AMMONIA AS AN INTERMEDIATE
IN NITRATE REDUCTION BY BACTERIA

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PREFACE

Ranking alongside photosynthesis as a phenomenon essential for life as we know it, is nitrogen assimilation by green plants and various microorganisms. Its importance is readily evident when we consider the enormous amounts of nitrogen applied to our agricultural fields annually, in the form of chemical fertilizer.

Within the past fifteen years the metabolic pathway of carbon in the photosynthetic cycle has been fairly completely elucidated. The mechanism of nitrogen assimilation which appears at least to be a much simpler process, has successfully evaded the efforts of many workers over a considerable length of time. Much of the early failure was due undoubtedly to lack of suitable techniques. In addition, the absence of specific criteria has led to many varied and conflicting results and conclusions. At the present time there are many unanswered questions in this field of research.

The experiments reported in this thesis were an attempt to shed light upon the possibility that ammonia might be an intermediate compound in the reduction of nitrate by soil microorganisms.

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INTRODUCTION

It was recognized rather early in the study of bacterial nutrition that many bacteria are able to utilize inorganic nitrate as the sole source of nitrogen. This process involves a reduction of the highly oxidized nitrogen of nitrate to its most reduced form as the amino group of amino acids. An alternative metabolic reduction results in the production of gaseous nitrogenous compounds and molecular nitrogen. This process, commonly referred to as denitrification, can be carried out by certain species of bacteria. The more important process from the point of view of the microorganism is the former, i.e. the formation of amino acids. In spite of a great deal of interest and activity in this field of research, various aspects of the pathway of reduction still remain obscure. Much of the earlier work has been inconclusive and contradictory and, as a result, there has been a great deal of confusion in this particular aspect of bacterial metabolism. Since the advent of the use of the stable isotope of nitrogen, N^{15}, as a biochemical tracer, much of the uncertainty in specific aspects of the work previously done has been eliminated. Recent studies using purified enzymes have also resulted in a better understanding of the biochemical mechanisms involved in biological nitrate reduction.

The first and easily recognizable reduction product is nitrite. This step is so well established that the bacteriological test for nitrate reduction by bacteria involves testing the medium for the presence of nitrite. A discussion of the properties of the enzyme system involved in the formation of nitrite from nitrate by *Bacterium coli* is given by Yamagata (1).

Much less certain are the intermediate compounds between nitrite and amino acids. There is a long list of experiments performed by numerous workers which seems to implicate ammonia as an intermediate in the reduction process by a wide variety of organisms. Bach and Desbordes (2) observed that when *Aspergillus* is maintained on an acid medium (pH 1.6-2.4), the amount of nitrate which disappears is equal to the amount of ammonia formed. Ammonia production by *Escherichia coli* on nitrate medium was reported by Aubel (3) and Korsakova (4). *Clostridium welchii* can effect the reduction of nitrate to ammonia with the aid of molecular hydrogen (5). In addition, Meiklejohn (6) has observed ammonia formation by a number of strains of bacteria isolated from sewage.

However, the mere appearance of ammonia in cultures falls far short of being convincing evidence of its position as direct intermediate in the reductive path. Much of this kind of evidence advanced in support of ammonia as an intermediate in the fixation of atmospheric nitrogen has

been sharply criticized by Burk and Horner (7). Their experiments indicated that the ammonia reported to be present in cultures of Azotobacter, fixing nitrogen, was derived in all probability from decomposition of cell nitrogen and not to any measurable extent by direct synthesis from molecular nitrogen.

Aside from the actual detection of ammonia in cultures, another kind of evidence that has been presented involves a study of the comparative utilization of ammonia versus nitrate. Thus in 1940, Pratt et al. (8) reported that cells of Chlorella vulgaris seem to show a very pronounced preferential absorption of ammonium ions in the presence of nitrate. These authors conclude from their work that support is gained for the notion that nitrate must be reduced to the ammoniacal form in order to be available for participation in the metabolic reactions of Chlorella cells. It has been shown by Virtanen et al. (9) that the soluble nitrogen-fraction formed in cells of low-nitrogen Torula utilis, both in nitrate and ammonium solutions, has a similar composition. Kinetic studies by Lewis and Hinshelwood (10) compared the rate of utilization of ammonia by coliform bacteria with its possible rate of formation from nitrate. The results indicated that while ammonia is being utilized by the cells, the reduction of nitrate and nitrite is inhibited. The addition of ammonia to a culture of Bacterium lactis aerogenes leads

to an almost complete inhibition of nitrite removal which does not restart until the amount of ammonia remaining is negligible. The above studies support the intermediate role of ammonia in nitrate reduction.

In the past few years, the heavy stable isotope of nitrogen, N^{15}, has been used extensively in an attempt to clarify the somewhat questionable evidence presented previously. In regard to nitrogen fixation, the following statement of Zelitch (11) sums up the advances that have been made: "There now appears to be little doubt that ammonia is a key intermediate in biological nitrogen fixation, both aerobic and anaerobic...". The chief support for ammonia's role in nitrogen fixation comes from the following kind of experiment. A comparison is made of the relative abundance of isotopic nitrogen in various fractions of compounds in organisms grown on both molecular nitrogen and ammonium nitrogen, both enriched with N^{15}. The organisms that have been tested in this fashion include the anaerobe, _Clostridium pasteurianum_ (12), the aerobe, _Azotobacter vinelandii_ (13), the photosynthetic bacteria, _Chromatium_, _Chlorobacterium_, and _Rhodospirillum rubrum_ (14), soybean root nodules (15), and the blue-green algae, _Nostoc muscorum_ (16). The results were essentially the same regardless which organism was used. Of the amino acids, glutamic acid contained the greatest abundance of isotope, followed by aspartic acid, when

either nitrogen or ammonia was the nitrogen source.

An observation made by Burris and Wilson (17) seems to indicate that there is a meeting of the pathways of molecular nitrogen fixation and nitrate reduction. They showed that aerated cultures of *Azotobacter vinelandii* previously grown either on atmospheric nitrogen or potassium nitrate, assimilated ammonium ion in preference to nitrate when supplied with ammonium nitrate. Thus *Azotobacter vinelandii* has pre-formed enzyme systems which are capable of utilizing ammonia immediately to the exclusion of other nitrogen compounds. Vickery et al. (18) with tobacco and MacVicar and Burris (19) with tomato, reported that when these plants are grown with $N^{15}H_4^+$ as the nitrogen source, the highest concentration of label appears in glutamic acid followed closely by aspartic acid, a result which is similar to that found in the previously mentioned studies of nitrogen fixation. Mendel and Visser (20) showed that in tomato plants, the distribution of N^{15} followed the same pattern in all organs of intact plants, whether the nitrogen source was N^{15}-labeled ammonia or N^{15}-labeled nitrate; in all cases the ammonium fraction of the plant contained the highest atom % excess of N^{15}. Additional work of this kind with isotopic nitrogen by Magee (16) in studies of *Nostoc muscorum* and by Eberts et al. (21) lend additional support to ammonia's role as an intermediate.

Another reason for ammonia's appeal as an intermediate

is its recognized ability to react with members of the Citric acid cycle to produce amino acids. Kornikova et al. (22) demonstrated in the case of Escherichia coli, the production of glutamic acid from α-ketoglutaric acid and of aspartic acid from malic acid. Enzyme preparations from Bacillus subtilis were shown by Kritsman et al. (23) to form amino acids from ammonia and keto acids. Other reports of amino acid formation by bacteria include those of Nisman et al. (24) and Yakobson et al. (25).

