very small which minimized evaporation of water and hence concentration of the nutrient ingredients.

The nitrogen determinations which were made on the samples taken from these cultural columns consisted of nitrate, nitrite, ammonia, and Kjeldahl-nitrogen. Total nitrogen was determined by employing Devarda's alloy (2). This compound contained 50 per cent copper, 45 per cent aluminum and 5 per cent Zinc. Devarda's alloy reduced nitrate and nitrite to ammonia which was then determined by the micro-Kjeldahl method. The use of Devarda's alloy in conjunction with the micro-Kjeldahl nitrogen determination served to check the results of the other analyses for all types of nitrogen by yielding a total nitrogen content. If the figures obtained by the separate tests, when totaled, did not equal the concentration obtained by using Devarda's alloy, then a rerun was made on the separate nitrogen compound determinations in an attempt to correct the error. A description of the procedure for a sample of nitrate was given by Hillebrand (2).

There are many modifications of the original micro-Kjeldahl method that may be used for the determination of nitrogen. Niederl (4) described the process as a test for aminoid nitrogen. The principle involved was one in which the aminoid nitrogen was decomposed in the presence of concentrated sulfuric acid and a suitable catalyst to quantitatively yield ammonium sulfate. After liberation with strong alkali, the total ammonia was distilled into an excess of 0.01 N acid. The excess acid was titrated back with 0.01 N alkali. The procedure without the introduction of reducing agents (such as Devarda's alloy) would be limited to ammonium salts, amines, amino acids, ureas, thio ureas, and acid amides provided that their structure was not too complex. Proteins yielding to decomposition, as well as hydrolysis products of a similar generic structure, were also subject to analysis by the same method. It was found in these investigations that the addition of reducing agents such as glucose would increase the number of nitrogen-containing substances that could be accurately determined. Some of the compounds that could be subjected to analytical procedures were nitroso(beta-nitrosonaphthol) and nitro compounds, aromatic amines, hydroxyl amines (oximes), hydrazines (substituted hydrozones, osazones) and some azo and diazo compounds.

Pelczar (5) described an additional method for micro-Kjeldahl determination of small quantities of nitrogen. He employed a special digestion mixture composed of copper sulfate, selenous acid, sodium sulfate and sulfuric acid in defined quantities. An indicator solution was used in the digested mixture to show when adequate base had been added to permit distillation of the free ammonia. An additional indicator was added to the receiver flask to show presence or absence of distilled ammonia. In the samples tested by this method it was found that the yield was not as high as it should have been. It was presumed that the digestion mixture was not strong enough to convert the nitrogen compounds into ammonium sulfate. Thus, it became necessary to combine the methods in order to obtain dependable results. The procedure was as follows: a 5 ml. sample of medium was added to a 100 milliliter Kjeldahl digestion flask. Then, 50 milligrams of copper sulfate (anhydrous), 30-50 of selenous acid, 100 mg of sodium sulfate, 2 milliliters concentrated sulfuric acid and 1 carborundum boiling chip were also added as a mixture. This material was digested slowly over an electrical heating unit until the solution became clear. It was then made basic with NaOH, distilled into boric acid and titrated with 0.01 N HCl.

The advantages of the micro-Kjeldahl method were: speed and simplicity (especially for multiple determinations),

convenience for the determination of nitrogen in aqueous solutions, and accuracy with heterocyclic compounds. These compounds, in the micro-Dumas method, form nitrogenous charcoals which are difficult to burn. For this reason the determinations were done with the micro-Kjeldahl and not the micro-Dumas method.

The analyses for ammonia were accomplished by use of the direct Nesslerization method cited in Standard Methods (1). In order to obtain a solution of the medium under consideration which contained substances such as ammonia in a working range, a 1:1000 dilution was usually made. This dilution, in all cases, reduced the amounts of substances causing interference to a minimum so that no interference was noted in the color development. A working standard curve was developed prior to analyses of the media. The Nessler reagent test was run on a Klett-Summerson Photoelectric Colorimeter utilizing a 420 millimicron filter.

The determination of nitrate was accomplished with the phenoldisulfonic acid method listed in Standard Methods (1) and run with the aid of a standard curve on the Beckman Model DU Specrophotometer.

The other source of nitrogen under consideration, nitrite, was quantitatively determined with the sulfanilic acid, naphthylamine hydrochloride, sodium acetate test

listed in Standard Methods (1). Determinations were done on the Klett-Summerson Photoelectric Colorimeter using a 540 millimicron filter.

All pH measurements were done with the aid of the Beckman Zeromatic pH meter.

Measurement of the amount of growth of at least one strain of actinomycete was accomplished by the sonic degeneration of the mycelial mat in a Raytheon Sonic Oscillator at 10KC for 20 minutes after which the resulting solution was measured at 301 millimicrons against a medium blank in the Beckman Model DU Spectrophotometer.

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CHAPTER III

RESULTS

In order to learn the relative growth rates of the seven strains of actinomycetes investigated in this study, it was deemed essential that comparative observations should be made on synthetic medium $M1B_2$. The results indicated that the liquid medium was not as efficient for general growth as the solid medium, but contained adequate nutrients to support certain of the strains (Table III). It was also noted in the agar controls that the quantity of growth was so small as to be practically negligible. The information obtained from the study made it possible to predict the amount of growth anticipated for each strain over a period of time on the standard synthetic medium. Isolates, NT 15, 59, 22, and 5 exhibited such poor growth that they were deleted from the detailed investigations.

Investigations on the growth of certain strains in the absence and presence of oxygen were undertaken in order to show if anaerobic growth would occur. In Table IV, the results showed that oxygen was desirable in producing moderate growth, although Strain S grew almost as well under an atmosphere of nitrogen. It should be observed that all colony development was subsurface and that no floating mats

TABLE III

COMPARISON OF GROWTH AND RELATED CHARACTERISTICS OF REPRESENTATIVE STRAINS OF ACTINOMYCETES ON SOLID AND LIQUID SYNTHETIC MEDIA

Strain	59	Olney	Cooper	22	15	S	62
Color	cream	white	cream	cream white	white	cream white	grey
Solid Synthetic Medium	XXXX	XXX	XXXX	XXXX	0	X	XXXX
Agar Control	0	0	0	X	0	X	X
Liquid Synthetic Medium							
a) Subsurface	0-X	X	X	X	X	X	XXX
b) Surface	0	X	XX	0	0	X	XXXX
Pigment Production @ 30 Days							
a) Liquid	0	0	XXXX	0	0	0	XXX
b) Agar Control	0	0	0	0	0	0	0
c) Solid	X	X	XXXX	X	XXX	X	XXXX

0 = No Increase in Growth
X = Slight Increase in Growth
XX = Moderate Increase in Growth
XXX = Rapid Increase, Good Increase Evident
XXXX = Prolific Growth Evident

TABLE IV

RELATIONSHIP OF AEROBIC AND ANAEROBIC GROWTH OF VARIOUS STRAINS OF ACTINOMYCETES

Atmosphere	Helium	Nitrogen	Oxygen
Strain "S"	X	XX	XX
Strain "62"	X	X	XX
Strain "15"	0-X	X	XX

0 = No Increase in Growth
X = Slight Increase in Amount of Growth
XX = Moderate Increase in Amount of Growth

or aerial mycelia were formed. It was presumed that only primary stages were present; however, these results were important since no other specific references indicated the development of these actinomycetes in anaerobic situations.

As mentioned in the description of laboratory methods in these investigations, some means of growth evaluation was desirable throughout the study. Customarily, investigators have reported growth development by filtering the actinomycetes from the medium, drying the mycelia and obtaining dry weight in terms of mg/l. While that method was employed in these studies, additional methods were also used for enumeration of actinomycetal growth. Figure 5 illustrates per cent transmission and optical density measurements on gradient samples of sonically degenerated mycelial mats suspended in the original medium. Since the maximum absorption occurred at 301 millimicrons, all readings were made on a Beckman Model DU Spectrophotometer at that setting. After several attempts to obtain dependable data, the method was discarded in favor of dry weight techniques. It was shown by the increase in optical density readings (Table V) that additional investigations might prove that this method would be a profitable one. At the same time the above results were being obtained, similar sets of flasks were used to determine growth in terms of dry weight as mg/l. Each series of flasks were incubated for a period of 30 days, two flasks

TABLE V

ANALYSIS OF GROWTH INCREASE WITH STRAIN "S" ACTINOMYCETE USING MODEL DU BECKMAN SPECTROPHOTOMETER AND SYNTHETIC MEDIUM $M1B_2$

Days Growth	Wavelength Millimicrons	Optical Density	Per Cent T
0	301	.352	44.4
1	301	.363	43.2
2	301	.378	41.9
3	301	.416	38.3
4	301	.429	37.2
5	301	.434	36.8
6	301	.460	34.7
7	301	.495	32.0
8	301	.501	31.5
9	301	.512	30.9
10	301	.519	30.3
11	301	.523	30.0
12	301	.533	29.3
13	301	.541	28.8
14	301	.550	28.2
15	301	.559	27.6*
16	301	.620	24.0
17	301	.637	23.0
18	301	.641	22.8
19	301	.710	19.5
20	301	.791	16.2

*Abrupt increase in pigment production at this point resulted in interference in readings.

being used daily for analytical procedures. In addition to dry weight of the mycelial mats, residual inorganic nitrogen and organic nitrogen were also determined. It was observed from these data that adequate growth could be obtained in a period of 15 days. In order to understand the data and their obvious variations, one should recall the fact that each set of samples was from different flasks and, therefore, would not have a constant growth rate (Table VI). All completed data seemed to be sufficiently dependable to be quoted as averages in terms of dry weight of growth obtained in a specific medium during a known period of time.

