STUDIES ON THE BIOCHEMICAL MECHANISM OF NITRATE REDUCTION IN BACTERIA

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By

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PREFACE

Second in importance only to photosynthesis and the utilization of carbon compounds by heterotrophic organisms are those biological reactions involving the fixation of nitrogen and the utilization and transformation of nitrogen compounds. Basically, all plant and animal life is dependent upon the maintenance of a dynamic state of equilibrium between those processes tending to fix atmospheric nitrogen and convert it into a form usable by plants and other organisms for growth and development, and those processes tending to convert fixed nitrogen into N_2 (denitrification). Despite the tremendous importance of these reactions in the "over-all" economy of all living forms, portions of this cycle have not been extensively studied from a biochemical point of view.

The experiments on nitrogen changes reported in this thesis, employing the use of N^{15} tracer techniques, were conducted with widely distributed nitrate reducing organisms commonly found in soils. Information concerning the mechanism of the reduction of nitrate may provide means of controlling the denitrification process which presumably occurs in soils containing readily decomposable organic matter.

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INTRODUCTION

The problem of how micro-organisms and green plants utilize both nitrate, the highest oxidation product of nitrogen, and ammonia, the fully reduced nitrogen compound, is still a central question in the assimilation of nitrogen. The mechanisms of nitrate reduction and molecular nitrogen fixation have consistently been an object of great interest. In the course of years since Gayon and Dupetit (7) performed their classical experiments in 1886, a very large number of investigators have studied the various aspects of the process of reduction of nitrate. This interest was doubtless due to its importance in understanding the nitrogen cycle of nature and to its agronomic importance. Unfortunately there are still wide gaps in our knowledge of the chemical reactions underlying the process of denitrification and nitrate assimilation by bacteria. Moreover, a number of contradictory observations which are difficult to interpret now confront investigators of this phenomena.

That nitrate may be reduced and the resulting compounds utilized for the synthesis of nitrogenous constituents of cells, and for cell growth and multiplication has been known for many years. The effect of oxygen on such processes has been of considerable interest from the beginning. Weissenberg (31) in 1897 tested and demonstrated the ability of three denitrifying bacteria to reduce nitrate and nitrite in shallow layers of medium exposed to air as well as in the complete absence of

oxygen. He found that complete denitrification occurred in anaerobic cultures; aerobically, nitrate was reduced only as far as nitrite.

Seiser and Walz (25) in 1925 observed a considerable nitrogen loss from a nitrate-containing culture of Pseudomonas putida exposed to air, though this was less than under anaerobic conditions. Lloyd and Cranston (12) some five years later measured the gas exchange that occurred when denitrifying cultures were grown in air or in a nitrogen atmosphere in a closed system. They observed a large nitrogen evolution under anaerobic conditions and an almost equally large oxygen uptake in air. They concluded that nitrate was only slightly attacked aerobically, although some nitrogen was lost from the medium even under their most aerobic conditions.

Quastel and Whetham (22) in 1924, Quastel et al. (21) in 1925 showed that nitrate could take the place of molecular oxygen in certain facultative anaerobes. Strickland (26) later showed in Escherichia coli that different enzymes are concerned in the activation of oxygen and nitrate. In the presence of oxygen, only a small part of the oxidation was caused by nitrate enzyme. Since oxygen was reduced ten times as fast as nitrate, it caused a noncompetitive inhibition of nitrate reduction.

Using two unidentified strains of Pseudomonas, Meiklejohn (14) showed that nitrate was reduced to nitrite and nitrogen gas under both aerobic and anaerobic conditions in a simple medium containing adequate amounts of suitable organic matter. Korsadova (9) also recently reported that when an organic source was supplied in an amount 5 to 10 times in excess of that required to reduce the available nitrate, the reduction of the latter by Achromobacter siccum and Pseudomonas aeruginosa was as complete

under aerobic as under anaerobic conditions. Similarly, previous studies in this laboratory using N^{15} tracer techniques by Bowers and MacVicar (2) have indicated that in solution cultures of heterogenous soil bacteria containing nitrate a loss of approximately 34% of the total nitrogen added as nitrate was found to occur during the first 24 hours under normal aerobic conditions of incubation. Broadbent (3), after conducting denitrification studies, on light textured soils by varying the flow of air with various amounts of oxygen, concluded that low oxygen concentration in the soil atmosphere is not a necessity for denitrification and that denitrification rates are affected more by the quantities of nitrate and oxidizable carbon in the soil than by partial pressures of oxygen.

Van Olden (19) in 1940 using Microccus denitrificans observed that the ability of these washed bacteria to produce nitrogen from nitrate is dependent upon their previous history. He concluded that "nitrate reductase" is an adaptive enzyme in the sense of Karstrom (8) which develops under conditions suitable for denitrification.

Sacks and Barker (23), using an isolate from soil designated by them as Pseudomonas denitrificans, observed that at oxygen tensions of about 5 percent, nitrate could be reduced only as far as nitrite, which accumulates in the medium, and that at partial pressures of oxygen in excess of 5 percent denitrification failed to occur. In this range of oxygen tensions, both the accumulation of nitrite and the rate of denitrification are greatly affected by relatively small changes in the oxygen level. They concluded that oxygen affects nitrate reduction and denitrification by suppressing nitrate and nitrite-reducing enzyme systems and that when these systems are present it decreases the rate of the reduction process by interfering with the enzymes.

Likewise, Lewis and Hinshelwood (10) in one of their experiments observed that when the oxygen supply was lowered the reduction of nitrate by Bacterium lactis aerogenes increased. Sudden aeration of an anaerobically growing culture led to a catastrophic drop in the rates of reduction of nitrate and nitrite. These authors introduced the hypothesis that some cell metabolite, denoted XH_2, which is easily oxidized, was responsible for such a phonomenum. An increase of this metabolite will increase the rate of nitrate reduction while a decrease of XH_2 will have the reverse effect. The growth rate in all experiments appeared to be determined by the rate of nitrite reduction. It may be inferred, therefore, that the rate of nitrite reduction is not affected in the same way as nitrate reduction.

Marshall and MacVicar (13) in an extensive series of experiments conducted in these laboratories, followed nitrate reduction both by determining the amount of reduced nitrogen appearing in the cells and the amount of nitrate lost from the culture. Incubation of Pseudomonas fluorescens under conditions of either normal incubation in still cultures or vigorous aeration resulted in a rapid decrease in nitrate from the culture. Determination of the total nitrogen in the cells and in the culture when the nitrate ion in the culture medium had been decreased to a low level, revealed a deficit which was not eliminated by vigorous aeration. To this extent the observations of previous workers were confirmed under conditions precluding the development of low oxygen tensions. These workers suggest that in view of these observations, nitrate disappearance, presumably by denitrification, occurs under aerobic as well as anaerobic conditions.

An investigation of the literature also indicates that there is

a lack of agreement among the various workers in the field as to the role of ammonia in nitrate reduction and assimilation. The hypothesis rather generally accepted by earlier investigators since the end of the last century was that nitrate as well as molecular nitrogen is reduced to ammonia, this being accordingly a central nitrogen compound in the amino acid synthesis. The validity of this attractive hypothesis was doubted very early. Thus, Meyer and Schulze (15) introduced as early as 1884 the idea that hydroxylamine could be the nitrogen compound which arises both from ammonia and nitrate. Later the observation of Blom (1) on the formation of hydroxylamine or oxime-nitrogen in cultures of Azotobacter using nitrate or molecular nitrogen, as well as the findings of Virtanen and Laine (29), (30), led again to the hydroxylamine theory. Chemically, the amino acid synthesis from hydroxylamine could be explained as a reduction of oximes resulting from the reaction between hydroxylamine and á-keto acids.

Virtanen and Csaky (27) noted that yeast and bacterial masses, especially Torula, suspended in nitrate solution under strong aeration produced a considerable amount of oxime-nitrogen within 15 minutes, after which time a drop occurs. The authors conclude that the cells evidently utilize oxime-nitrogen, since its amount decreased during the experiment. They also point out that these findings do not give conclusive evidence of the significance of oxime-nitrogen nor explain its mode of formation, because oxime-nitrogen is produced also from ammonium nitrogen by the action of many aerobic micro-organisms. Thus in both instances oxime-nitrogen might arise only after ammonia formation. Should this be the course of reaction, the oxime formation noted in nitrate reduction would be no proof of the formation of hydroxylamine as an intermediate.