In spite of all the evidence which implicates ammonia as an intermediate in the reduction process, a number of experiments point to hydroxylamine or oximino nitrogen as possible intermediates. Indeed, Csaky (26), from a study of the comparative effectiveness of various inhibitors such as sodium fluoride, salicylaldehyde, and 8-oxyquinoline upon ammonia versus nitrate assimilation, concludes that in the case of Azotobacter, the assimilation of nitrate does not necessarily proceed through ammonia. Various workers such as Blom (27), Lindsey and Rhines (28), and Virtanen and Jarvinen (29), have reported the presence of free or bound hydroxylamine in cultures of bacteria actively reducing nitrates. On the other hand, the significance of the presence of hydroxylamine in various cultures is difficult to evaluate. Burk and Horner (30) attribute the occurrence of hydroxylamine to decomposition of cell protein. One of the big obstacles to acceptance

of hydroxylamine as an intermediate is its extreme toxicity and lack of utilization by microorganisms as shown by Weinrich (31), Segal and Wilson (32), and Novak and Wilson (33). Wood reports (34) that Azotobacter can utilize oximinosuccinic and oximinoglutaric acid in contradiction to the results reported by the Wisconsin workers. Quastel et al. (35) have shown that certain soil organisms are able to utilize pyruvic oxime. Rosenblum and Wilson (36) have postulated that the traces of oxime nitrogen found in Azotobacter cultures arise from hydroxylamine formed through side reactions and that since these oximes cannot be utilized by this organism, they accumulate and are readily detected in the medium. However, since _Clostridium pasteurianum_ can utilize oximes, they would be absent from cultures of this bacterium.

Previous work at this laboratory has supported the view that ammonia is a key intermediate in nitrate reduction by _Pseudomonas fluorescens_ (37,38). The first part of the work reported in this thesis is an attempt to verify this conclusion by an observation of the pattern of ammonia and nitrate utilization as exhibited in amino acid synthesis. Thus, if ammonia is an intermediate, the relative abundance of isotopic nitrogen in various amino acids should be similar whether the organism is grown on N^{15}-enriched ammonium or nitrate ion. The second part deals with a study of the utilization of nitrate, enriched with N^{15},

in the presence of ammonium ion by a bacterium (designated BMY soil isolate) isolated from soil by a worker in this department.

I. STUDY OF AMINO ACID SYNTHESIS

MATERIALS AND METHODS

Organism-- The organism used in this study was a strain of *Pseudomonas fluorescens*, NRRL-B-6, an isolate of the Northern Regional Research Laboratory.

Culture Methods-- The organism had been trained, by extended serial subculture, to grow on a medium having the following composition per liter: KH_2PO_4, 3.25 g.; K_2HPO_4, 4.25 g.; $NaNO_3$, 1.0 g.; glucose, 20.0 g.; $MgSO_4 \cdot 7H_2O$, 0.4 g.; $FeSO_4 \cdot 7H_2O$, 0.02 g.; NaCl, 0.02 g.; $MnSO_4 \cdot 7H_2O$, 0.08 g.; and biotin, 0.1 mg. The pH of the media was 6.8. The organism was maintained on agar slants of the above medium. When subcultures were desired, the bacterium was transferred to 20 ml. of the above medium in 50 ml. erlenmeyer flasks. Serial transfers were normally made every 24 hours until the organism was used to inoculate a larger experimental culture.

In the experiments, the large cultures were grown in 12-liter Pyrex glass bottles. Aeration was obtained by passing compressed air through a fritted-glass dispersion tube at a rate sufficient to fill the culture with small air bubbles. The stream of compressed air had been previously bubbled through water to minimize evaporation of water from the medium and filtered through a cotton-filled tube.

Six or eight-liter cultures were inoculated with a large volume (350-500 ml.) of a vigorously growing suspension of organisms. Small samples were removed periodically and analyzed for nitrate concentration by the brucine sulfate method (37). Upon depletion of the original nitrate present, the cells were centrifuged down using a steam driven Sharples Super Centrifuge, and roughly divided into two equal portions. One portion was resuspended in a liter of fresh medium containing 100 mg. of nitrogen as $(N^{15}H_4)_2SO_4$ (36.5 atom % N^{15} excess). The other half of the centrifuged cells was suspended in a liter of medium containing 100 mg. of nitrogen as $KN^{15}O_3$(36.5 atom % N^{15} excess). The two inoculated flasks were aerated vigorously for 2 hours. At the end of this time, both cultures were killed by the addition of 6 ml. of 12N H_2SO_4 to each flask followed by autoclaving for ten minutes.

<u>Isolation of Amino Acids</u>-- The cells were removed from the medium by centrifugation and were washed four times with distilled water. The cellular mass was then hydrolyzed by boiling with 6N HCl under reflux for 24 hours. The hydrolysate was extracted three times with 20 ml. portions of ether to remove fats, and then separated from the humin by filtration through filter paper. The filtrate was clarified by treatment with a small portion of Norite decolorizing carbon and concentrated in vacuo to about 20 ml. The concentrated amino acid solution was yellow in color and

contained 0.8-3.0 mg. of N per ml. Isolation of the amino acids from the hydrolysate was effected by column chromatography using Dowex-50 as the exchange resin and various concentrations of HCl as the eluting solvent. The method was essentially that described by Moore and Stein (39). In several instances, fractions were re-chromatographed on a second column of alumina with water and 1N HCl as the eluting solvents.

The chromatography tubes used in these experiments had ground-glass joints and a coarse sintered-glass plate which supported the column of resin. A glass-wool plug was placed between this plate and the resin. The large column used with Dowex-50 was 5.5 cm. in diameter and 60 cm. in length. The solvents used for elution were stored in 18-liter bottles with polyethylene tubing forming the connections to the top of the column.

The Dowex-50 resin was prepared by conversion to the sodium salt with 2N NaOH followed by repeated washings with distilled water. The resin was then converted to the acid form with 1.5N HCl. In preparing the column, the resin was poured into the tube as a slurry and packed by slight suction. The layer of liquid was never allowed to fall below the top of the resin. Upon obtaining the desired height of resin in the tube (approximately 50 cm.), the column was connected to the reservoir of 1.5N HCl and mounted above the automatic fraction-collector.

The fraction-collector used in this study operated upon a time-flow principle. Depending upon the setting of the timer mechanism, the delivery tube which received the eluate from the column moved into position over the next receiving tube after a given interval of time. Either fifteen or thirty minute intervals were found to be the most useful for our purposes. The rate of flow of solvent through the column was adjusted to the desired value, the lowest being 60 ml. per hour and the highest being 120 ml. per hour.

Certain fractions, namely those containing aspartic acid, threonine, serine, and glutamic acid, had to be re-chromatographed since effective resolution of these amino acids was not achieved on the Dowex-50. A much smaller column (0.8 by 15 cm.) of alumina was used in the separation of the neutral amino acids, threonine and serine from the acidic aspartic and glutamic acids (40). The alumina (Harshaw Activated Chromatographic Alumina) was first washed with 1N HCl and then with distilled water. The washed alumina was then added to the chromatography tube in a slurry and allowed to settle under the influence of gravity. The neutralized samples to be chromatographed were added to the top of the column and first eluted with distilled water (CO_2-free) until about 200 ml. had passed through and then the solvent was changed to 1N HCl, again passing through about 200 ml. When distilled water con-

taining CO_2 was used, the CO_2 became adsorbed on the alumina and then was released as bubbles of gas when the solvent was changed to HCl, and caused a troublesome blockage of solvent-flow in the tube. Serine and threonine were eluted with water, and aspartic and glutamic acids came through with HCl.