Since the investigations involved the relationship between inorganic nitrogen assimilation, growth rate and the production of organic nitrogen, it was desirable to show that synthetic medium $M1B_2$ was optimum, or another type of medium must have been formulated. Strain S was chosen for a series of flask studies in which four different media were evaluated. These were previously described in the chapter on methods under the title of Medium #1, Medium M1B, Medium $M1B_1$, and $M1B_2$. Medium $M1B_1$ had as its major source of nitrogen the ammonia ion added as ammonium chloride. On this medium the total quantity of growth obtained was 36 mg/l over a period of 30 days (Figure 4). Although no nitrate was added as such, some apparently was available

TABLE VI

RELATIONSHIP OF GROWTH OF STRAIN "S" IN FLASK MEDIUM #1 ON ROTARY SHAKER

Days	NO_3	NO_2	NH_3	Kjeldahl Nitrogen	Growth mg/L
1	2500	0.0	1280	1395	1.0
2	1250	0.0	1275	1325	8.0
3	1255	0.5	1290	1350	16.0
4	1209	1.5	1295	1400	10.0
5	1320	1.5	1350	1450	15.0
6	1375	1.5	1357	1465	16.0
7	2333	1.3	1251	1484	8.0
8	2550	1.0	1355	1495	8.0
9	1385	1.0	1462	1585	20.0
10	1659	1.0	1450	1993	24.0
11	1960	0.6	1420	2116	25.0
12	2100	0.5	1395	2280	40.0
13	2105	0.3	1385	2300	45.0
14	2300	0.2	1340	2480	35.0
15	2666	0.0	1314	2520	54.0
16	2580	0.25	1290	2520	35.0
17	2670	0.6	1282	2522	40.0
18	2720	1.3	1270	2525	42.0
19	2730	1.5	1270	2531	39.0
20	2750	2.0	1268	2535	28.0
21	2760	2.6	1265	2540	32.0
22	2760	2.8	1263	2548	48.0
23	2800	3.3	1258	2553	40.0
24	2800	3.6	1252	2559	38.0
25	2820	4.0	1242	2560	49.0
26	2830	4.2	1240	2565	27.0
27	2824	4.8	1232	2569	38.0
28	2660	5.0	1200	2200	30.0
29	1820	5.4	1120	1950	65.0
30	900	5.6	1005	1680	80.0

from other chemical sources. As shown in Figure 3, Medium M1B was tested with the same strain, using a total of 30 flasks over a period of 30 days in which the growth obtained was 52 mg/l. While this appeared to be good growth, further experiments, as illustrated in Figure 1, yielded a growth of 75 mg/l in a period of 30 days with a similar set of samples. The loss of ammonium and nitrate in this series was adequate to account for the increase in organic nitrogen. In a study of Strain S in Medium $M1B_2$, the growth in a period of 30 days was 140 mg/l. This clearly demonstrated, in so far as this investigation was concerned, that the synthetic medium employed developed maximum growth with proper utilization of ammonium prior to nitrate. The nitrite consumption or production was very small.

As previously described, it was found from these studies that flasks were inadequate for comprehensive investigations that would include enough parameters to capably judge inorganic nitrogen assimilation. All subsequent work was completed in the large cultural columns. In order to set up a series of studies that would yield adequate information, a synthetic medium was prepared similar to $M1B_2$, except that all sources of inorganic nitrogen were deleted. After the columns were established and inoculated, it was found that there was some available nitrate which apparently was introduced in other inorganic components even though the assay

on the containers did not indicate its presence. There was no ammonia or nitrite available, but the nitrate amounted to 250 ppm. Figure 5 illustrates the rapid assimilation of nitrate by "Cooper" strain with a comparable production of organic nitrogen. Similar sets of columns were prepared containing nitrate with minimum quantities of ammonia. Here again, it was demonstrated that the nitrate assimilation was almost a straight-line curve. The small quantities of available ammonia disappeared, which accentuated the assimilation of the nitrate. The amount of organic nitrogen formed was very similar to that shown in Figure 5. A third set of columns was employed to learn if ammonia, alone or with minimal quantities of nitrate, was capable of producing maximum organic nitrogen. It would appear from results shown in Figure 7 that ammonia was more readily assimilated than nitrate and might readily result in a more rapid and greater development of available organic nitrogen.

Four strains of actinomycetes were chosen for detailed studies to learn if there were any specific isolate differences to their inorganic nitrogen assimilation. Those employed in the sequence were #62, Streptomyces griseus, "Cooper" strain and "Olney" strain. All of these isolates were cultured in regular $M1B_2$ medium and permitted to grow for a period of 30 days. During this time daily analyses were made

on samples removed from the columns. While the curves were variable in certain instances, they all indicated the same type of growth by the actinomycetes; namely, rapid consumption of ammonia followed by reduction in nitrate and increase in organic nitrogen. The strain showing the greatest organic nitrogen production was Streptomyces griseus, followed by "Olney" strain, Strain #62, and finally the "Cooper" strain. One must assume from these results that any substrate or environment, either supporting or containing ammonia, would give adequate nitrogen for available growth. It should also be observed that the addition of nitrate would increase the yield in the actinomycete population.

Figure 12 illustrates one of the parameters included in the investigations having to do with the pH of the various media during a 30-day study period of the different strains in different columns. Since ammonia may be discharged as a gas when the pH exceeds 7.5, these data were added to illustrate that few conditions existed when ammonium could be liberated from the media. It must, therefore, be concluded from these results that the ammonium lost from the medium was assimilated by the growing actinomycetes.