Virtanen and Jarviren (28), believing the question to be of utmost importance in elucidating the mechanism of nitrogen fixation, examined the formation of oxime-nitrogen by Aztobacter vinelandii in nutrient solutions containing as a source of nitrogen either ammonium salts or nitrate salts or in a nitrogen-free medium. Oxime-nitrogen was formed in every case, and it was observed that oxime-nitrogen was formed much more rapidly from molecular and nitrate nitrogen than from ammonium nitrogen. Nitrogen was assimilated by cells from ammonium-nitrogen media as rapidly as or more rapidly than from nitrate-nitrogen media or by fixation of molecular N_2. They concluded from the findings of this experiment that nitrate is not, at any rate entirely, reduced to ammonia but that hydroxylamine preceding it forms oximes with carbonyl compounds.

On the other hand, Novak and Wilson (18) in a series of carefully controlled trials demonstrated that nitrogen in the form of oximes of pyruvic, oxaloacetic, and a -ketoglutaric acids is not available to Aztobacter vinelandii. Experiments by Burris and Wilson (4) on the utilization of nitrate by Aztobacter suggest that nitrate is reduced to ammonia before it is assimilated by the cells. In a mixture of nitrate and ammonium salts, in which a part of the ammonium nitrogen was N^{15}, the synthesis of ammonium ion from nitrate could be demonstrated.

Lewis and Hinshelwood (11) observed that the addition of ammonia to a medium inoculated with Bacterium lactis aerogenes lead to an almost complete inhibition of nitrate and nitrite reduction, which would not restart until the amount of ammonia remaining was negligible. If the addition was made before growth had started, little or no reduction occurred. Imperfect aeration was found to reduce this inhibitory action

of ammonia on the nitrate reduction process and the concentration of nitrite built up in the medium. The delayed recovery suggested that during the growth in ammonia, some substance was formed which inhibited the reduction of nitrate to nitrite. It was concluded from these data that the ratio of X/XH_2, oxidized and reduced form of the metabolite previously discussed, was high and under these conditions nitrate and nitrite were not reduced. In these terms the authors concluded, the inhibition and recovery process described were generally understood.

Marshall and MacVicar (13), using isotopic techniques, clearly showed that nitrate-nitrogen assimilation and incorporation into cellular components was dramatically halted by the addition of NH_4-N in an aerated culture of Pseudomonas fluorescens, although NO_3-N and NH_4-N were disappearing at a rapid rate. These findings, therefore, give additional support to the concept that denitrification in this organism is not exclusively an anaerobic mechanism.

In view of the discordant results thus reported, the object of this study was to investigate further the effect of oxygen tension and ammonia supply on nitrate reduction in bacteria.

EXPERIMENTAL

Materials

The organisms used in these studies were a strain of Pseudomonas fluorescens from the stock collection of the Department of Agricultural Bacteriology, University of Wisconsin; a strain of Pseudomonas denitrificans, an isolate obtained by Division of Plant Nutrition, University of California; and a strain of Escherichia coli from the stock collection of Department of Bacteriology, Oklahoma Agricultural and Mechanical College. These organisms were trained to grow in a simple medium having the following composition per liter: KH_2PO_4, 3.96 gm.; $NaNO_3$, 1.0 gm.; glucose, 20.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.4 gm.; $FeSO_4$, 0.02 gm.; NaCl, 0.02 gm.; $MnSO_4$, 0.08 gm.; and biotin, 0.1 mg. The solution was then brought to a pH of approximately 6.8 with KOH, using bromthymol blue as an indicator. A precipitate formed upon autoclaving, but this did not interfere except to make turbidimetric measurements of growth impractical. Transfers were normally made every 48 hours except when a large culture was to be inoculated; in this case, the organism was subcultured every 24 hours for three days prior to the experiment. Ten ml. of cell suspension obtained this way was used as an inoculum in each case.

In the experiments, one liter of nitrate media was placed in a two liter flask fitted with a fritted glass dispersion tube. A stream of air was then passed through a tightly packed cotton filter before it entered the medium. Air sufficient to churn the medium as well as

disperse minute bubbles of air throughout was passed through the medium during incubation at room temperature. Upon depletion of the original nitrate, detected by the brucine sulfate method (13), (6), the culture was immediately sampled. Usually 25 mg. of N was then added in the form of $KN^{15}O_3$, containing approximately 30 atom % N^{15} excess, and 25 mg. N as $(NH_4)_2SO_4$ and the culture again sampled. Sampling was repeated at definite time intervals thereafter over a period of 6-8 hours. Samples were obtained by withdrawing 25 ml. of the culture and pipetting into 2 ml. of boiling 0.8N H_2SO_4. Samples were then brought to a boil, stoppered immediately and reserved for analysis. At the completion of sampling, the samples were transferred quantitatively to 50 ml. plastic centrifuge tubes and the cells packed by centrifugation. The supernatant was decanted to 250 ml. volumetric flasks. The cells were washed three times with distilled water by centrifugation and the washings added to the supernatant. The supernatant and washings were then autoclaved and reserved for analysis. The packed cells were transferred into semi-micro Kjeldahl flasks, made distinctly basic (pH 8-9) with N/14 NaOH and boiled for at least five minutes to remove occluded ammonia.

Methods

Cellular nitrogen was then determined by a semi-micro modification of the Kjeldahl procedure. The Kjeldahl titration mixtures from the cellular nitrogen analysis were prepared for conversion as suggested by Schoerheimer (24), and the conversion apparatus for N^{15} was similar to that described by him. N^{15} analyses were made on a Westinghouse mass spectrometer under the supervision of Glenn Hallmark of the Department of Electrical Engineering, A. and M. College of Texas.

Analysis for ammonia was made on 50 ml. aliquots of the supernatant after dilution to 250 ml. The samples were rendered strongly alkaline and aerated into 5 mls. of N/7 H_2SO_4 for one and one half hours at elevated temperatures. The distillate was brought to a volume of 100 ml. and ammonia was determined by Nesslerization of 10 ml. aliquots.

Analysis for nitrate was made by a modification (13) of the brucine sulfate method developed by Gad (6). This method was decided upon because of the desirability of a rapid colorimetric method for nitrate determination in the presence of glucose. Recoveries on known samples have been obtained consistently with an accuracy of about 5 %. The method has further been substantiated by checking it against the aeration procedure using Devarda's alloy to reduce nitrates to ammonia.

Total nitrogen was determined in the cell-free supernatant by the semi-micro Kjeldahl procedure. Prior to digestion the nitrate present was reduced by a semi-micro modification (13) of the iron procedure as developed by Fucher and Vickery et al. (20).

RESULTS AND DISCUSSION

Previous evidence from the literature strongly indicates that under suitable conditions either ammonia or oxygen may arrest nitrate reduction. However, from the findings by Marshall and MacVicar (13) and other data it appears that Pseudomonas fluorescens, adapted to nitrate nutrition by long serial sub-culture in a nitrate medium, reduced nitrate to some gaseous nitrogen compound in the presence of ammonia under aerobic conditions, and to some other substances as yet uncharacterized. These substances seem to be utilizable by the cells for the synthesis of cellular constituents. To confirm such observations of these workers was the first purpose of these studies.

Cultures of Pseudomonas fluorescens, previously conditioned to nitrate by long serial sub-culture in media containing nitrate as the sole source of nitrogen, were grown in large flasks and rapidly aerated, as has been previously described. Experiment 1 was designed to determine the rate at which NO_3-N was being reduced and assimilated in the cells, thus 1000 ml. of nitrate medium was inoculated with a 21 hour culture. The culture was incubated at room temperature until the original NO_3-N was exhausted. The time required for exhaustion of the nitrate was 23 hours. At this time 50 mg. of N as $KN^{15}O_3$ containing 31.4 atom % N^{15} excess was added. Aliquots of 25 ml. were taken at definite intervals, as previously described. Analysis was made for NO_3N in the cell-free medium and cellular nitrogen was determined by semi-micro Kjeldahl procedure. The Kjehdahl titration mixtures were prepared for

N^{15} analysis, as described by Schoerheimer (24), and forwarded to the A. and M. College of Texas for mass spectrometric analysis. The N^{15} data showed a parallel rise to the cellular nitrogen increase. Thus it was clearly shown, by the use of N^{15} tracer Techniques in Experiment 1 (Figure I), that under conditions of vigorous aeration nitrate was being reduced and that assimilation of nitrate to cellular constituents occurred. But it can also be clearly seen that although 5.0 mg./100 ml. of NO_3^- disappeared, only 2.4 mg./100 ml. appeared in the cells. This indicated that 52% of the nitrogen was unaccounted for at 300 minutes. N^{15} recovered was also incomplete, showing roughly a 41% recovery. This indicates that denitrification, accumulation of previously discussed reduced substances in media, or both, occurred to a great extent. A study as to what extent each occurred is presented later.