Graphs were drawn plotting the fraction number versus the % transmission of the ninhydrin-treated aliquots. Since the concentration of amino acid is inversely proportional to the % transmission, a succession of peaks was obtained whose shape and height was dependent upon the quantity of amino acid present. Except in a few instances, each peak represents only one of the separated amino acids.

Analytical Methods-- The eluted fractions were tested for their amino acid concentrations by the ninhydrin method as developed by Moore and Stein (41). 0.1 ml. aliquots were removed from each tube for analysis. The HCl was evaporated from the samples in a vacuum dessicator, using two infra-red light bulbs as a heat source. The samples never exceeded a temperature of 90 degrees Centigrade, and complete evaporation of 40 samples usually was accomplished in about an hour's time.

The isolated amino acids were identified by means of comparative one-dimensional descending paper chromatography, using a solvent system composed of water-saturated butanol and propionic acid in the ratio 7:3 (v/v) (42). A mixture

of isovaleric and isobutyric acids in the ratio 5:5 (v/v) was used in the identification of leucine and isoleucine since these amino acids do not have significantly different R_f values in the previously mentioned solvent system (43). The techniques employed in these identifications were essentially those of Block (44).

Analysis for nitrate was made by a modification (37) of the brucine sulfate method developed by Gad (45). This method allows a rapid colorimetric estimation of nitrate in the presence of quantities of glucose which would interfere with the analysis by other common colorimetric methods.

Analyses for N^{15} in the various amino acids were made on a Westinghouse mass spectrometer under the supervision of Dr. Glen Hallmark of the Department of Electrical Engineering, A. & M. College of Texas. The amino acid samples were first evaporated to a conveniently small volume, digested, and analyzed for nitrogen by a semi-micro Kjeldahl procedure. The released ammonia was converted to gaseous nitrogen in an apparatus similar to that used by Rittenberg et al. (46).

RESULTS AND DISCUSSION

As was previously indicated, it might be presumed that if an organism exhibited the same pattern of synthesis of amino acids regardless whether it is cultured on nitrate or ammonium ion as the sole source of nitrogen, one is used sequentially to the other. To determine whether nitrate and ammonium ion do exhibit this similarity of utilization, the following experiment was performed:

Six liters of nitrate media was inoculated with 500 ml. of a 42 hour culture of Pseudomonas fluorescens. After aeration for 20 hours, most of the original nitrate had been exhausted and at this time the cells were harvested by centrifugation in an International Centrifuge (size 2) at 5,000 r.p.m. The cells were divided into two equal portions, resuspended in fresh media containing in one case $KN^{15}O_3$ and in the other $(N^{15}H_4)_2SO_4$, and harvested after aeration for two hours. The cells were then hydrolyzed in HCl, and the hydrolysates treated as described earlier. The hydrolysates, which had been concentrated down to a small volume, were diluted to 25 ml. and one ml. samples removed for Kjeldahl analysis. The results from these analyses were as follows:

1) culture grown on $N^{15}O_3$ contained 17.0 mg. nitrogen
2) culture grown on $N^{15}H_4$ contained 27.7 mg. nitrogen

The solutions were then chromatographed using a flow rate

of about 60 ml. per hour.

The results are shown in figures 1 and 2. The order of displacement of the various amino acids followed that given by Stein and Moore (39). The first peak in each case was a complex containing serine, threonine and aspartic acid. The second peak contained glutamic acid. The fractions representing these two peaks were evaporated to a small volume and rechromatographed on a column of alumina. A graph representing this latter separation is not shown. The peak which followed valine and is marked _unknown_ does not correspond to any position for a known amino acid. Furthermore, this fraction gives a green color with the ninhydrin reagent rather than the customary blue resulting from reaction with an amino acid. This substance has not been identified.

As can be seen, there has been in most cases a sufficient separation of the various amino acids so that pure samples of each could be analyzed for N^{15}. Accordingly, samples of each of the amino acids were converted to molecular nitrogen and sent to Texas A. & M. for mass spectrometric examination. Due to difficulties encountered in the conversion process, the samples contained excess air and carbon dioxide. Since these substances interfere with the analysis for N^{15}, no reliable isotopic data was obtained in this first experiment.

A second experiment was therefore conducted in much the same manner as the first. Eight liters of nitrate