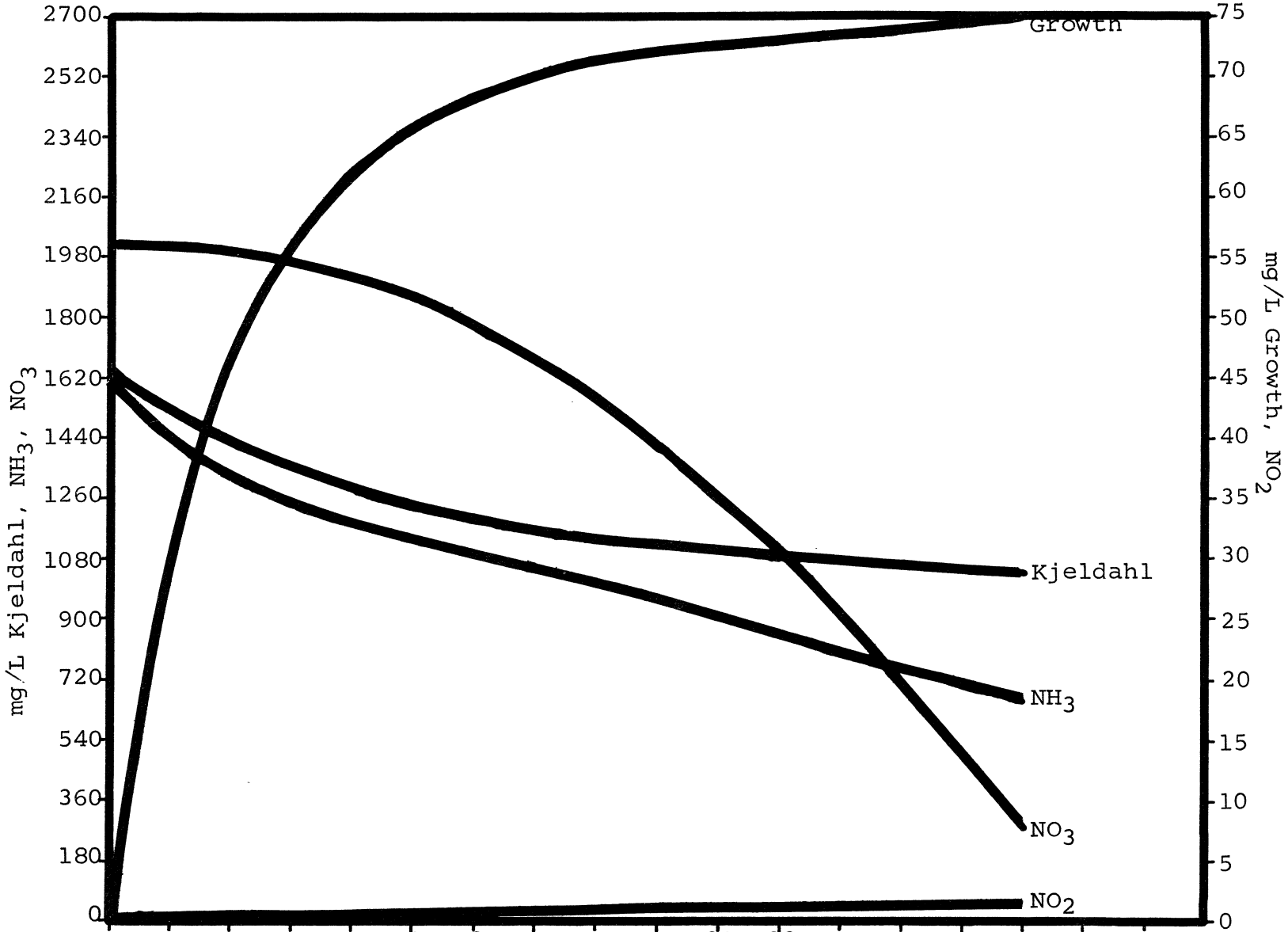

Figure 1--Relationship of growth and inorganic nitrogen uptake in Flask Medium #1, Strain "S", flask series.

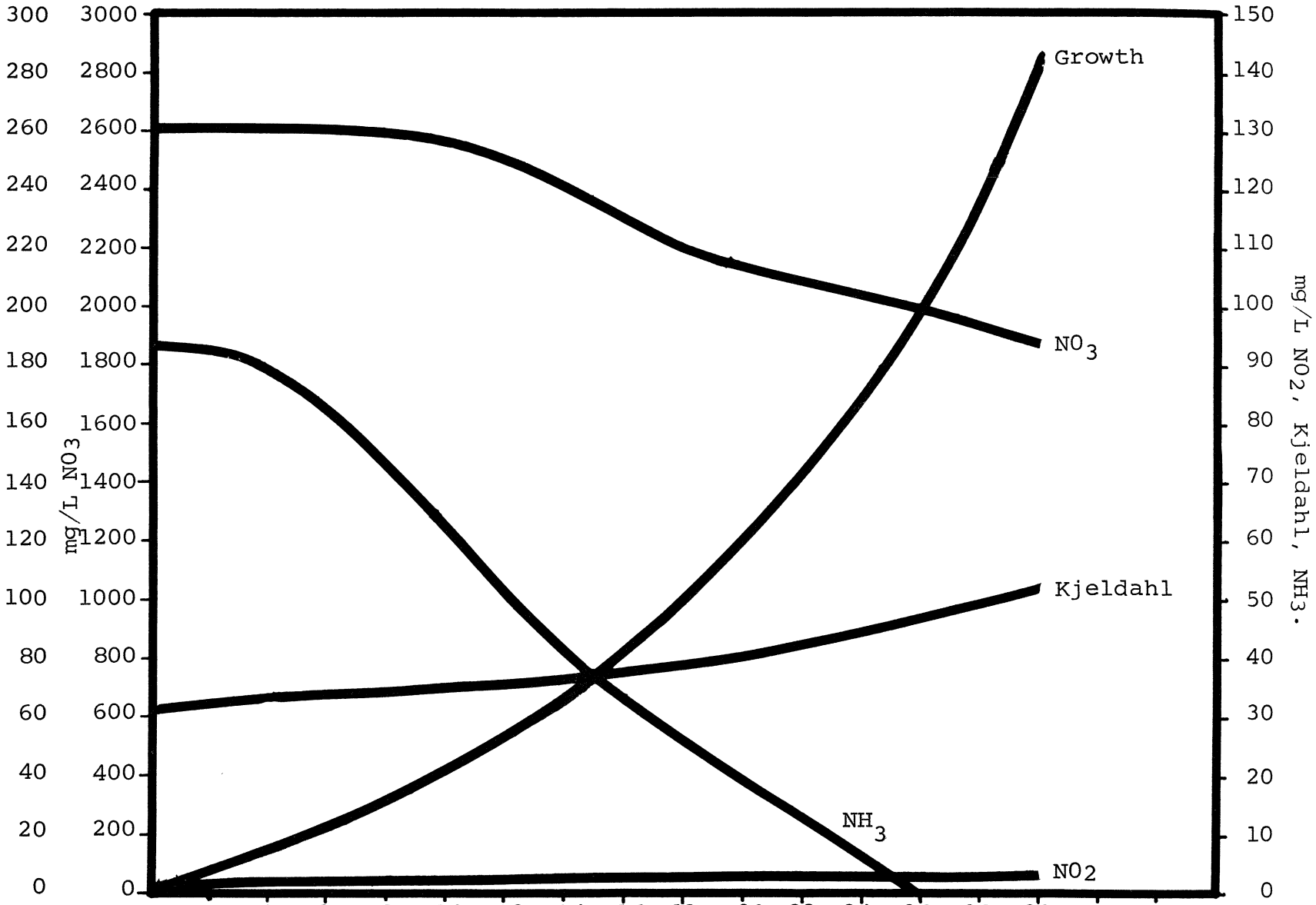

Figure 2--Relationship of growth and inorganic nitrogen uptake in Medium MlB_2, Strain "S", flask series.