This continued reduction of nitrate and the failure of cellular nitrogen to increase proportionally made it desirable to fix the pathway of reduction as well as confirm previous results. Since ammonia has also been ascribed an important role in this process, it would be suspected that if NH_4^-N and NO_3^-N were supplied to an actively growing culture on nitrate nutrition at the time when the original supply of NO_3^-N was depleted, the organism should preferentially use NH_4^-N, if ammonia was an obligate intermediate in the process of nitrate assimilation. Nitrate reduction should be halted until the level of ammonia reached a very low level at which time nitrate reduction should be resumed. Experiments 2 and 3 were conducted under conditions similar to those described for Experiment 1. The cultures were incubated at room temperature until the original NO_3-N was exhausted. At this time $KN^{15}O_3$ containing 31.4 atom % N^{15} excess equivalent to 25 mg. of nitrogen and

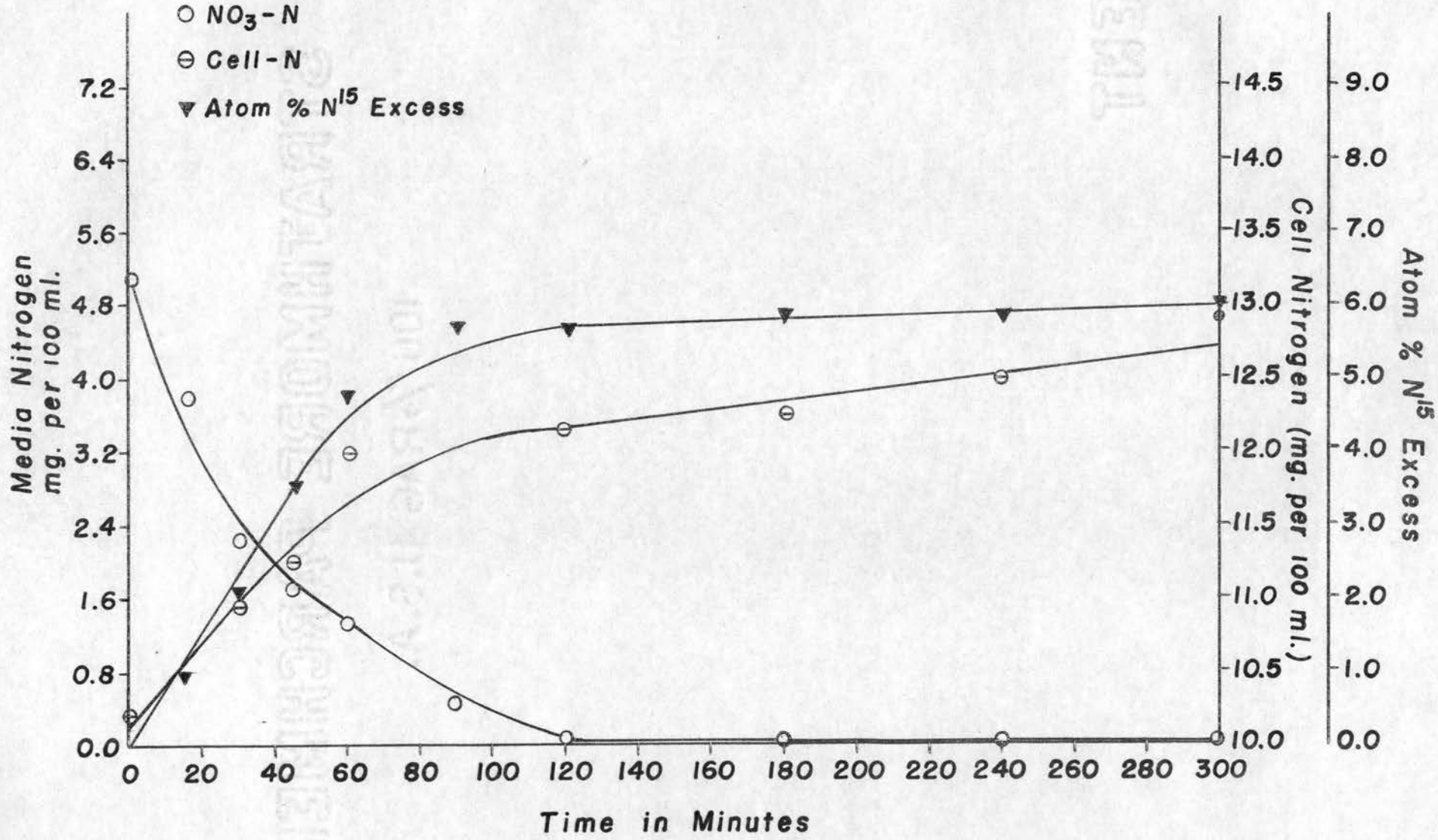
FIGURE I

Relation of Cellular Nitrogen to Nitrate

Disappearance in an Aerated Culture

of Pseudomonas fluorescens

5 mg./100 ml. of N as $KN^{15}O_3$ Added


○ NO_3-N
⊖ Cell-N
▼ Atom % N^{15} Excess
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
Time in Minutes
0.0
0.8
1.6
2.4
3.2
4.0
4.8
5.6
6.4
7.2
10.0
10.5
11.0
11.5
12.0
12.5
13.0
13.5
14.0
14.5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

25 mg. of nitrogen as $(NH_4)_2SO_4$ were added in Experiment 2, while in Experiment 3, 50 mg. of nitrogen was added as $N^{15}H_4NO_3$ containing 34 atom % N^{15} excess. During the ensuing period, samples were taken at definite intervals and analyzed for the following components: cellular nitrogen, NH_4-nitrogen of the media, and NO_3-nitrogen of the media. These data are presented in Figures II and III for the labeled nitrate and ammonia, respectively. It can be seen that nitrate continued to disappear from the media in the presence of equal amounts of ammonia and under conditions of vigorous aeration. The rate of disappearance of NH_4-N was approximately parallel to the increase in cellular nitrogen. The ratio of NH_4-N disappearance to cellular nitrogen increase was so near unity that one might assume all of the cellular nitrogen was being derived from NH_4-N. To substantiate such an assumption, that ammonia was being preferentially used for synthesis of cellular constituents, one only has to inspect the isotope data. Thus, rapid incorporation of N^{15} into the cells occurs when the $N^{15}H_4$-nitrogen is present; when the nitrate was labelled, however, only relatively slow incorporation of the isotope into the cells was found. The rate of incorporation of ammonia and nitrate at the end of 260 minutes was 8.93 and 1.15 atom % N^{15} excess, respectively; yet nitrate in the media continued to be reduced in both experiments. This suggests the presence in the media of a form of nitrogen (neither nitrate nor ammonia) capable of contributing nitrogen to protein synthesis. On the basis of the increase in cellular nitrogen over this time, these calculations also suggest that ammonia nitrogen almost exclusively was being used for synthesis of cellular materials.

The observations of Sacks and Barker (23) that an isolate from soil,

FIGURE II

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Pseudomonas fluorescens

2.5 mg. /100 ml. of N as $KN^{15}O_3$ and 2.5 mg. /100 ml. of N as $(NH_4)_2SO_4$ Added