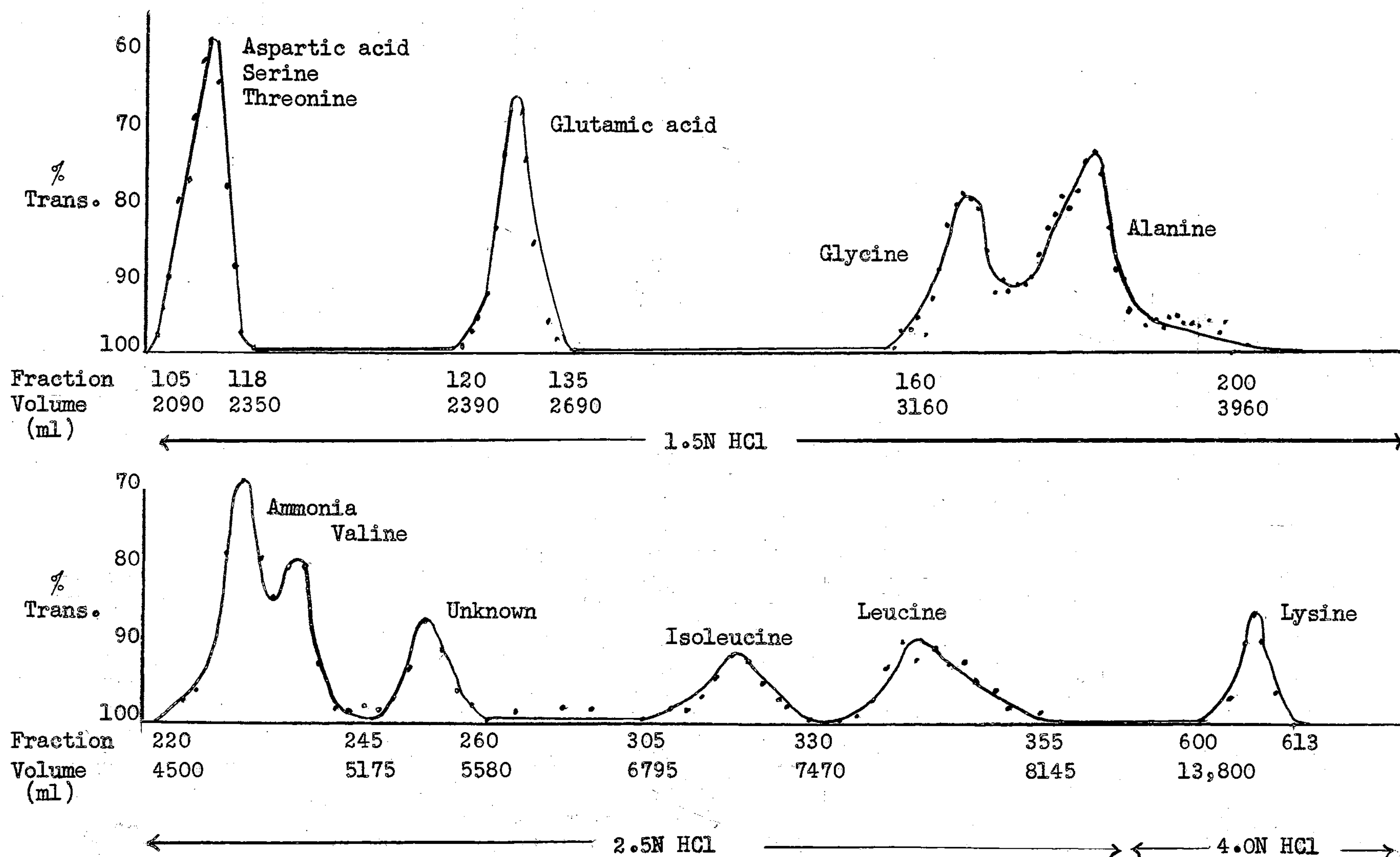

Fig. 1. Fractionation of bacterial hydrolysate on Dowex-50; culture grown with $N^{15}O_3$ as nitrogen source

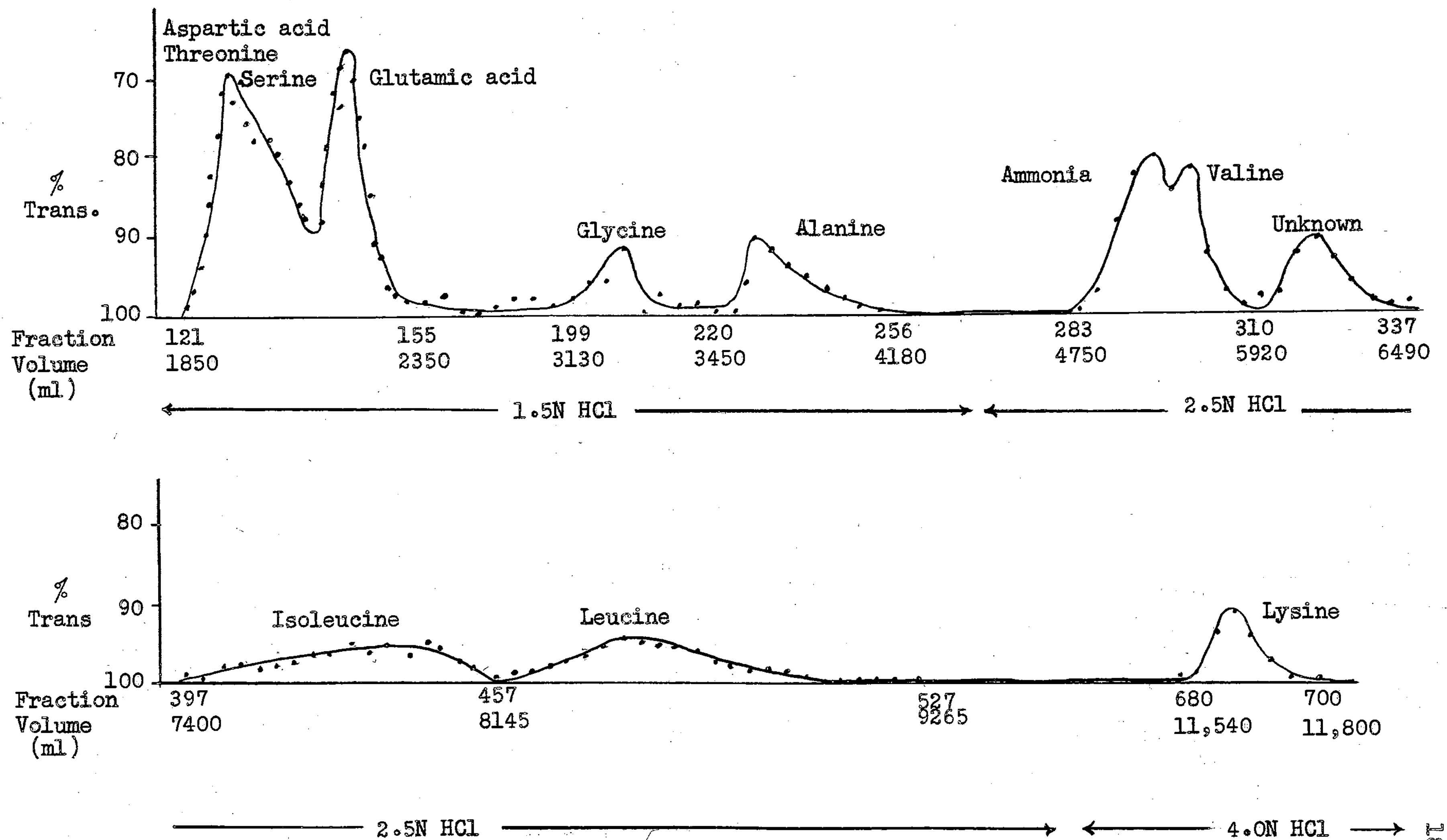

Fig. 2. Fractionation of bacterial hydrolysate on Dowex-50; culture grown with $N^{15}H_4$ as nitrogen source.

medium was inoculated with 350 ml. of a 13 hour suspension of Pseudomonas fluorescens and aerated vigorously. After 13 hours of growth, the cells were centrifuged down in a Sharples Super Centrifuge at about 40,000 r.p.m. The cells were then treated in an identical manner as in experiment 1. Analyses of aliquots of the hydrolysates for nitrogen and N^{15} gave:

1) culture grown on $N^{15}O_3$ contained 57.6 mg. nitrogen; 9.6 atom % N^{15} excess.

2) culture grown on $N^{15}H_4$ contained 47.6 mg. nitrogen; 11.0 atom % N^{15} excess.

The hydrolysates were then chromatographed using a somewhat faster flow rate (90-120 ml. per hour) than previously. The results are shown in figures 3-8. They are similar to those of the first experiment. The substance giving the green color with ninhydrin did not show up in this experiment. It would be difficult to surmise the reason for this without a knowledge of the chemical nature of the material in question. A number of other amino acids appeared which did not do so in the first experiment. These are tyrosine, histidine and arginine. This may be ascribed to greater sensitivity of the ninhydrin reagent, which was achieved by adjusting the pH more closely to the recommended value of 5. It is not known why peaks for tyrosine and arginine were not forthcoming in figure 3 as well as in figure 4. The graphs in figures 5-8 indicate that

there is an efficient separation of aspartic and glutamic acids from serine and threonine. Due to operational difficulties, isotope data for the various amino acids is not available at this time and hence it is not possible to make any noteworthy conclusions concerning the pattern of amino acid synthesis.