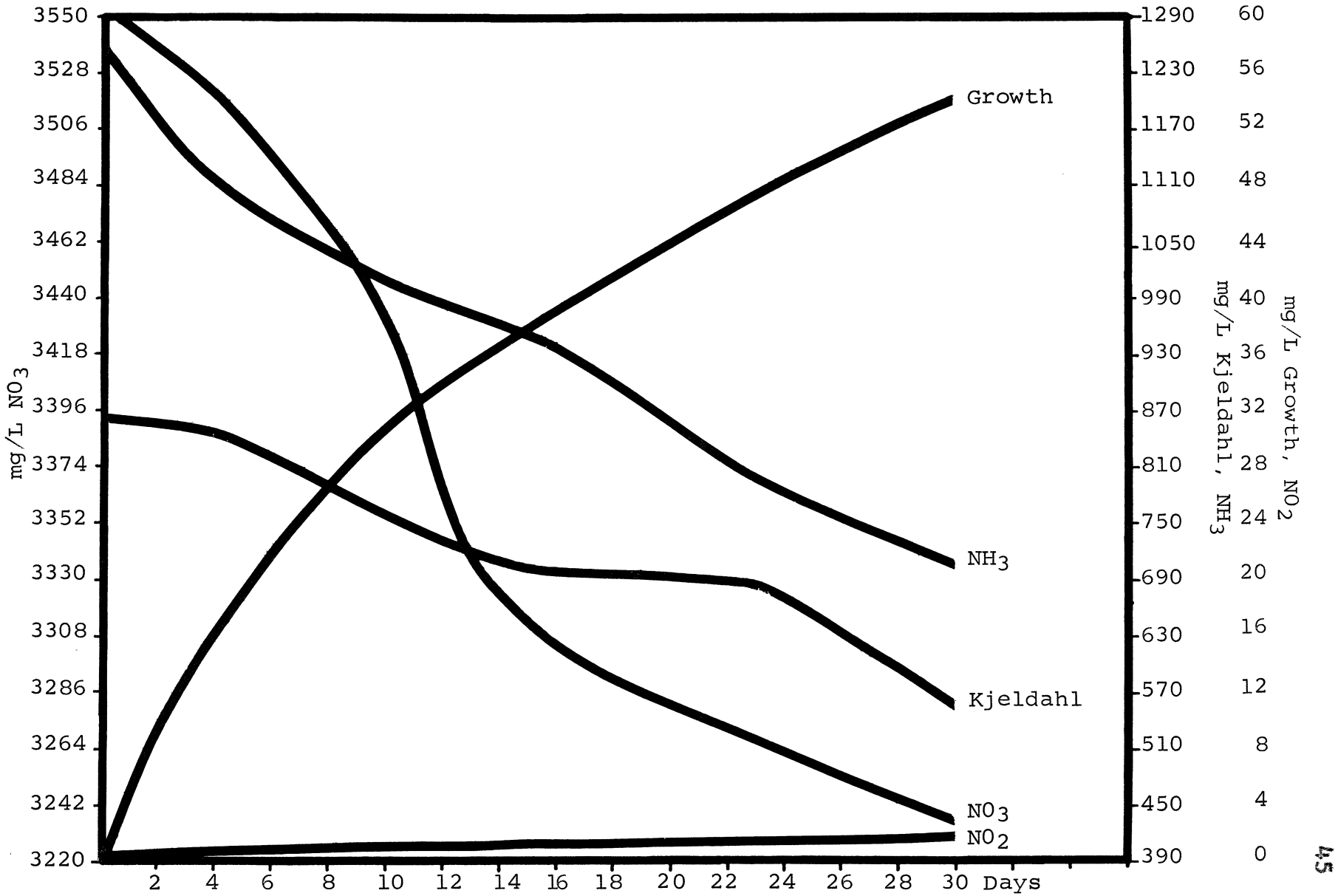

Figure3--Relationship of growth and inorganic nitrogen uptake in Medium M1B, Strain "S", flask series.

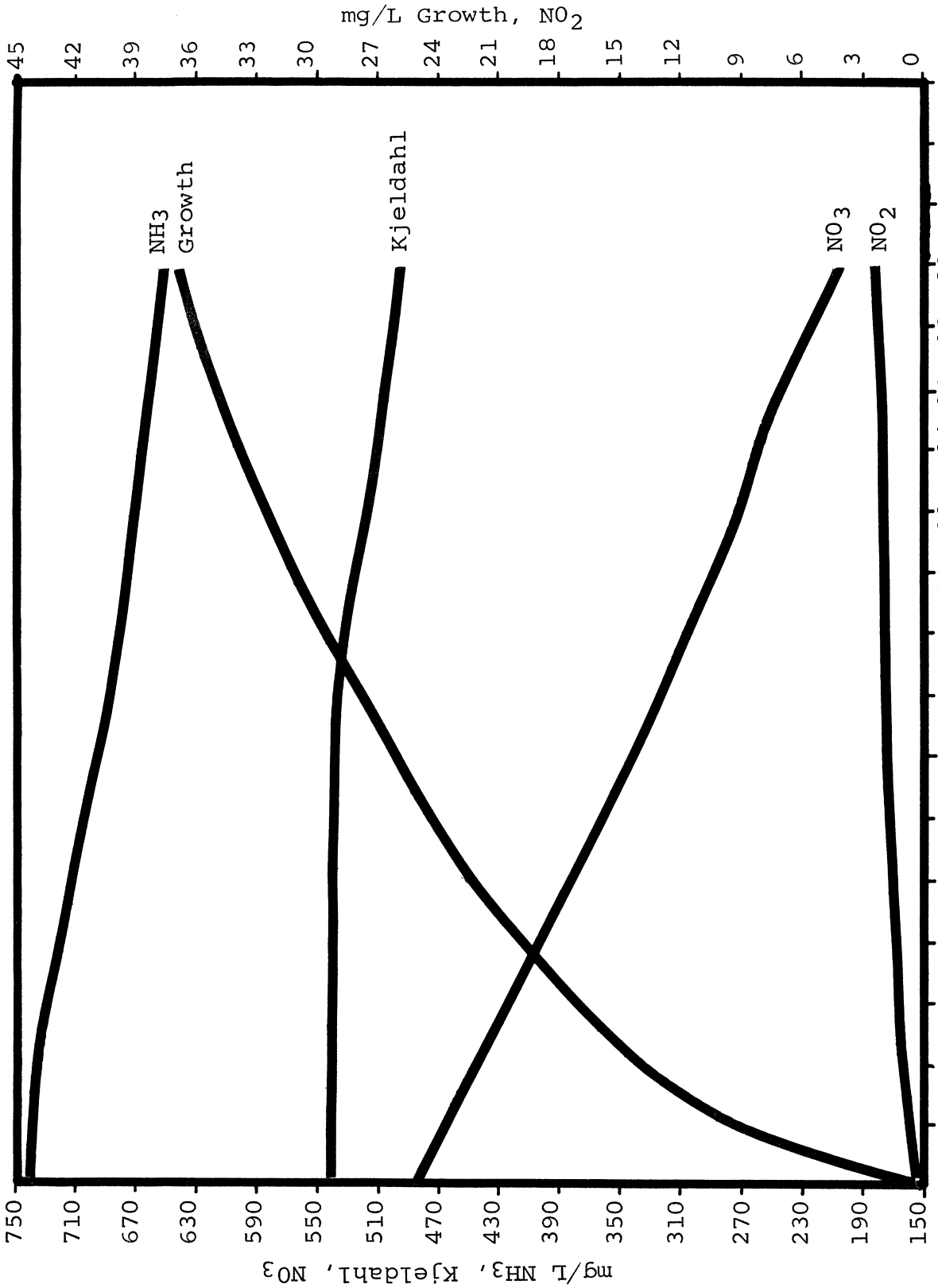

Figure 4--Relationship of growth and inorganic nitrogen uptake in Medium M1B1, Strain "S", flask series.

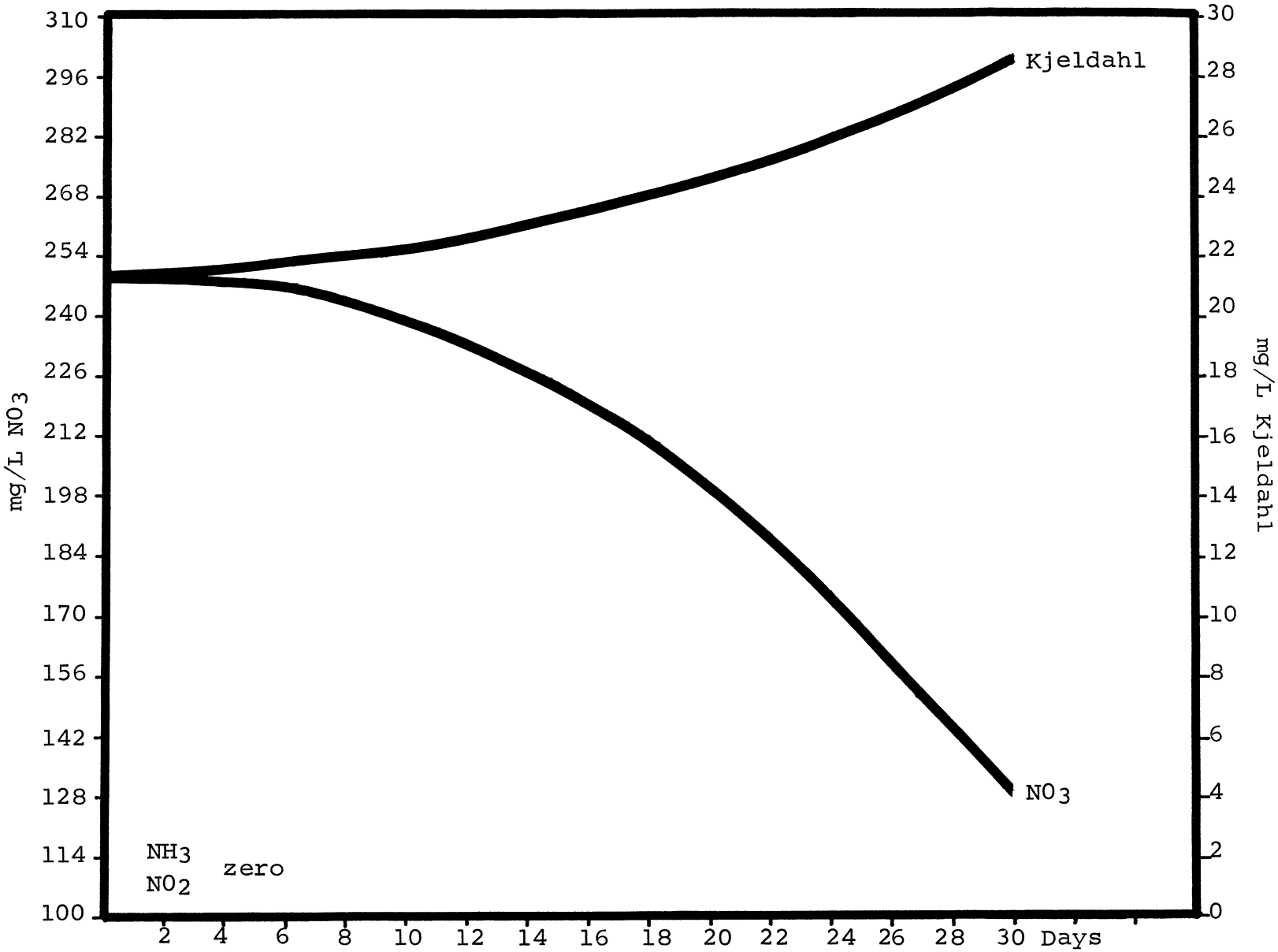

Figure 5--Relationship of inorganic nitrogen and organic nitrogen in cultural column employing "Cooper" strain. No nitrogen added separately. Only that available in other components of the medium, MlB_2.