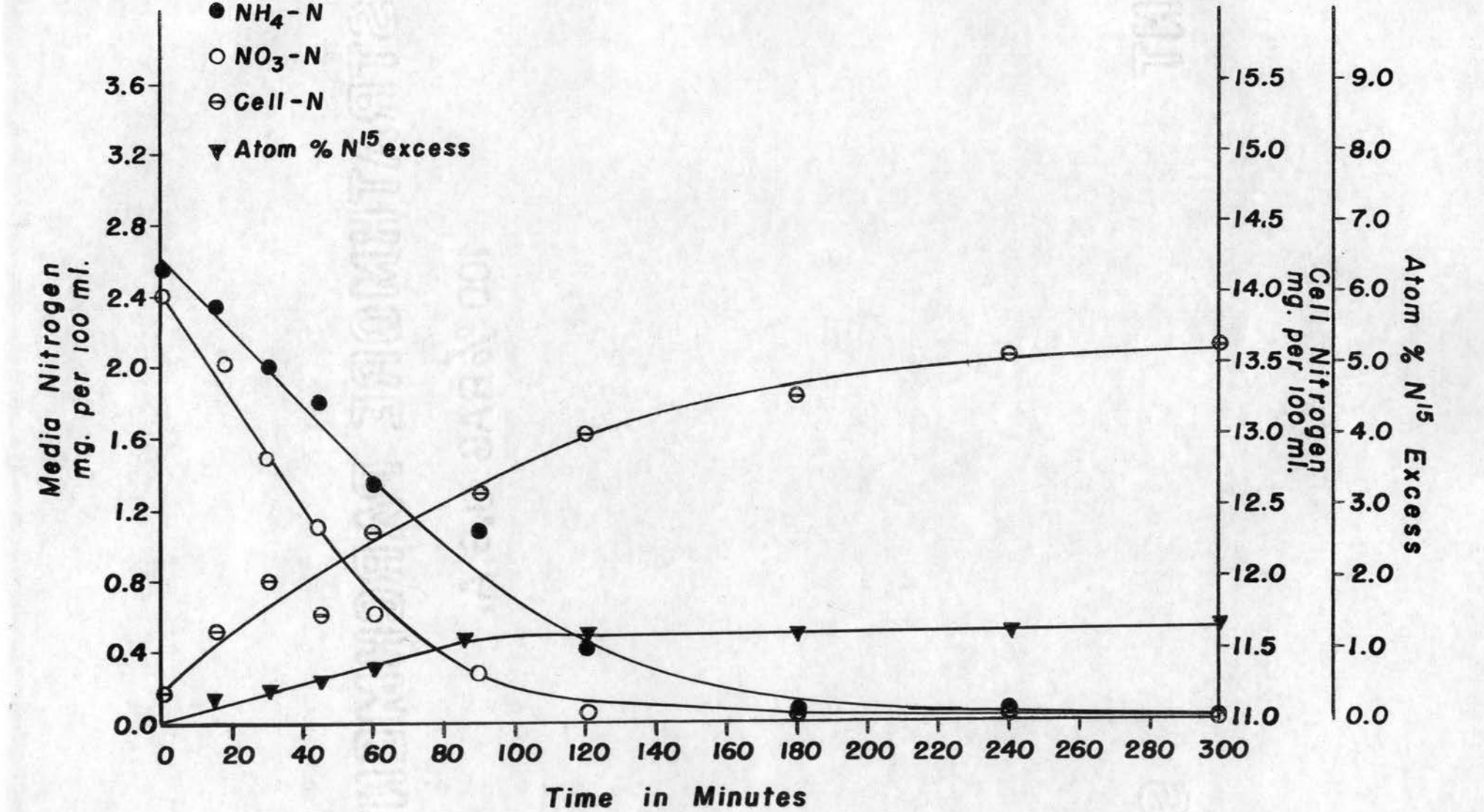
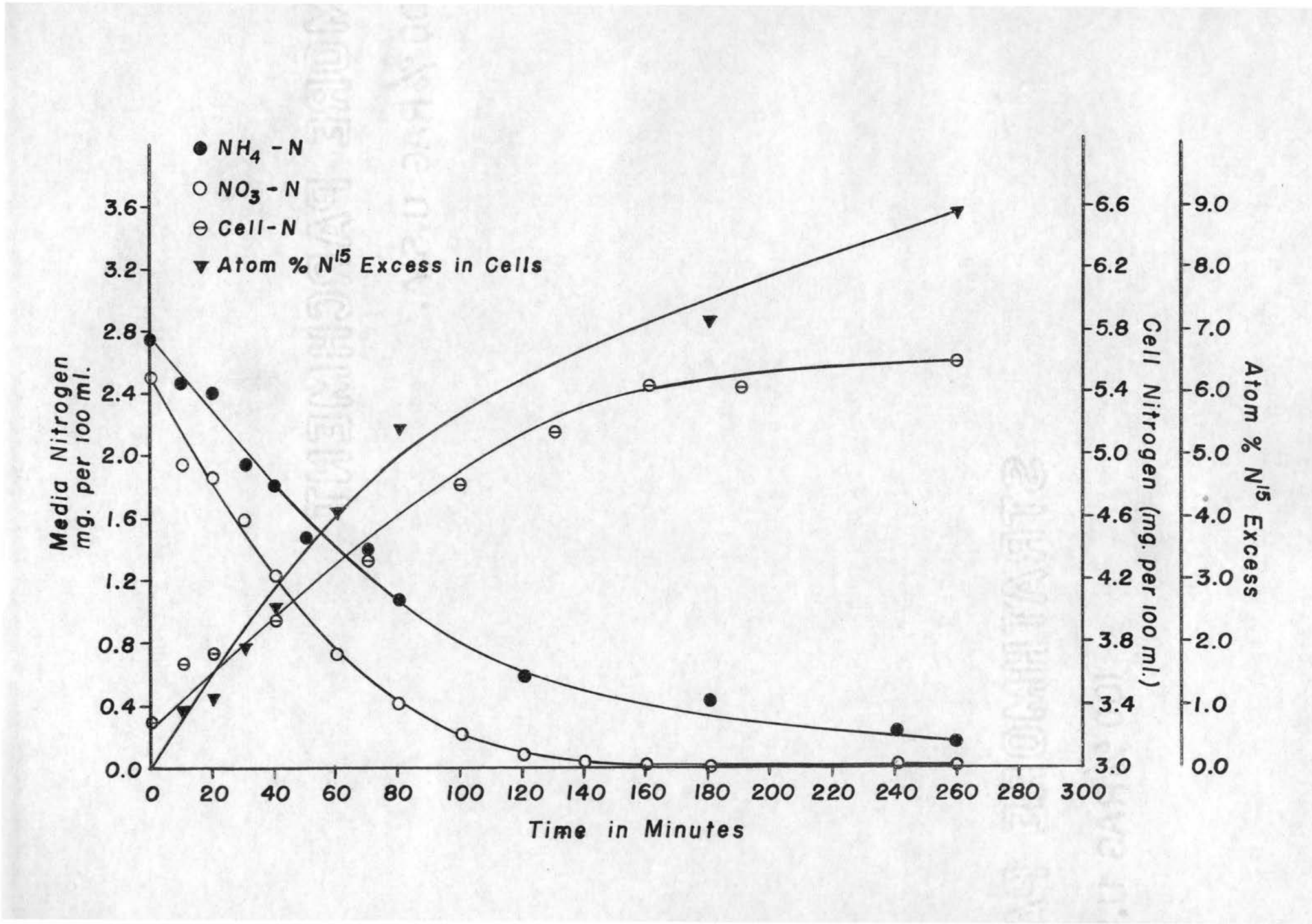

● NH_4-N
○ NO_3-N
⊖ Cell-N
▼ Atom % N^{15} excess
Media Nitrogen mg. per 100 ml.
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
15.5
15.0
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
Cell Nitrogen mg. per 100 ml.
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Atom % N^{15} Excess
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Time in Minutes

FIGURE III

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Pseudomonas fluorescens

5.0 mg. /100 ml. of N as $N^{15}H_4NO_3$ Added


● NH_4 - N
○ NO_3 - N
⊖ Cell-N
▼ Atom % N^{15} Excess in Cells
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
Time in Minutes
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
3.0
3.4
3.8
4.2
4.6
5.0
5.4
5.8
6.2
6.6
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

designated by them as Pseudomonas denitrificans failed to cause denitrification at partial pressures of oxygen in excess of five percent, were of interest in contrast to the results obtained by us with Pseudomonas fluorescens. It may be noted that Sacks and Barker did not measure residual nitrate in the media, but based their conclusions on nitrite determinations and manometric measurements of nitrogen evolution under anaerobic conditions. A culture of the isolate was obtained (through the kindness of Dr. Barker), and experiments similar in design to those described for Pseudomonas fluorescens were performed, after placing the culture on nitrate as its sole source of nitrogen for four months. In Experiment 4, $KN^{15}O_3$ containing 31.4 atom % N^{15} excess equivalent to 50 mg. of nitrogen was added, while in Experiment 5, $KN^{15}O_3$ containing 31.4 atom % N^{15} excess equivalent to 25 mg. of nitrogen and 25 mg. of nitrogen as $(NH_4)_2SO_4$ were added under conditions of vigorous aeration. The data obtained from representative experiments of this type are presented in Figures IV and V for the labelled nitrate alone, and for nitrate in presence of ammonia, respectively. Examination of the data presented, (chemical and isotopic), in this figure indicates that there is considerable similarity between these two species. It has been our observation however, that Ps. denitrificans appears to be somewhat more sensitive to lowered O_2 tensions; growth was greatly improved by agitation or by aeration.