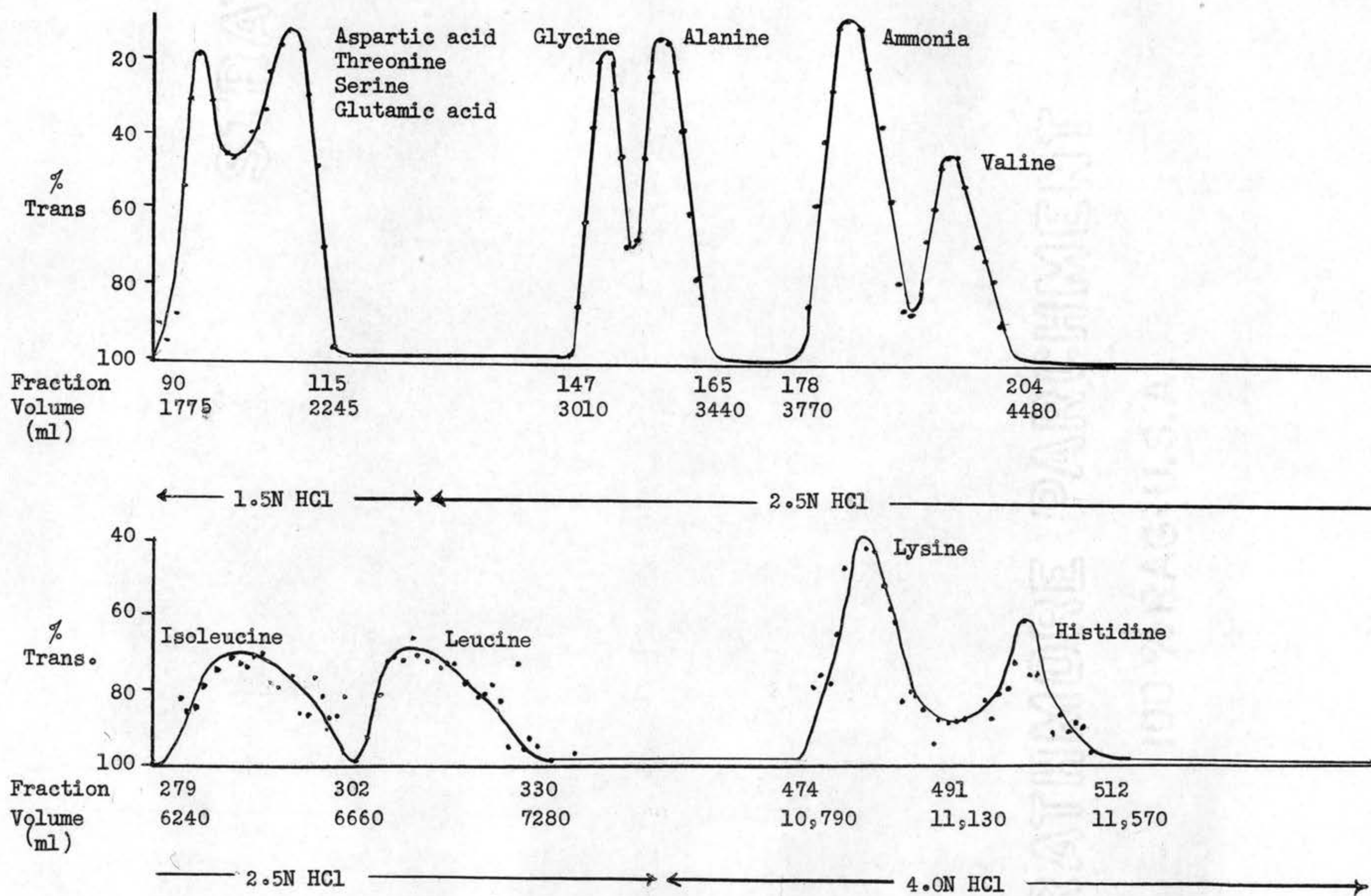

Fig. 3. Fractionation of bacterial hydrolysate on Dowex-50; culture grown with $N^{15}O_3$ as nitrogen source.

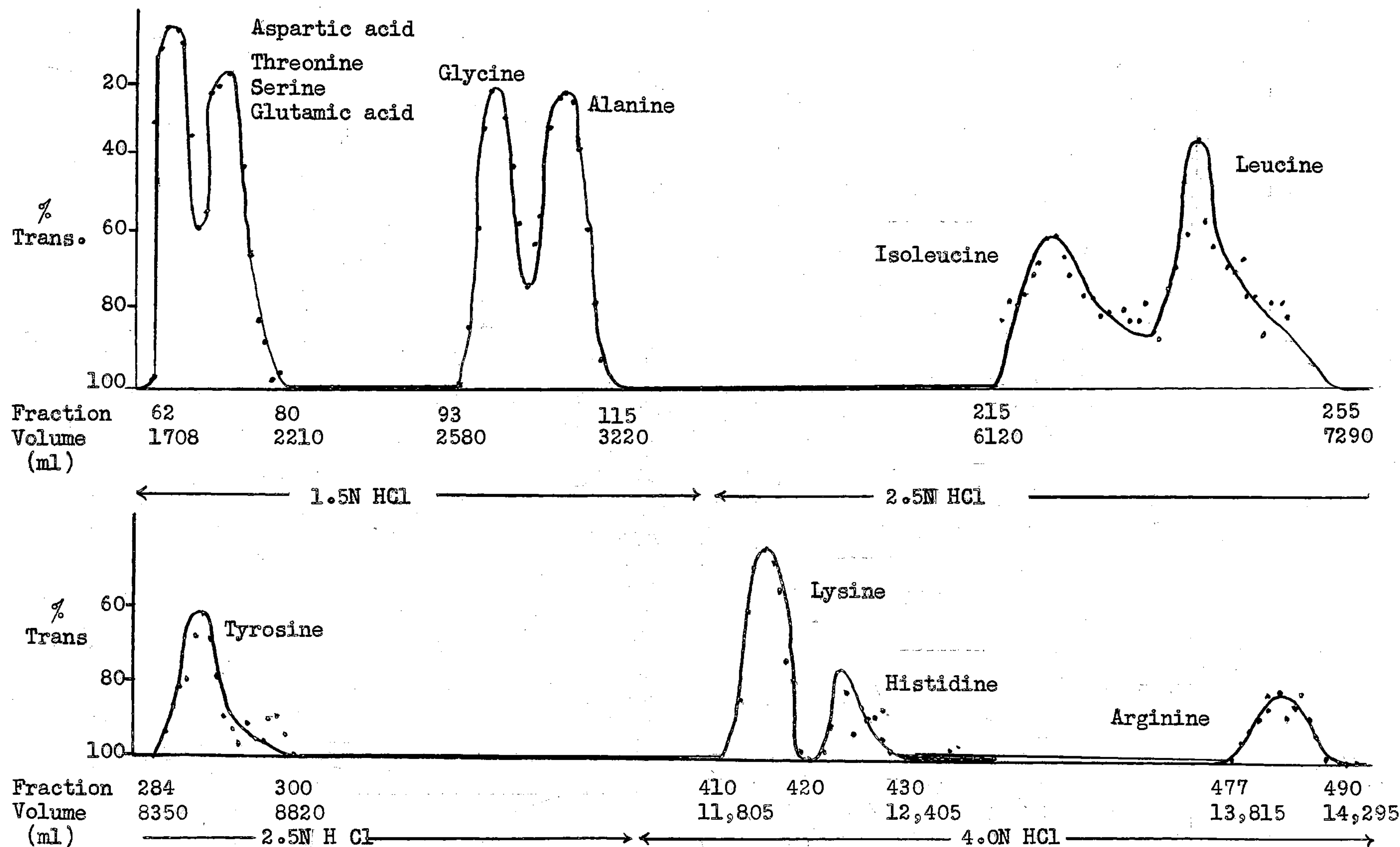

Fig. 4. Fractionation of bacterial hydrolysate on Dowex-50; culture grown with $N^{15}H_4$ as nitrogen source.