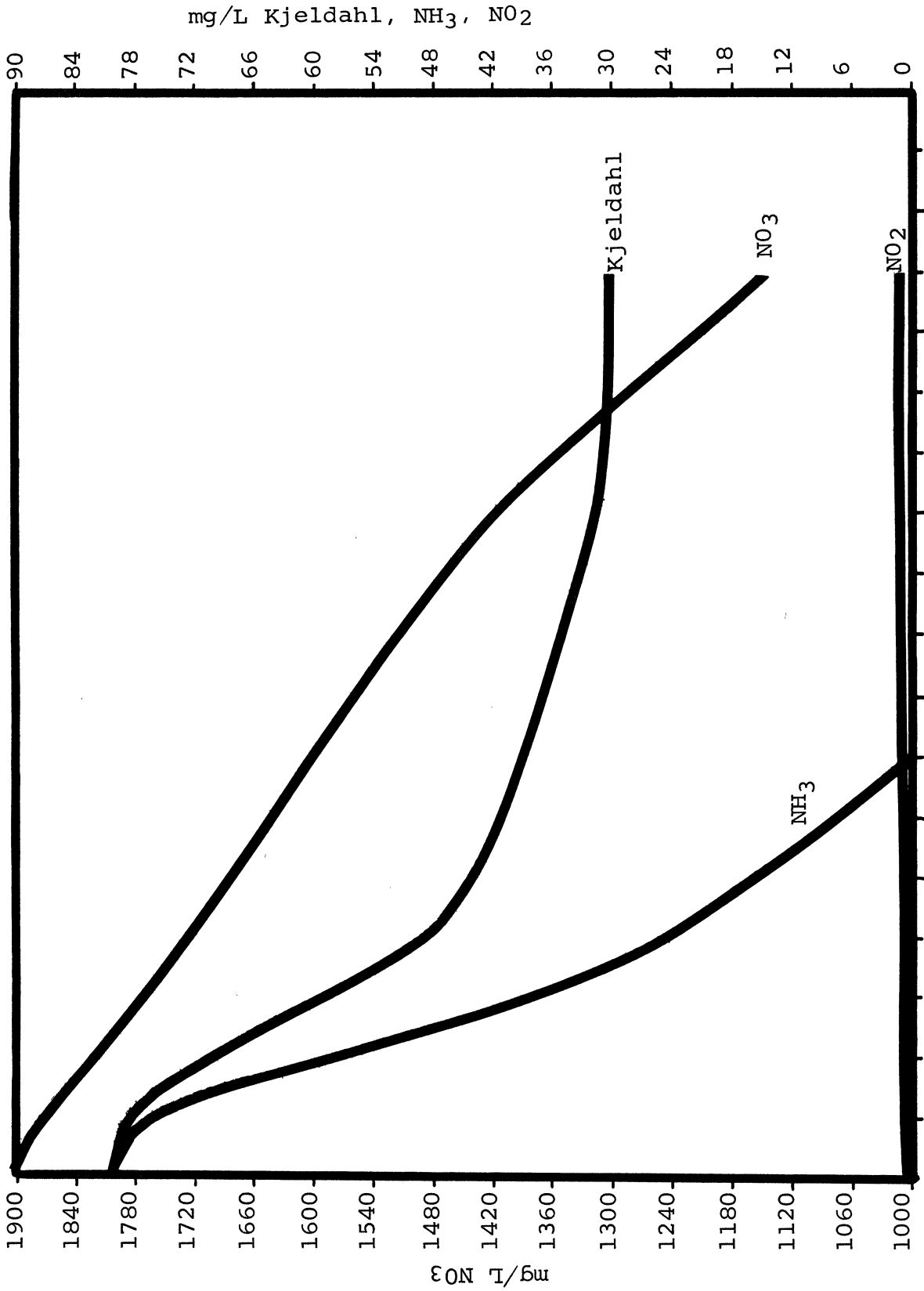

Figure 6--Relationship of inorganic and organic nitrogen in cultural column employing "Cooper" strain. Medium MlB_2.

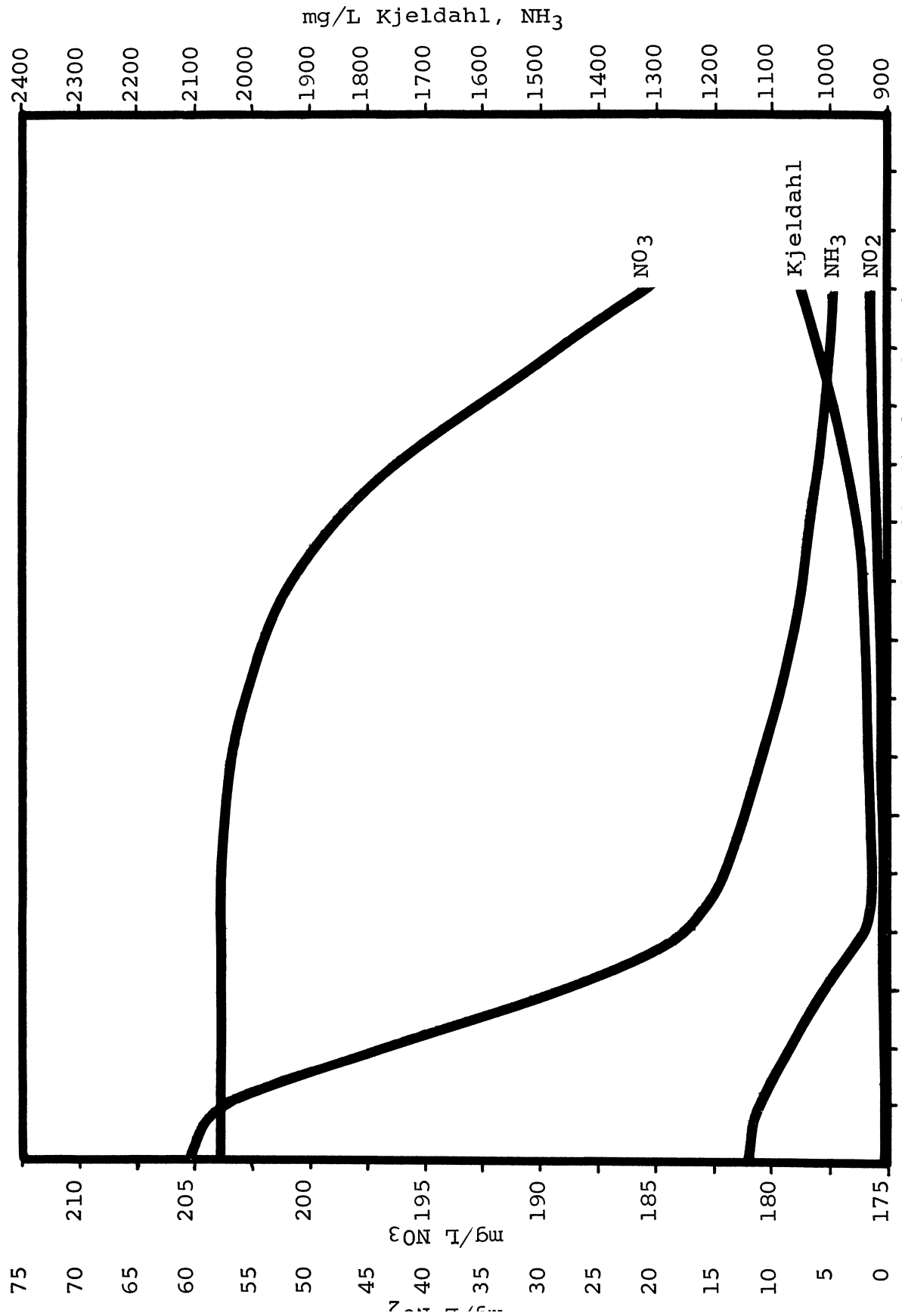

Figure 7--Relationship of inorganic and organic nitrogen in cultural column employing "Cooper" strain. Medium M1B1.