Several explanations can be advanced for the apparent differences of results obtained with this organism by us and the California workers. It is not believed that lack of adequate O_2 supply could be one of these, since the most vigorous rates of aeration were maintained in all investigations. It might be that some of the well known difficulties of

FIGURE IV

Relation of Cellular Nitrogen to Nitrate

Disappearance in an Aerated Culture

of Pseudomonas denitrificans

5 mg. /100 ml. of N as $KN^{15}O_3$ Added

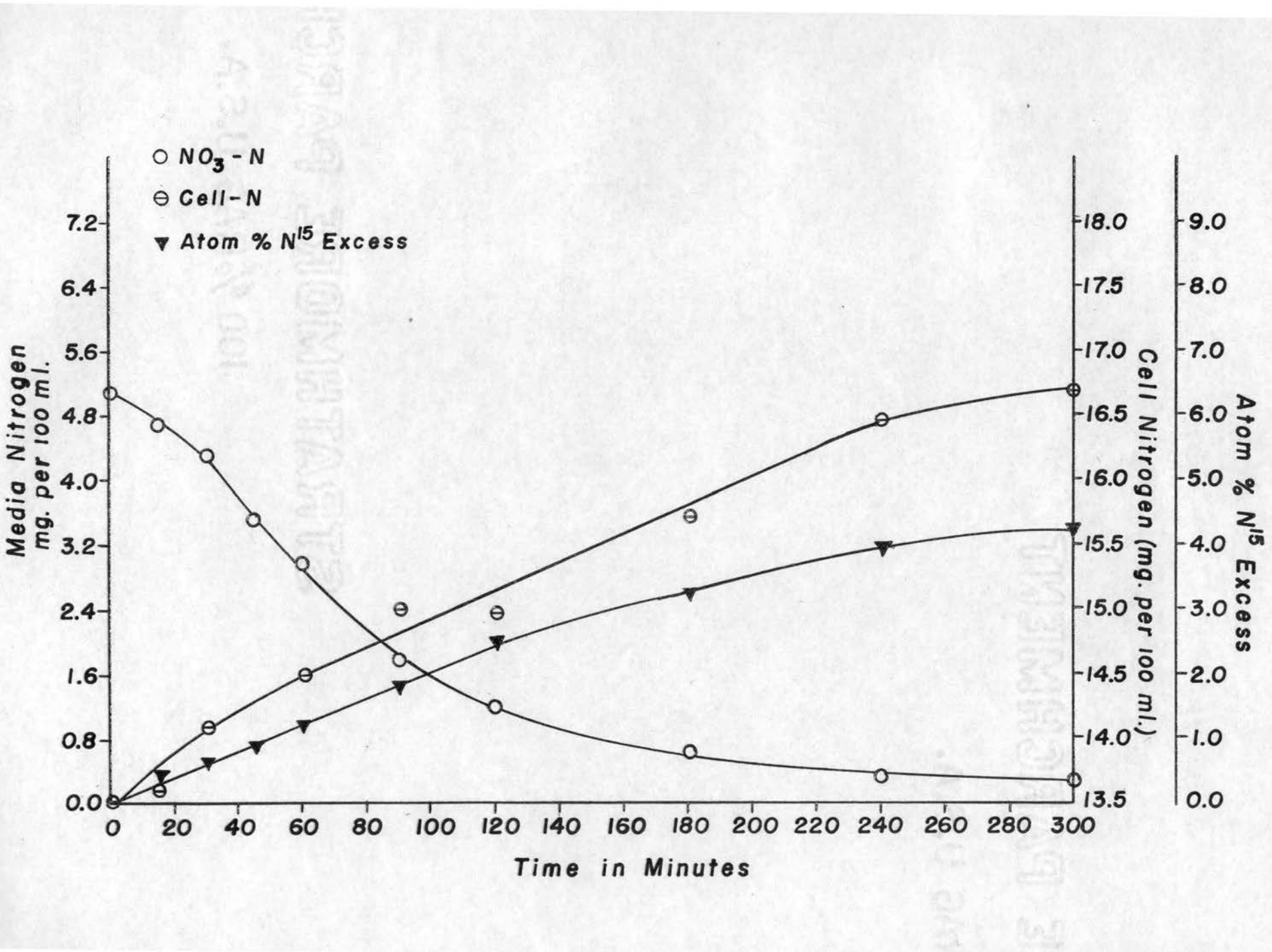
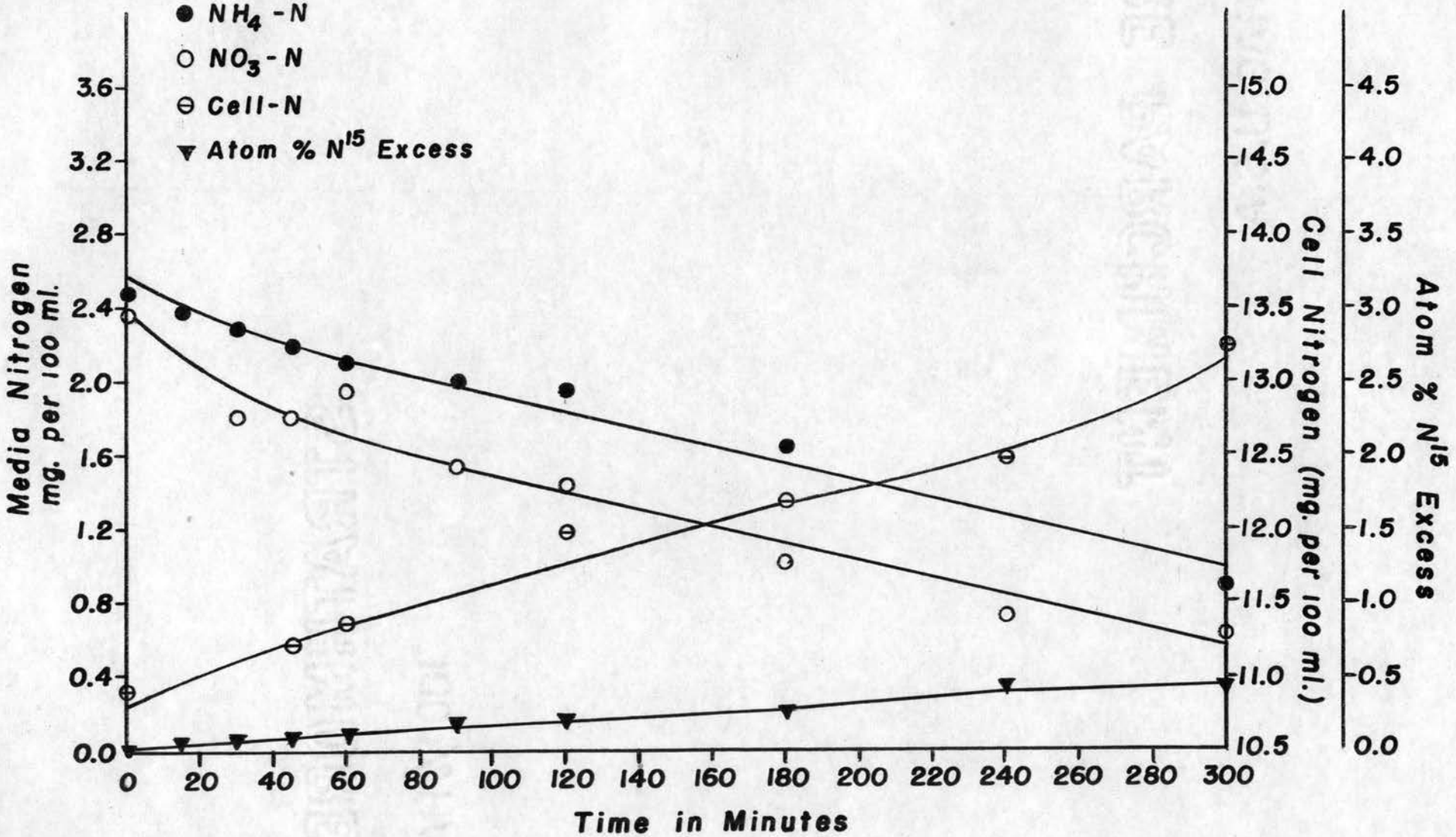

○ NO_3 - N
⊖ Cell - N
▼ Atom % N^{15} Excess
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
7.2
6.4
5.6
4.8
4.0
3.2
2.4
1.6
0.8
0.0
18.0
17.5
17.0
16.5
16.0
15.5
15.0
14.5
14.0
13.5
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Time in Minutes