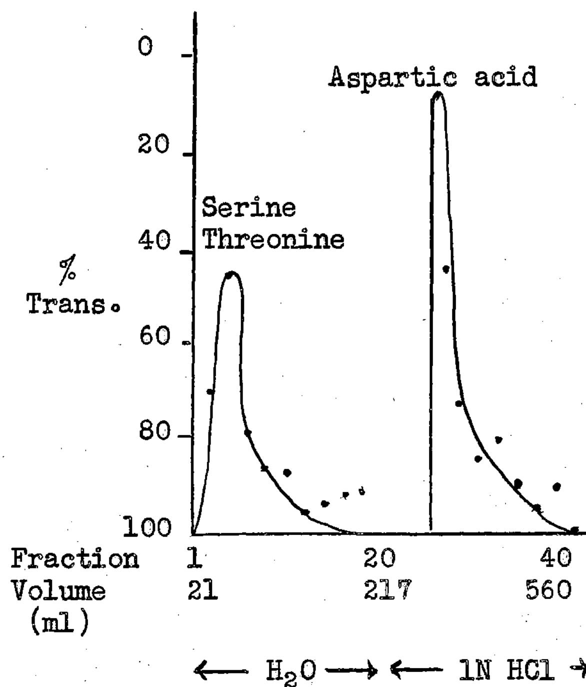

Fig. 5. Fractionation on alumina of fractions 91-101 of graph in Fig. 3.

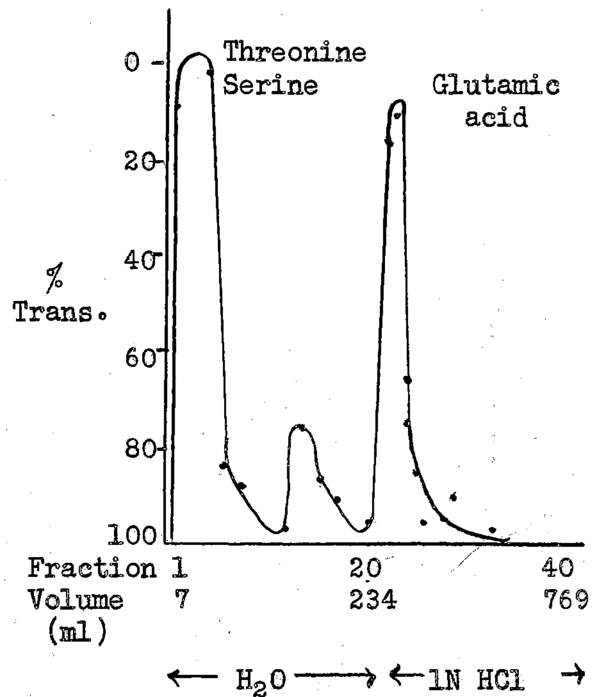

Fig. 6. Fractionation on alumina of fractions 102-113 of graph in Fig. 3.

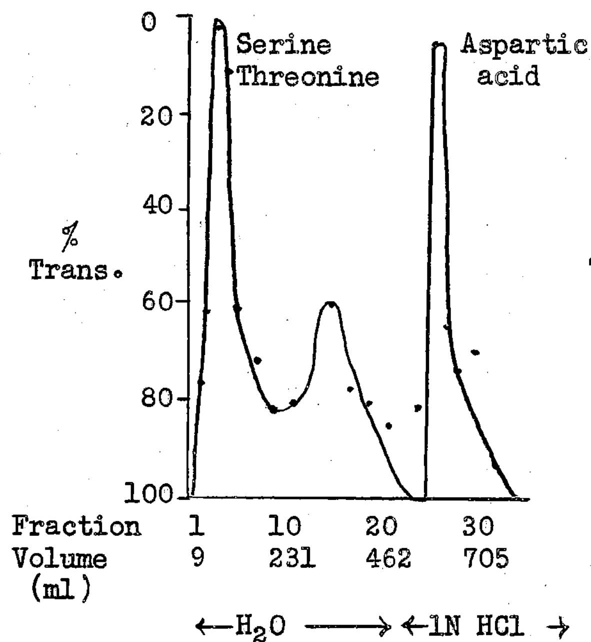

Fig. 7. Fractionation on alumina of fractions 62-68 of graph in Fig. 4.

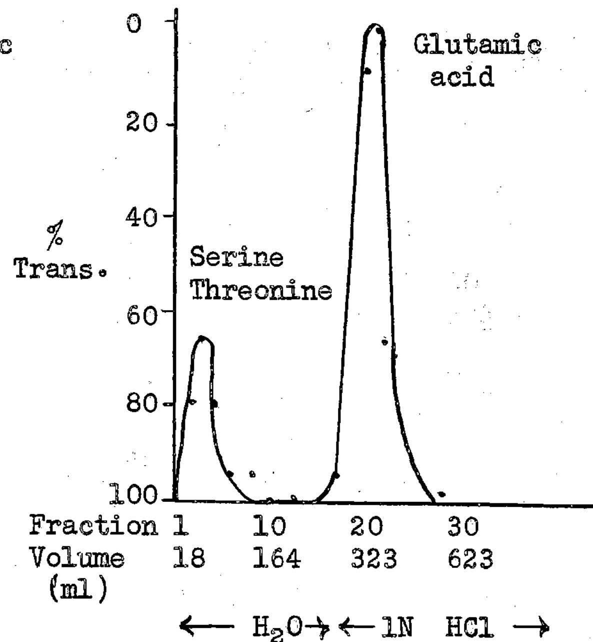

Fig. 8. Fractionation on alumina of fractions 69-79 of graph in Fig. 4.

Summary

A study has been made of the pattern of amino acid synthesis by a strain of *Pseudomonas fluorescens* when cultured on either NH_4^+ or NO_3^- as the nitrogen source, using N^{15} as a tracer. The constituent amino acids of the bacterial protein were isolated by selective adsorption on a ion-exchance resin (Dowex-50) using various concentrations of HCl as the eluting solvent. A column of alumina was used to separate aspartic and glutamic acids from serine and threonine, since these amino acids were not resolved by the Dowex-50.

Due to difficulties experienced in the conversion of the ammonia in the Kjeldahl titration solutions of the isolated amino acid samples to molecular nitrogen, no isotope data is as yet available and hence final conclusions as to the outcome of the experiment will await the reporting of these results.

II. UTILIZATION OF NH_4^+ IN THE PRESENCE OF NO_3^- BY A SOIL BACTERIUM

INTRODUCTION

Previous experiments in this laboratory with *Pseudomonas fluorescens* and *Bacillus subtilis* showed that these organisms are able to reduce nitrate rapidly in the presence of ammonia (37, 38). Burris and Wilson (17) have shown that in the case of *Azotobacter vinelandii*, aerated cultures previously grown either with molecular nitrogen or with potassium nitrate assimilated ammonium ion in preference to nitrate when supplied with ammonium nitrate. In view of the different modes of nitrogen utilization as evidenced by the above results, it was thought desirable to examine the utilization of nitrate in the presence of ammonia by a newly isolated bacterium which was being used for other studies in this laboratory and the nitrogen metabolism of which was of interest to the research group. This organism is of special interest because of its ability to utilize ortho-nitrobenzoic acid as a sole source of nitrogen. It was hoped that a knowledge of the metabolism of inorganic nitrogen by this organism might shed light upon its mode of utilization of ortho-nitrobenzoic acid.