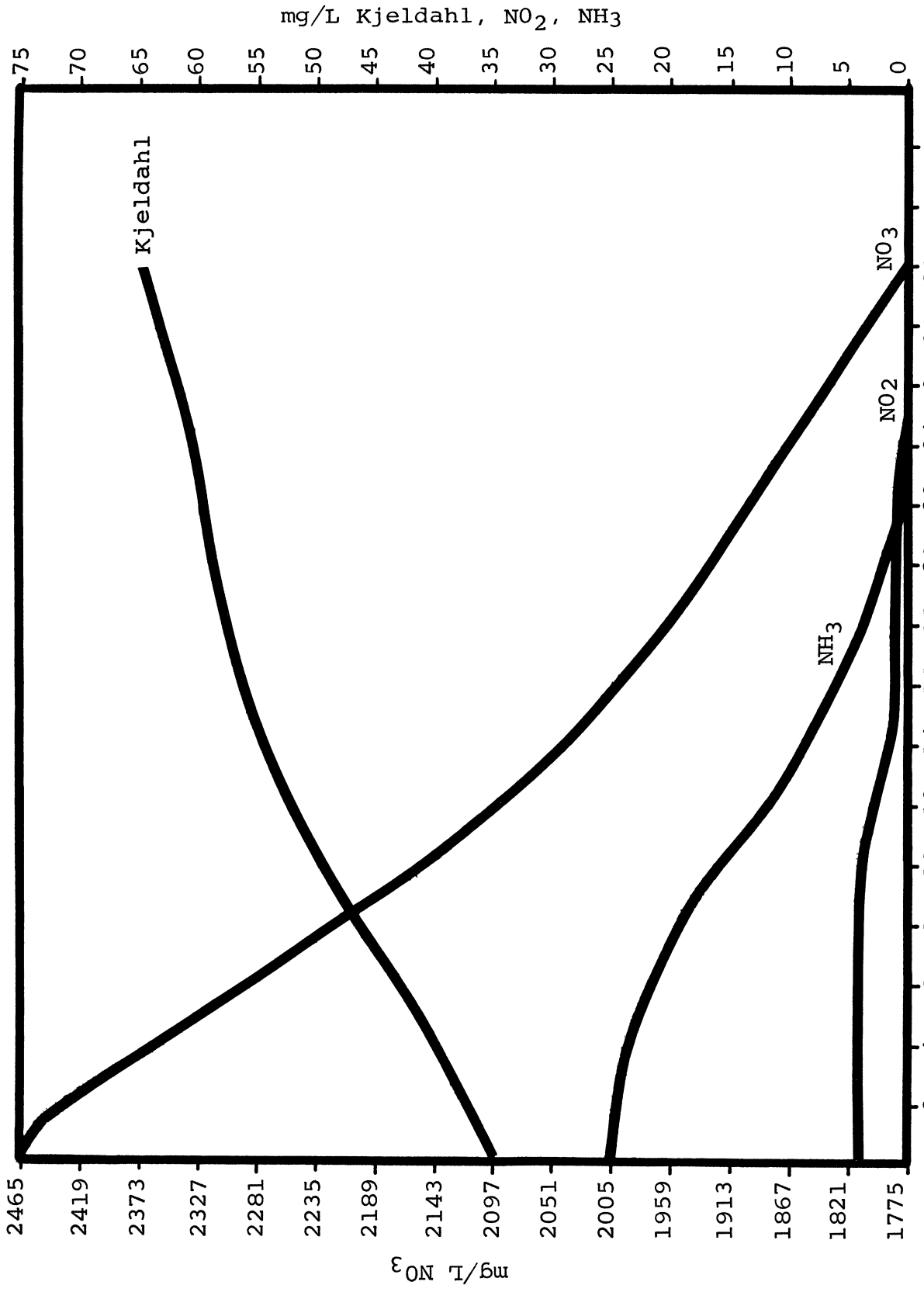

Figure 8--Relationship of inorganic nitrogen uptake in cultural column employing Strain #62. Medium M1B2.

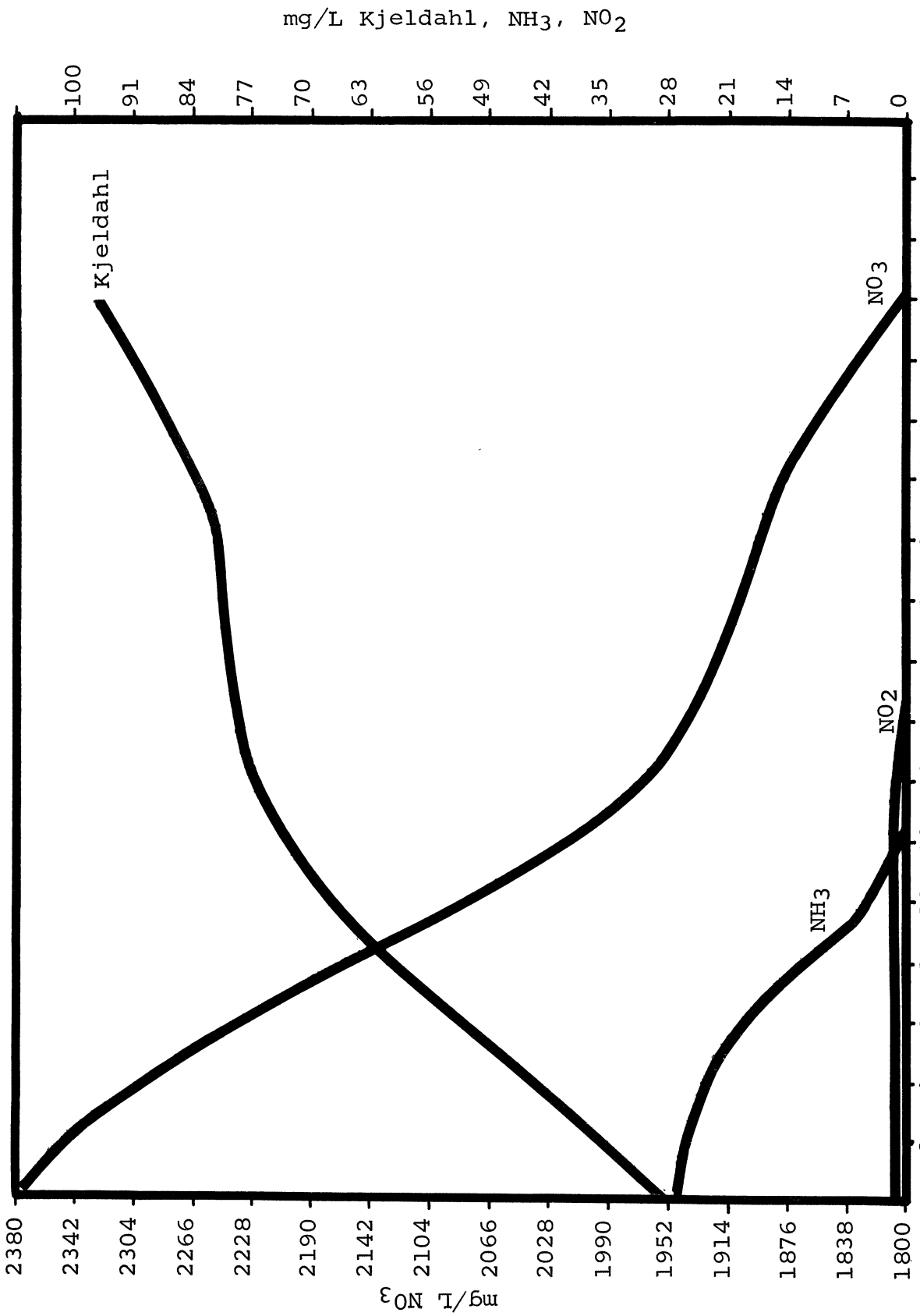

Figure 9--Relationship of inorganic nitrogen uptake in cultural column, strain used - Streptomyces griseus, Medium $M1B_2$.