FIGURE V

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Pseudomonas denitrificans

2.5 mg. /100 ml. of N as $KN^{15}O_3$ and 2.5 mg. /100 ml. of N as $(NH_4)_2SO_4$ Added


● NH_4 - N
○ NO_3 - N
⊖ Cell-N
▼ Atom % N^{15} Excess
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
Time in Minutes
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
10.5
11.0
11.5
12.0
12.5
13.0
13.5
14.0
14.5
15.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

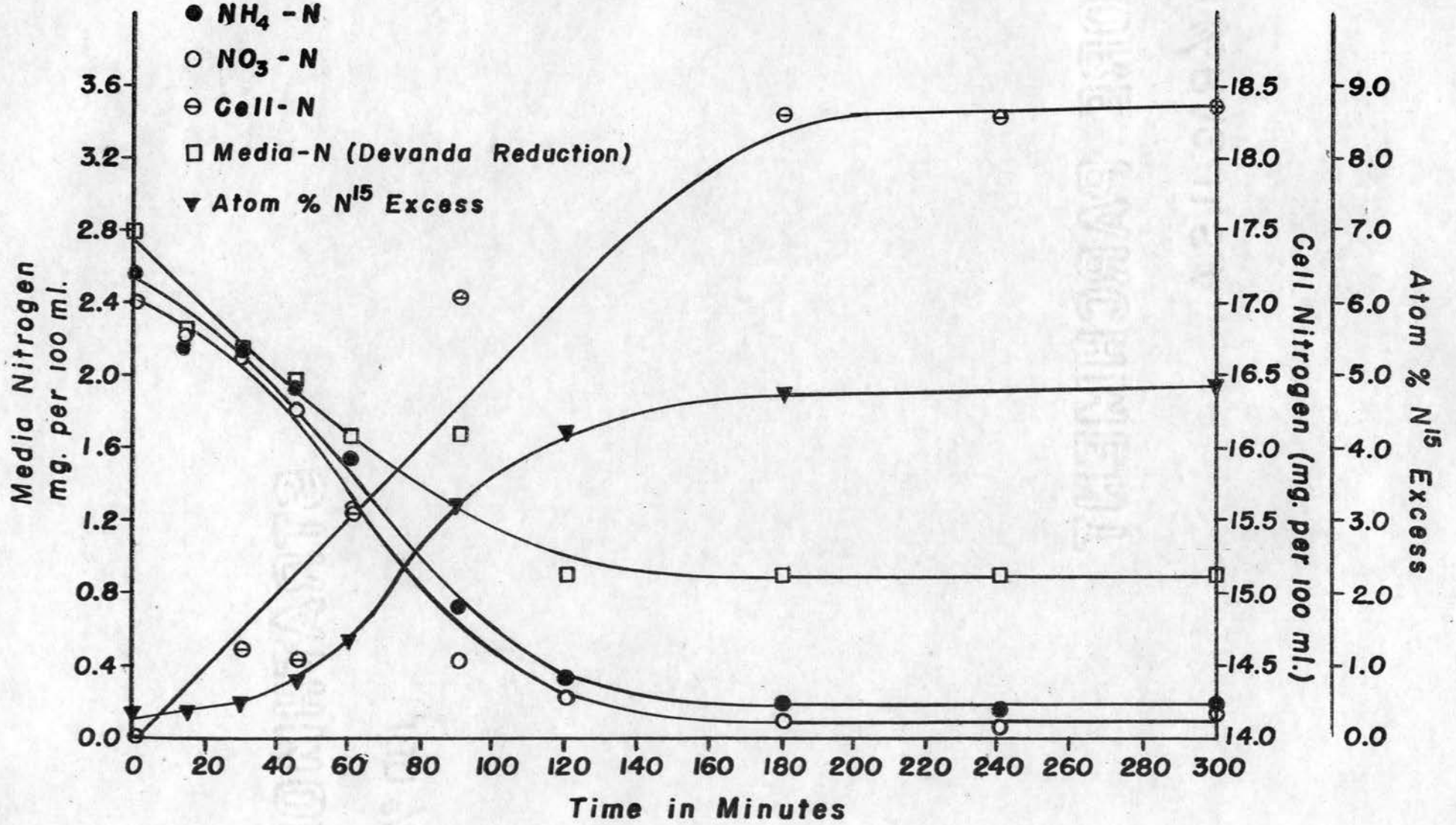
manometric measurements may have introduced experimental errors not detected. Therefore, the explanation of Sacks and Barker, in which they attribute the numerous observations of denitrification in the literature to inadequate rates of O_2 supply, is questioned. It should also be pointed out that the measurement of nitrite accumulation or disappearance may not be an exact measure of nitrate reduction, since the time of formation and disappearance of such an intermediate may be so short that this method of detection is not applicable. It is equally possible that ample supply of O_2 depressed denitrification, but did not inhibit the reduction of nitrate to some intermediate compound in the reduction path. Some evidence that this may indeed be the case is the accumulation under aeration of some reduced form of nitrogen in the medium to which nitrate has been supplied. In Experiment 4 to which $KN^{15}O_3$, containing 31.4 atom % N^{15} excess, it was found that 14.76 atom %N^{15} excess, roughly one-half of the nitrate added, still remained in the medium at 180 minutes, yet virtually all of the nitrate had disappeared.

Experiment 6 was designed to compare the rates of disappearance of NO_3-N and NH_4-N and the rate of cellular synthesis of Pseudomonas fluorescens to that of another organism, Escherichia coli. A 1000 ml. aerated culture in a 2000 ml. flask was set up and $KN^{15}O_3$, containing 31.4 atom %N^{15} excess, equivalent to 25 mg. of nitrogen and 25 mg. of N as $(NH_4)_2SO_4$ were added under conditions similar to those described previously. Examination of these data (Figure VI) shows that the addition of NH_4-N did not materially affect the disappearance of NO_3-N, but cellular nitrogen sharply reduced its incorporation into cellular components. After NH_4-N was reduced to about 1/3 the initial level (60 minutes), nitrate incorporation into cells increased and continued throughout the course of the experiment.

FIGURE VI

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated culture of Escherichia coli

2.5 mg./100 ml. of N as $KN^{15}O_3$ and 2.5 mg./100 ml. of N as $(NH_4)_2SO_4$ Added


● NH_4 - N
○ NO_3 - N
⊖ Cell - N
□ Media - N (Devanda Reduction)
▼ Atom % N^{15} Excess
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
Time in Minutes
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
14.0
14.5
15.0
15.5
16.0
16.5
17.0
17.5
18.0
18.5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Cellular nitrogen accounted for approximately 86% of the total nitrogen added at zero time. From the isotope data it can also be seen that nitrate assimilation was relatively slow until the ammonia concentration became low, yet analysis for NO_3-N in the media indicated that nitrate was disappearing at a rapid rate. Although these results differ somewhat from previous studies with Pseudomonas species, this rapid disappearance of nitrate from the media with little N^{15} incorporation into the cells until the NH_4-N became low, lends further support to the presence of a soluble reduced form of nitrogen in the media (neither nitrate or ammonia) capable of contributing nitrogen to protein synthesis.

Upon examining these data it was deemed desirable to check the NO_3-N values by Devarda's Method. It was found upon analysis that the values showed the same trend, but were comparatively high. At 120 minutes where NO_3-N by the brucine sulfate method had reached virtually an insignificant value, it was found that 0.9 mg. N/100 ml. of media remained as determined by Devarda's reduction method. An explanation was sought of this anomaly since the brucine sulfate procedure has been shown previously to give good correlation with values obtained with Devarda's alloy reduction. A reasonable explanation would be that some compound other than nitrate was being converted by either the strongly basic or vigorous reducing conditions to give ammonia. This is supported in a later experiment (Experiment 7). The exact nature of this compound or compounds are unknown.

Further examination of the results showed that little or no denitrification occurred. At 120 minutes both NO_3-N and NH_4-N had reached insignificant levels by the brucine sulfate method, but 0.9 mg. (18%) was found in the media by Devarda's reduction method. Cellular nitrogen

during this time showed an increase of 4.30 mg. which accounts for approximately 86% of the nitrogen in the NH_4-N and NO_3-N present at zero time. Such a recovery (104%) shows clearly that no denitrification occurred with Escherichia coli. These findings are in agreement with Lewis and Hinshelwood (11).