MATERIALS AND METHODS

Organism-- The organism used in this study was an unidentified strain of bacteria isolated from soil, designated for sake of convenience as BMY soil isolate.

Culture Conditions-- The organism was trained to grow on a simple medium having a composition identical to the one described earlier in this thesis. Preliminary studies indicated that there was some factor other than nitrogen supply limiting growth when this medium was used. As a result, in the two experiments reported, a modified medium was used, with the following composition per liter: KH_2PO_4, 3.96 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; potassium gluconate, 10 g.; $NaNO_3$, 0.20 g.; $CaCl_2$, 20 drops of 1% solution; $FeCl_3$, 10 drops of 1% solution; biotin, 1 mg. The medium was brought to a pH of approximately 6.8 with KOH, using bromthymol blue as an indicator.

Culture Methods-- The apparatus used in the experiments was similar to that previously described. One liter of nitrate medium was placed in a two-liter filter flask fitted with a fritted-glass dispersion tube. The medium was then inoculated with 10 ml. of a 24 hour suspension of the organism and incubated at 25° C with vigorous aeration. Upon depletion of the original supply of nitrate-nitrogen, as evidenced by serial analysis by the brucine sulfate method (37),

a 50 ml. sample was removed. Then 12.5 mg. of N as $KN^{15}O_3$ containing 36.5 atom % N^{15} excess, and 12.5 mg. of N as $(NH_4)_2SO_4$ were added to the medium and the culture again sampled. Subsequently, sampling was repeated at definite intervals over a period of six hours.

Sampling Procedures-- Samples were obtained by withdrawing 50 ml. of the culture and pipetting into 2 ml. of boiling 0.8N H_2SO_4. The sample was then brought to a boil, stoppered, and reserved under refrigeration for analysis. At the completion of sampling, the samples were transferred to plastic centrifuge tubes and the cells packed by centrifugation. The supernatant was carefully transferred via pipette to 250 ml. volumetric flasks. The cells were then washed three times with distilled water and the washings added to the supernatant which was then autoclaved and reserved for analysis.

Analytical Procedures-- The packed cells were transferred to semi-micro Kjeldahl flasks, made distinctly basic (red color with phenolphthalein) with N/14 NaOH and boiled for at least five minutes to remove any occluded ammonia. Cellular nitrogen was determined by a semi-micro modification of the Kjeldahl procedure. Total nitrogen in the cell-free supernatant was determined by a semi-micro modification of either the iron-sulfuric acid reduction procedure as developed by Pucher et al. (47) or the salicylic acid - sodium thiosulfate reduction method as found in

Methods of Analysis A.O.A.C. (48). The Kjeldahl titration solutions from the analyses for cellular nitrogen were reserved for N^{15} analysis.

Analysis for ammonia was made directly on the supernatant by nesslerization of 10 ml. aliquots without preliminary aeration into acid solution.

Nitrate was determined by a modification (37) of the brucine sulfate method developed by Gad (45).

RESULTS AND DISCUSSION

In the first experiment, a liter of nitrate medium was inoculated with 10 ml. of a 36 hour suspension of BMY soil isolate. After about 24 hours of aeration at room temperature, the original nitrate was exhausted and at this time 12.5 mg. of N as $KN^{15}O_3$ (36.5 atom % N^{15} excess) and 12.5 mg. of N as $(NH_4)_2SO_4$ was added to the medium. 50 ml. aliquots of the culture were removed periodically and the various analyses for NO_3-N, NH_4-N, cell-N, and total medium-N were performed on the collected samples. The Kjeldahl titration mixtures were reserved for N^{15} analyses in the case of the cellular nitrogen.

The results of this first experiment are shown in figure 1. As can be seen from the graph, there was a very rapid utilization of the ammonium ion whereas the nitrate remained at essentially its initial concentration in the medium until most of the ammonia had disappeared. Only then did the nitrate concentration decline and it was utilized at about the same rate as was the ammonium ion previously. Thus, the data seems to indicate that ammonium ion was in some way inhibiting the utilization of nitrate. Due to the lack of a sufficient amount of reliable data, particularly after 120 minutes, it is difficult to conclude whether or not denitrification took place. The method used for total medium-nitrogen in this experiment was the iron-reduction

method of Pucher et al. (47), and some difficulty was experienced with the analyses due to bumping and the formation of a large quantity of precipitated salts. Mass spectrometric analysis of the samples of cells for N^{15} content have not been completed. A value of 0.40 atom % N^{15} excess have been reported for the sample of cells corresponding to 300 minutes after the addition of isotope. This sample contained too much air to allow a correction for the dilution of the nitrogen in the sample. However, we can regard this value at least as a minimal one, and even if the dilution were three or four times, the actual value of the amount of N^{15} present in this sample would be relatively small, indicating that the incorporation of the labeled nitrate into cellular nitrogen was probably slight.

The second experiment was performed in order to confirm the previous findings. The various procedures used were identical with that of the previous experiment except that the analysis for total medium-nitrogen was a modification of that found in <u>Methods of Analysis A.O.A.C.</u> (48).

The results, shown in figure 2 are essentially the same as found in the previous experiment. Ammonia inhibits the utilization of nitrate, as would be expected to be the case if ammonia were an intermediate in the pathway of nitrate reduction. As before, it is not possible to determine from the data whether or not denitrification took place.