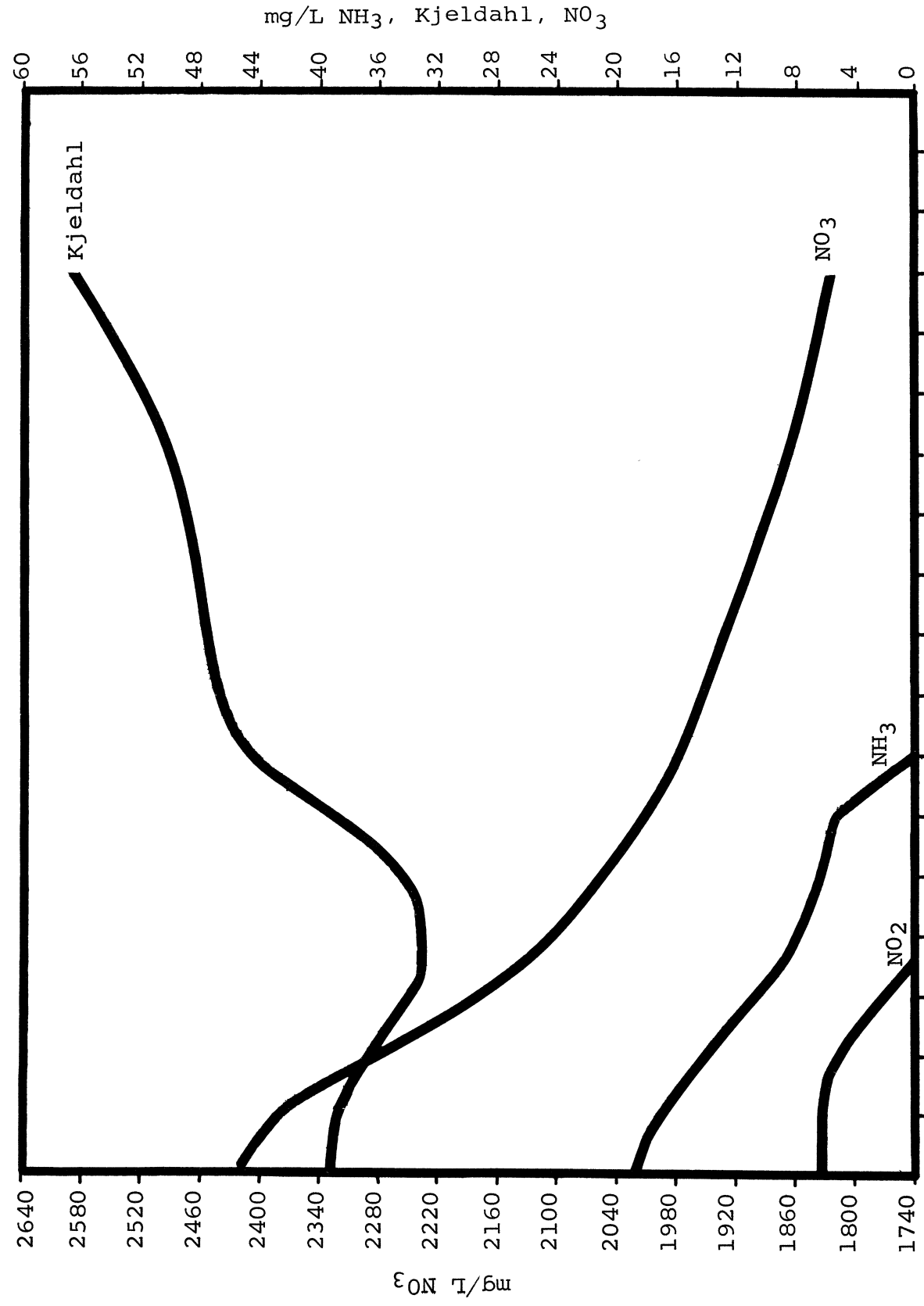

Figure 10--Relationship of inorganic nitrogen uptake in cultural column employing "Cooper" strain, Medium M1B2.

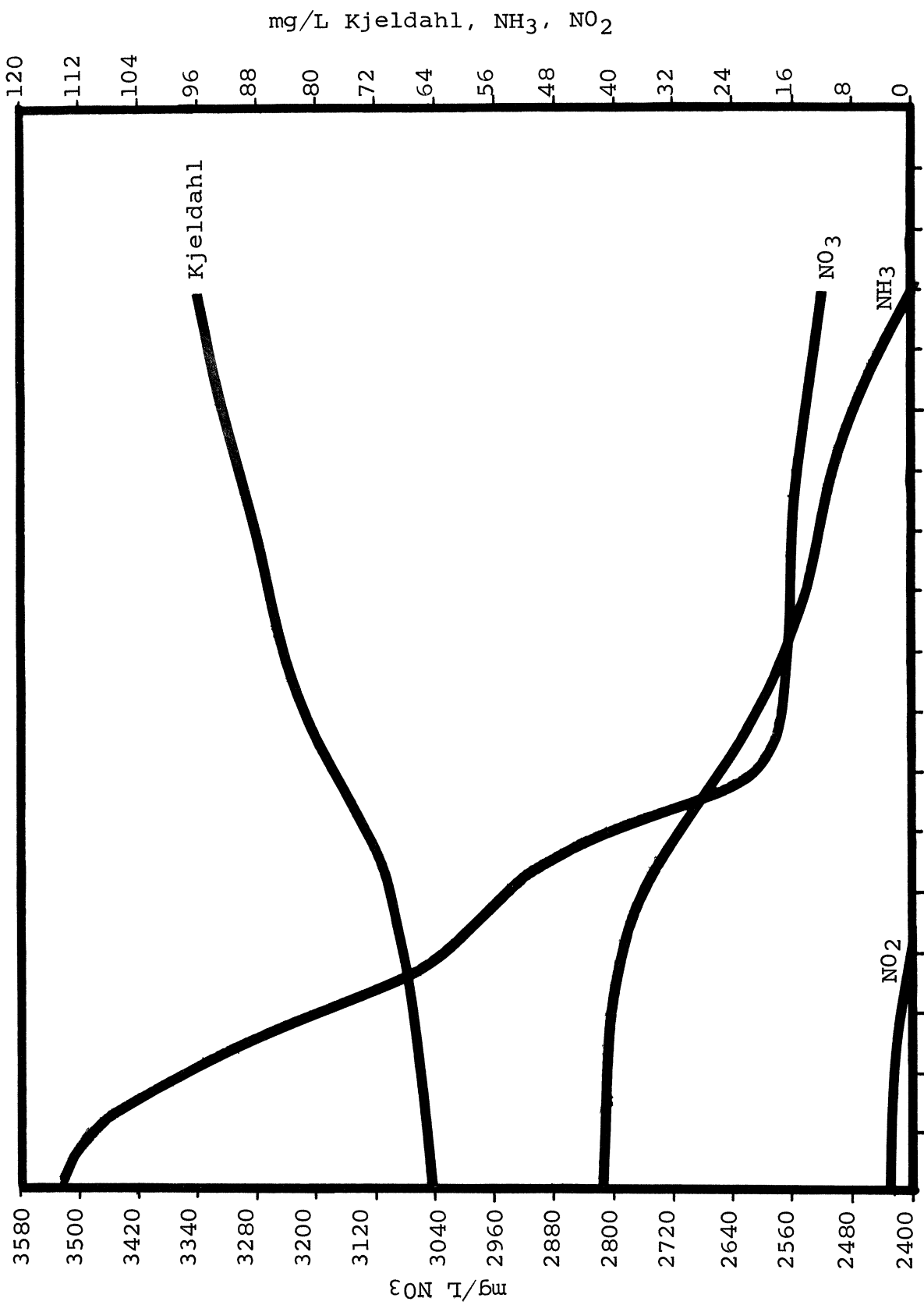

Figure 11--Relationship of inorganic nitrogen uptake in cultural column employing "Olney" strain, Medium M1B2.