In order that we might facilitate the elucidation of these reduced forms of nitrogen, it was deemed desirable to enlarge the size of our culture at this time, as well as the amount of nitrogen added per 100 ml. at zero time. With this proportional increase in resulting compound(s) it was anticipated that separation of such could be obtained in such purity that the amount of N^{15} could be determined in each compound(s). The determination of the form in which this residual medial nitrogen (presumably derived from nitrate) is present would be of great interest because of the possible light it might throw on the pathway of nitrate reduction. The determination of nitrogen balance was highly desirable also in this phase of work, as it had been found previously that all of the nitrogen disappearing as NH_4-N and NO_3-N was not appearing in the cells, but that there was, rather, a deficit of nitrogen that varied from 30-50 percent occurred in cultures of Pseudomonas fluorescens. Hence, in Experiment 7 a 24 hour, nitrate-adapted culture of Pseudomonas fluorescens was inoculated into a flask containing 6000 ml. of media, which was vigorously aerated. Upon depletion of the nitrate a sample was removed for analysis and $KN^{15}O_3$, containing 36.0 atom $\%N^{15}$ excess, equivalent to 300 mg. of nitrogen and 300 mg. of nitrogen as $(NH_4)_2SO_4$, were added to the culture. Serial samples were then collected during the next 360 minutes, and analyzed for nitrate, ammonia, total cellular nitrogen, and total nitrogen remaining in the

media (including any nitrogen present in oxidized form) by the iron-reduction method of Pucher, Leavenworth, and Vickery (20). The cells were harvested from the media remaining after samples were taken, steamed, and reserved for analysis. The results are represented graphically in Figure VII. At 360 minutes the NO_3-N had reached an insignificant value, while NH_4-N was at a somewhat higher level, but still declining. The increase in cell nitrogen was still increasing slowly, indicating the relationship of NH_4-N to cellular synthesis. Table 1 shows these relationships clearly.

Table 1

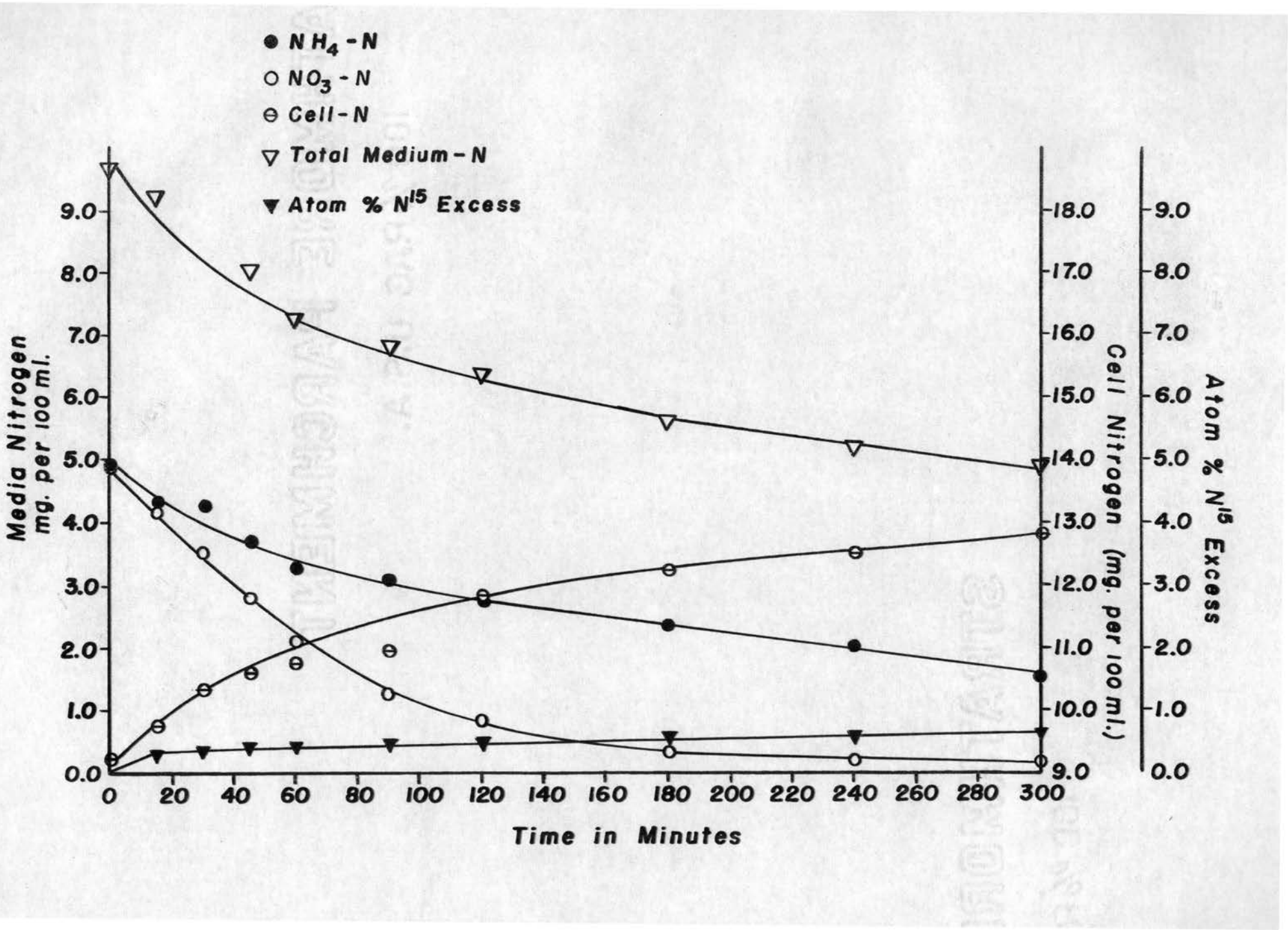
Nitrogen balance of culture of Pseudomonas fluorescens supplied with 5.0 mg. NH_4-N and 5.0 mg. NO_3-N per 100 ml.

Sample No.	Time (minutes)	Increase in cellular N	Corrected Residual media N	Recovery percent
1	0	0.23	9.60	98.3
2	15	0.52	9.10	96.2
3	30	1.10	8.20	93.0
4	45	1.31	8.00	93.1
5	60	1.80	7.20	90.0
6	90	2.20	6.80	90.0
7	120	2.52	6.40	89.2
8	180	3.00	5.60	86.0
9	240	3.20	5.20	84.0
10	300	3.60	4.90	85.0
11	360	4.02	4.20	82.0

Examination of these data shows that there was a consistent and regular loss of nitrogen with time. The level of nitrogen in the cell-free medium was still roughly two-fifths the value of zero time. Cellular nitrogen during this time showed an increase of 4.02 mg., which was closely related to the total ammonia nitrogen disappearance, and

FIGURE VII

Relation of Total Nitrogen Content of the Cell-Free Media to Other Nitrogen Changes in an Aerated Culture of Pseudomonas fluorescens 5.0 mg./100 ml. of N as $KN^{15}O_3$ and 5.0 mg./100 ml. of N as $(NH_4)_2SO_4$ Added


● NH_4 - N
○ NO_3 - N
⊖ Cell - N
▽ Total Medium - N
▼ Atom % N^{15} Excess
Media Nitrogen mg. per 100 ml.
Cell Nitrogen (mg. per 100 ml.)
Atom % N^{15} Excess
Time in Minutes
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

accounted for approximately 40% of the nitrogen in the NH_4-N and NO_3-N present at zero time. This indicated that 1/5 of the nitrogen added at zero time was lost by denitrification during 360 minutes. If the nitrogen lost by denitrification came from nitrate, as it presumably would, it would represent 40% of the NO_3-N added at zero time. From evidence of this type it is difficult to rationalize the assertion that losses of nitrogen (denitrification) are limited to anaerobic conditions. At this time there remained 4.20 mg. of nitrogen in the media; 1.26 mg. is accounted for by NH_4-N and NO_3-N, leaving 2.94 mg. as unknown. The determination of the form in which this residual medial nitrogen is of great importance because of the light it may throw on the reduction pathway. It seems to be derived chiefly from nitrate, as was illustrated by the large atom % N^{15} excess found in the media in Experiment 4 and others at the end of 300 minutes when the culture was supplied N^{15} in the form of $N^{15}O_3$-N. Microbiological assays (performed by Mr. Finn Wold), ninhydrin tests, and other data all tend to indicate that these nitrogen components were in part, at least, amino acids. But on the treatment of the concentrate of cell-free media remaining (after samples were taken) with Neuberg's Reagent (16), (17) very little N^{15} was found in the precipitate or filtrate; 0.87 and 1.37 atom %N^{15} excess respectively. Although isotope data have not been received to-date on the media previous to treatment, there seems little reason to doubt that it contained large quantities as was found in previous studies. These data indicated that some other reduced form of nitrogen was present which could easily be converted to NH_3 or was in a form easily lost while undergoing the series of steps in this procedure; one of which involved rendering the solution basic. Upon finding these results it