These results are in accordance with the hypothesis

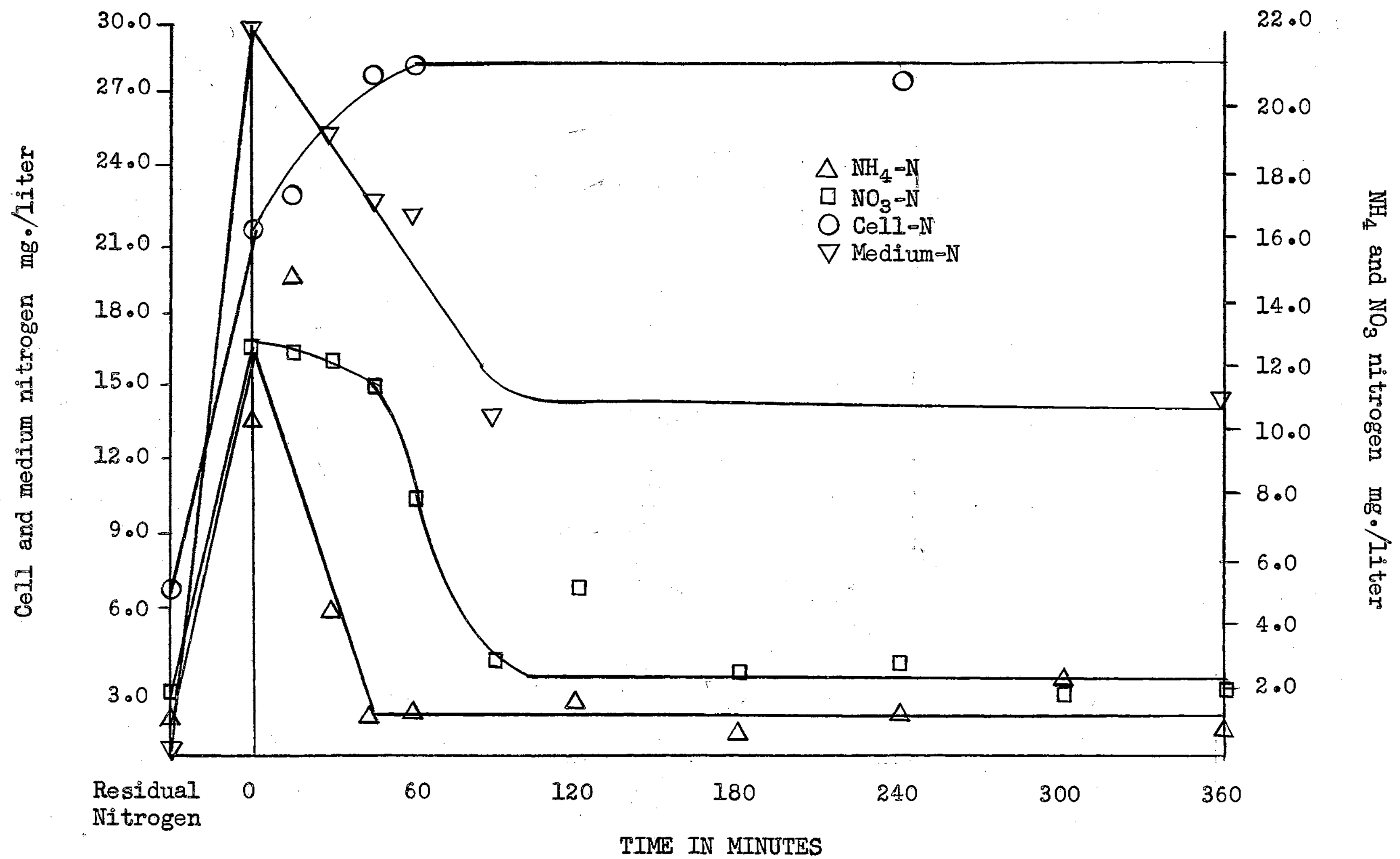

Fig. 1. Relation of cellular nitrogen to nitrate and ammonia disappearance in an aerated culture of __BMY soil isolate__; 12.5 mg. $N^{15}O_3$-N and 12.5 mg. NH_4-N added per liter at zero time.

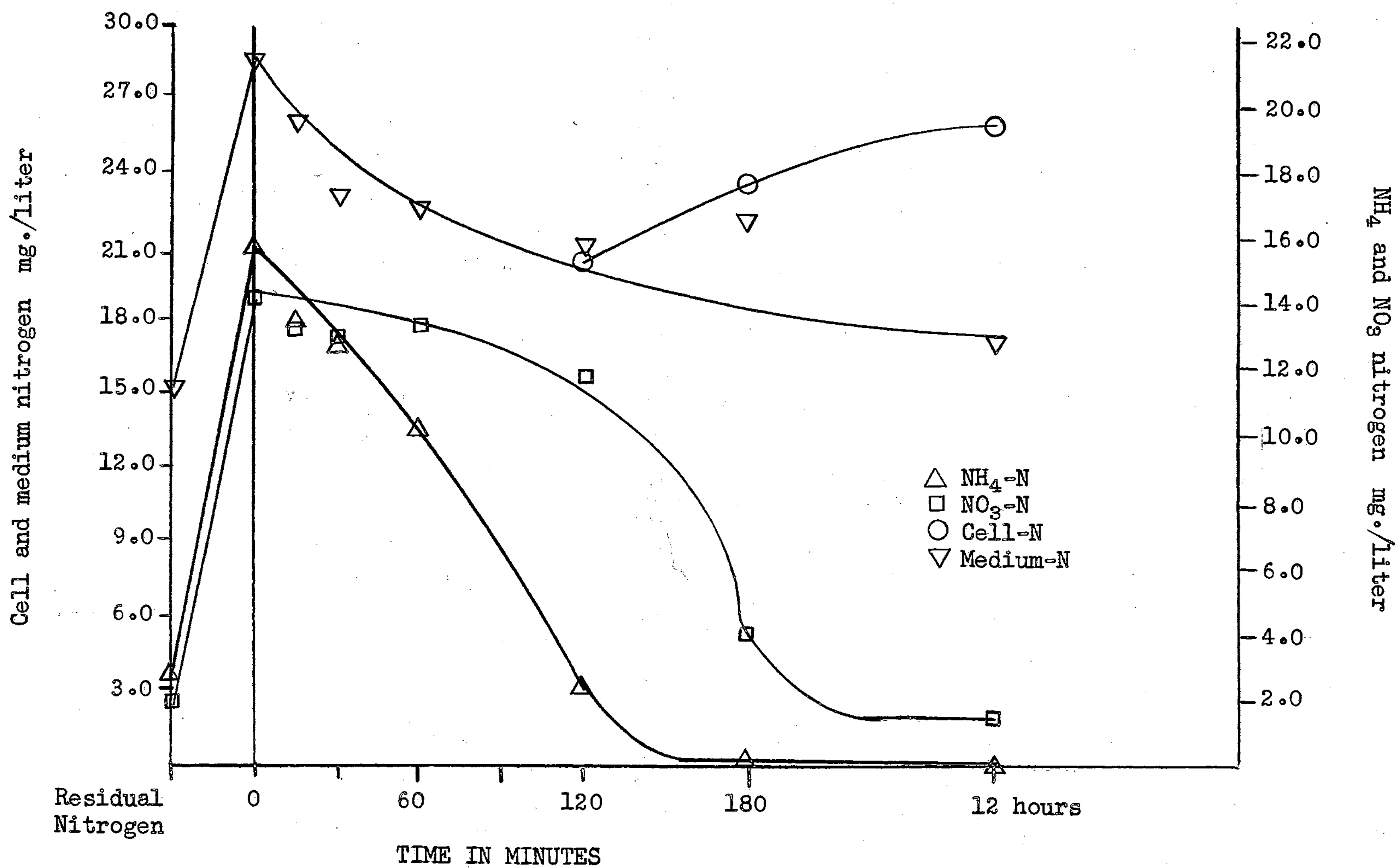

Fig. 2. Relation of cellular nitrogen to nitrate and ammonia disappearance in an aerated culture of BMY soil isolate; 12.5 mg. $N^{15}O_3$-N and 12.5 mg. NH_4-N added per liter at zero time.

that ammonia is an intermediate in bacterial nitrate reduction in this particular organism.

SUMMARY

Nitrogen changes in cultures of an unidentified soil bacterium, adapted to nitrate nutrition, have been followed by quantitative procedures and by the use of N^{15} as a tracer.

In aerated cultures to which $KN^{15}O_3$ and $(NH_4)_2SO_4$ were added simultaneously, after depletion of the initially present nitrate, extremely rapid disappearance of ammonium nitrogen occurred. The nitrate concentration remained constant until most of the ammonia had been utilized, after which the nitrate disappeared from the medium as rapidly as the ammonia had previously.

This study supports the view that ammonia is an intermediate in biological nitrate reduction in this particular organism.

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