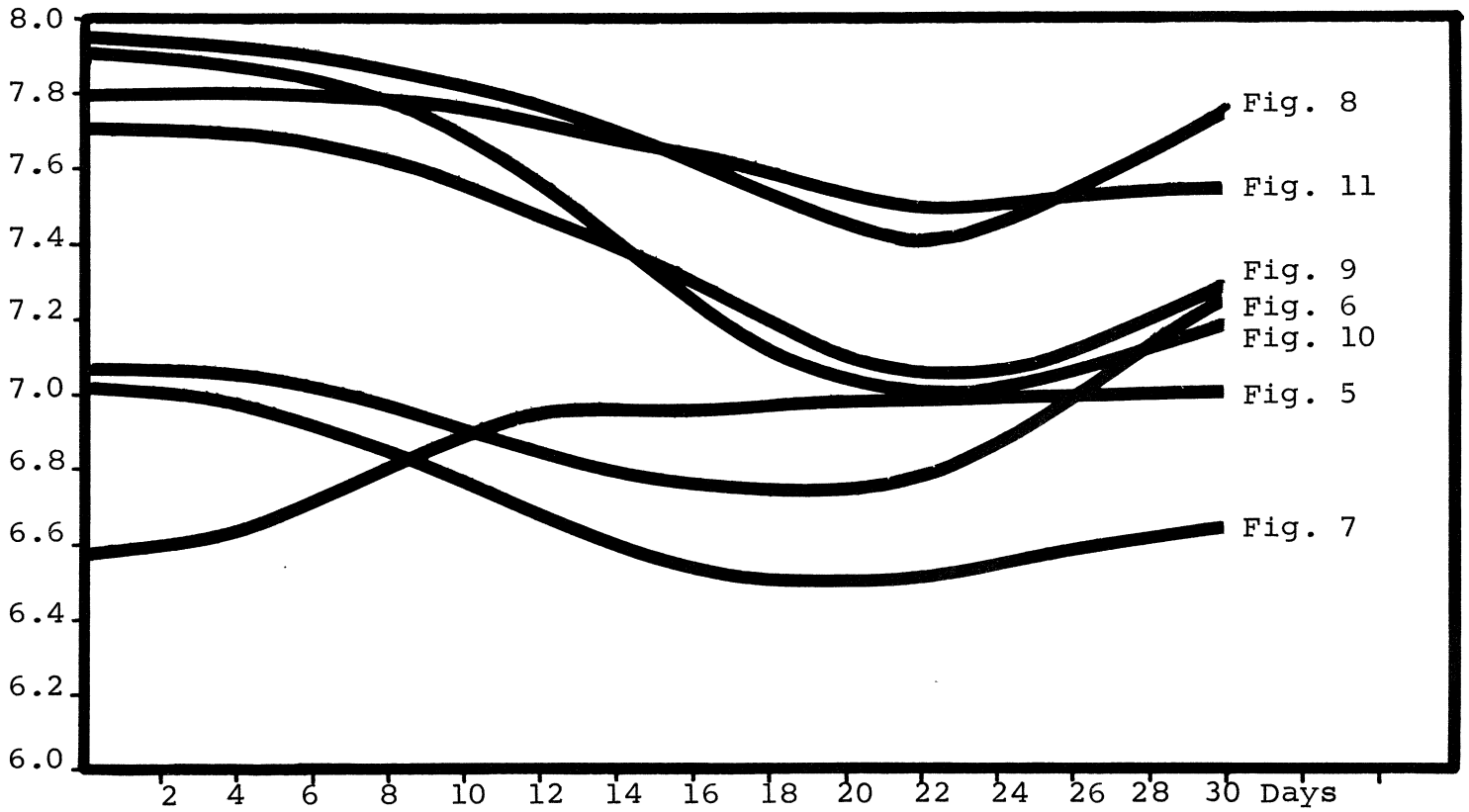

Figure 12--pH readings obtained by various experimental media and strains (Figure notations indicate previous graphs).

CHAPTER IV

DISCUSSION

The precise evaluation of the inorganic nitrogen assimilation during growth and development of the strains of actinomycetes used in this study yielded several factors for consideration. Suitable chemical tests for the types of inorganic nitrogen compounds under consideration were selected on the basis of accuracy and minimum detectable quantities. Of primary interest was the possible preference for one particular nitrogen source. As shown in Chapter III it appeared that ammonia was assimilated initially at a more rapid rate than nitrate when both sources were present in a medium.

At no time was the rate of assimilation of a specific nitrogen source the same for the different strains of actinomycetes tested. The cultural columns, described earlier, aided these investigations by providing a more convenient means for manipulation of the isolates. These columns simulated an environment in the laboratory that approximated as nearly as possible the natural conditions.

It is true that the amount of nutrition supplied these microorganisms in the different types of synthetic media surpassed in concentration that found in their native habitat.

Nevertheless, the organisms required higher concentrations and were, consequently, capable of utilizing each nutritive compound furnished them under these circumstances. In many cases, if lesser quantities of such ions as phosphate, potassium, calcium, sodium, magnesium, chloride, and sulfate were supplied, the amount of growth which resulted would not have been as high as if those ions had been supplied in high concentrations. The inoculated spores germinated into primary mycelia. Development of those primary mycelia occurred below the surface of the liquid medium. Growth on the surface of the liquid medium was that of secondary mycelia. At one particular point in the growth and development of the subsurface mycelia a series of spores were developed on the side of those same mycelia. Those spores were lateral spores and in many cases gave rise, upon germination, to surface growth. The aerial mycelia (those growing on the surface of the liquid medium) gave rise to chain spores which occurred at the end of the hyphae. This was usually considered a secondary type of growth, and this sequence of events should have occurred for the over-all metabolic life cycle to be accurately portrayed. A solid substrate on which the actinomycetes could adhere during their growth and development has been shown to yield greater quantities of growth. Nevertheless, a sample taken from such a container would not show a

true representation of the amount and kind of nitrogen present because of the difficulty in separating the mycelia from the solid substrate. Moreover, in many cases, analyses could not be run on solid substrate or medium.

The results obtained from larger growth chambers, as well as small chambers which were static, were more consistent than those obtained from agitated chambers. The possibility was that the continuous agitation was not conducive to a constant rate of growth, and, consequently, the amounts of inorganic nitrogen which were assimilated were varied. The type of growth which occurred in flasks on a rotary shaker varied significantly. Primary mycelia did not account for all of the increase in growth and development. Neither did aerial mycelia. Certainly there was evidence of both types present. It was noted, with great interest, that in spite of the continuous agitation of the medium there was a strong tendency on the part of some mycelia to adhere to the flask wall. That type of growth occurred at a level on the side of the flask which alternated between exposure to the air and exposure to the media, depending on the phase of the rotation cycle through which the shaker was passing.

For the most part ammonia was assimilated at a faster rate than nitrate. Reference is made to Figures 1 through 11. The indication was that ammonia, being the more reduced form of inorganic nitrogen available, was assimilated in

preference to nitrate. However, nitrate was utilized by all the strains tested in increasing amounts after the quantity of ammonia was reduced to a minimum.

Nitrite, apparently toxic to the actinomycetes in relatively high concentrations, did not increase significantly during the 30-day experiments. The data indicated that each strain of actinomycete, during its metabolism, either allowed a certain quantity of nitrite to exist or reduced the nitrite to ammonia which could then be utilized. This would suggest that a protective mechanism existed within the actinomycetes which prevented the accumulation of a toxic substance. In that case it was nitrite.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. Different types of growth chambers were investigated to determine the behavior of the actinomycetes in static and agitated flasks as well as in large cultural columns. The cultural columns proved to be the most useful since they provided an environment for a constant rate of growth and development of the particular actinomycete under consideration. More consistent and reliable data were obtained in the columns from the standpoint of inorganic nitrogen utilization and the formation of organic nitrogen.

2. The development of a medium which would support the growth of several strains of actinomycetes was paramount in the investigations. That medium, $M1B_2$, satisfied that requirement. Special attention was directed toward maintenance of all ions in solution so that the essential ingredients would be available and consequently could be subjected to analytical determinations. At the same time, it was necessary that the proper medium would furnish all necessary nutrients for spore germination, growth and development of the actinomycetes.

3. Nitrate was utilized by the strains tested at one of two rates. Some of the strains did not assimilate nitrate

rapidly for approximately six days after which time the increased assimilation was quite apparent. On the other hand, other strains tested utilized nitrate at roughly the same rate throughout the thirty-day period of investigation. The amounts of nitrate assimilated ranged from approximately 7 per cent to 80 per cent of that which was in the medium. When the concentration was high, the corresponding amount assimilated was not necessarily high.

4. Ammonia utilization varied also according to the strain being tested. In the majority of cases, 100 per cent of the ammonia present in the medium was utilized when it was present in low concentrations. The lowest figure in this category was approximately 15 per cent utilization followed by 50 per cent for one other strain of actinomycete when the ammonia was present in concentrations approaching 1000 mg/L.

5. Organic nitrogen increased detectably over the thirty-day investigation period. The largest increase in any one medium was 70 mg/L increase during the thirty-day period. The opposite extreme was also evidenced. Several strains deposited only a few milligrams into the medium.

6. Nitrite content of the media increased to a particular concentration which was not exceeded at any time during the thirty-day period. If, on the other hand, nitrite was

already present in the media from an external source then the concentration was reduced to zero well before the thirty-day time was reached.

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