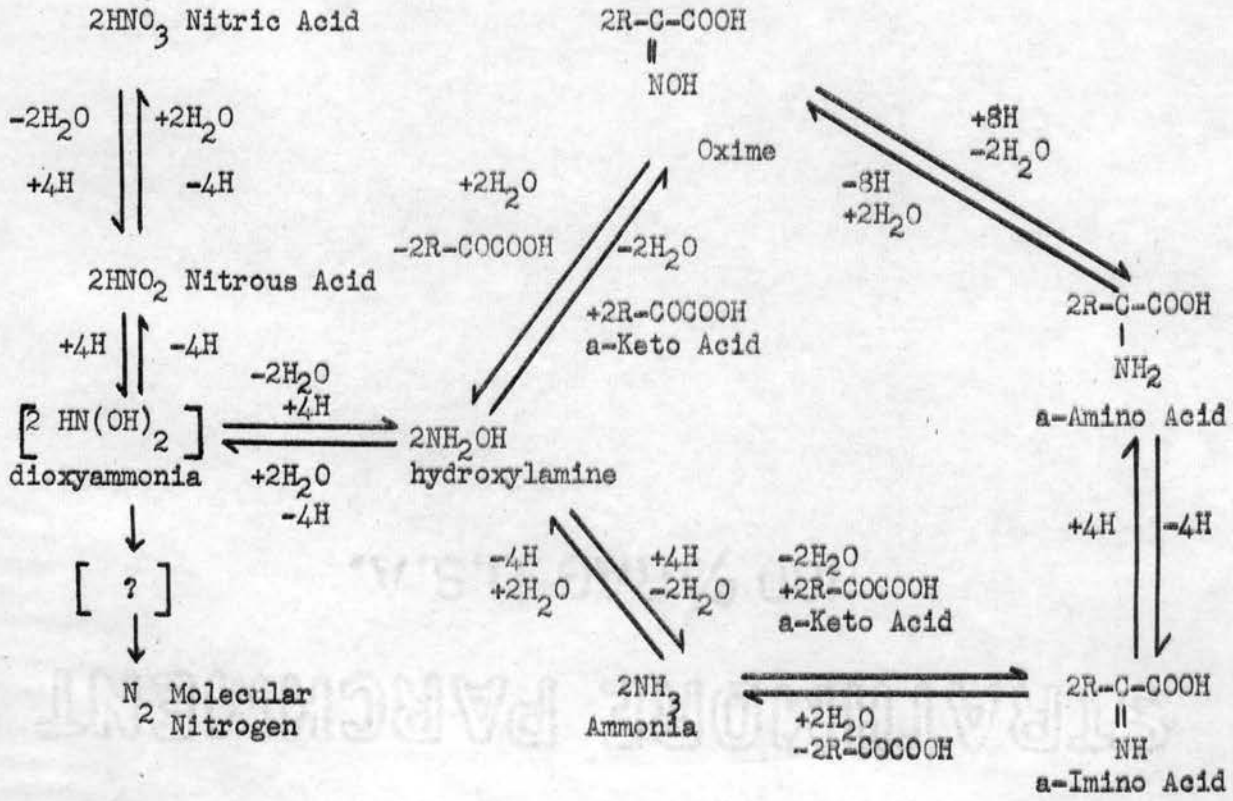
was decided to check what difference, if any, there would be if we rendered a sample basic and boiled it for thirty minutes before performing an iron-reduction. Hence, duplicate samples (240 minute sample) were taken; one was made basic and boiled for thirty minutes, then the two underwent reduction. The difference was 1.2 mg. /100 ml., excluding the remaining ammonia accounted for in the media by nesslerization. This clearly shows that 1.2 mg. N/ 100 ml. was in such a reduced form (not ammonia) that it could be converted into a gaseous compound and lost by basic treatment. This corresponds well to the value of 0.9 mg. N/100 ml. found in the Escherichia coli experiment with similar treatment. Such substances as amides, oximes, or hydroxylamine are implicated. Some evidence that this may be the case has been demonstrated with certain nitrogen fixers by Zelitch et al. (32).

Theoretical Discussion

The chemical mechanism of nitrate assimilation and denitrification has long been the subject of frequent speculation, but only within the past decade has experimental evidences in favor of various schemes been furnished. The proponents of nitrate assimilation represents two groups, those favoring hydroxylamine and those favoring ammonia as the key intermediate in assimilation. A proposed mechanism for the process is presented below:

FIGURE VIII

Proposed Pathways of Nitrate Assimilation and Denitrification



Virtanen has proposed that nitrogen is fixed as hydroxylamine, through some unknown intermediate compound, and combines with oxaloacetic acid to form oximinosuccinic acid, which in turn is reduced to aspartic acid. He has

unquestionably presented the most extensive supporting evidence to date. On the other hand, Burris and Wilson support the ammonia hypothesis. They have shown by isotope techniques that in Aztobacter cultures nitrate is reduced to ammonia which after combination with a-ketoglutaric acid and reduction gives glutamic acid.

Study of Figure 8 suggests that the two compounds (hydroxylamine and ammonia) may be part of the same mechanism in which the precise pathway eventually is dictated by the availability of the carbon chain functioning as the acceptor of fixed nitrogen. Hydroxylamine is separated from ammonia by a reductive step. Since it is most probable that nitrate reduction and assimilation is a stepwise process, hydroxylamine would logically occur in the production of ammonia. If hydroxylamine is converted to an oxime, it requires a reductive step in addition to that required for the formation of an amino acid via ammonia and a keto acid. Amino acid formation, either from hydroxylamine via the oxime, or from ammonia via the a-imino acid, appears feasible and there is no reason to assume that the pathways are mutually exclusive. Under particular conditions contributing to the formation of large quantities of oxaloacetic acid, the fixed nitrogen may be predominantely arrested at the hydroxylamine stage with the formation of oximinosuccinic acid which on reduction yields aspartic acid. On the other hand, in the absence of appreciable quantities of oxaloacetic acid, the reduction may proceed largely to ammonia which after combination with a-ketoglutaric acid and reduction gives glutamic acid. Virtanen's data, which have demonstrated the excretion of aspartic acid, isolation of oximinosuccinic acid, and the occurrence of oxaloacetic acid, have been used exclusively to favor the function of oxime. The studies with isotopes by Burris

and Wilson to date are strictly compatible with the ammonia hypothesis. These authors claim these data are highly suggestive of its dominance.

This mechanism was presented as a part of the theoretical pathway for nitrogen fixation in a review by Burris and Wilson (5). Evidence supporting this pathway is far from conclusive. Some of the results of these studies are in accordance with the view that such a mechanism might function in Pseudomonas under the conditions described in these experiments.

SUMMARY

Nitrogen changes in various micro-organisms adapted to nitrate nutrition have been traced by quantitative procedures and by utilization of the stable isotope of nitrogen, N^{15}. In aerated cultures containing nitrate as the sole source of nitrogen, nitrate disappeared rapidly from the culture within 24-26 hours after inoculation. When KNO_3 and $(NH_4)_2SO_4$ were added to the culture after the concentration of initially added nitrate had been reduced to a low level, nitrate continued to disappear from the culture at a rapid rate. The concentration of NH_4-N also decreased at a somewhat slower rate. Increase in total cellular nitrogen could usually be correlated closely to NH_4-N disappearance. That ammonia was rapidly assimilated into cellular components when NO_3-N and NH_4-N were present simultaneously, was demonstrated by the small appearance of isotope concentration when NO_3-N containing an excess of N^{15} was added, and by rapid incorporation of $N^{15}H_4$ into the cells.

Nitrogen balance studies, using both quantitative and isotope techniques, indicated that the organisms used in this study, adapted to nitrate nutrition by long serial sub-culture in nitrate medium, under aerobic conditions, reduced nitrate to a gaseous form of nitrogen and to some uncharacterized substances utilizable by the cells for cellular synthesis. This substance which accumulates in the medium, has been shown to be neither ammonia nor nitrite, and the present data indicates that it is not a simple amino acid (s).

This supports the view that (1) denitrification in these organisms are not exclusively an anaerobic mechanism (with the exception of Escherichia coli), (2) amino acid synthesis from nitrate and denitrification can occur simultaneously, and (3) that the synthetic process can proceed through two alternative mechanisms which are not mutually exclusive, and only one of which involves ammonia.

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