THE BIOCHEMICAL MECHANISM OF NITRATE REDUCTION IN BACTERIA

by

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INTRODUCTION

Since the observation of Goppelsröder (1) in 1862 that soil nitrate is reduced to nitrite, the processes of biological nitrate reduction, nitrogen fixation, denitrification, and nitrogen assimilation by various organisms have been extensively investigated. The bacterial nature of nitrate reduction was recognized by Shoenbein (2), in 1868, and by Meusel (3), in 1875. The classical experiments of Gayon and Dupetit (4), in 1882, on the biological nature of nitrate reduction, gave added impetus to the investigation of these problems. The agronomic import of the nitrogen cycle of nature accounts for the long-continued study of the phenomena associated with it. Despite the large number of investigations, however, present knowledge of the chemical reactions underlying these processes is very incomplete, and contradictory observations have been reported.

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LITERATURE REVIEW

Soil nitrogen losses other than those of leaching and crop removal are known to occur. The processes of immobilization or assimilation of inorganic nitrogen by soil microorganisms, ammonification, nitrate reduction and denitrification are known to be affected by a variety of chemical and physical factors. These factors have been the subject of extensive investigations for over fifty years.

Effect of Oxygen Tension on Nitrate Reduction

The effect of oxygen was of considerable interest to early investigators. Weissenberg (5), in 1897, 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 absence of oxygen. The completeness of the reduction under these two conditions was quite different. Complete denitrification occurred in anaerobic cultures, but in the aerobic cultures nitrate was reduced only as far as nitrite.

In 1925, Seiser and Walz (6) demonstrated a considerable nitrogen loss from a culture of *Pseudomonas putida* exposed to air, though this loss was less than that observed under anaerobic conditions. Using manometric techniques, Lloyd and Cranston (7) measured the gas exchanges occurring when denitrifying cultures were in air and in a closed system. They observed a large oxygen uptake under aerobic conditions, and an almost equally large nitrogen evolution under anaerobic conditions. They concluded that nitrate was only slightly reduced aerobically, although some loss was observed under aerobic conditions. In 1924, Quastel et al. (8, 9) demonstrated that nitrate could take the place of molecular

oxygen in certain facultative anaerobes. Similarly, Stickland (10), in 1931, studied the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by Escherichia coli. He showed that different enzyme systems are concerned in the reduction of molecular oxygen and of nitrate. In the presence of very small oxygen tensions, the reduction of nitrate to nitrite was inhibited markedly, and increasing the nitrate concentration by a factor of ten did not modify these results. Thus, it appears that under these conditions the inhibition of nitrate reduction in Escherichia coli by molecular oxygen is non-competitive.

Van Olden (11), in 1940, observed that the ability of washed cells of Micrococcus denitrificans 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 (12))which developes under conditions suitable for denitrification. Prolonged subculture on a nitrate medium would increase the denitrifying activity of the organism.

Using two unidentified strains of Pseudomonas, Meiklejohn (13) 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. At a C/N ratio of ten, better growth was obtained under aerobic than under anaerobic conditions. Likewise, Korsakova (14) reported that when the organic carbon source was five to ten times as great as that required for reduction of the available nitrate, the reduction of nitrate by Achromobacter siccum and Pseudomonas aeruginosa was not affected by aeration.

Lemoigne (15) found that a pure oxygen atmosphere greatly increased the lag period for Bacillus megatherium grown in a medium containing

nitrate as the sole source of nitrogen. This did not occur if there was an organic nitrogen source in the medium, or if the atmosphere contained less than 64% oxygen. They concluded that oxygen inhibited the mechanisms involved in the assimilation of nitrate, a conclusion that seems to harmonize with the findings of Weissenberg (5).

Sacks and Barker (16), using an isolate of soil designated by them as Pseudomonas denitrificans, observed that at oxygen tensions of about 5%, nitrate could be reduced only as far as nitrite, which accumulated in the medium. At lower oxygen tensions, there was an abnormally large accumulation of nitrite, but this was accompanied by denitrification. They interpreted their experimental results as showing that oxygen affects nitrate reduction in two ways: by suppressing the formation of the enzyme systems that catalyze these reactions, and by directly interferring with the action of the enzyme systems when they are present. The formation of the enzymes responsible for nitrite reduction is suppressed to a greater extent than those responsible for the reduction of nitrate to nitrite. On the basis of these data, the experiments of other investigators reporting contradictory results are criticized. It is claimed that other workers employed methods of aeration inadequate to keep the culture medium saturated with oxygen at atmospheric pressure, thus allowing the partial pressure of oxygen in some parts of the medium to be very low.

Very recently, Skerman et al. (17) investigated the influence of oxygen concentration on the reduction of nitrate by an unadapted, unidentified, species of Pseudomonas. These authors measured the actual concentration of oxygen dissolved in the medium by means of polarographic analysis. Varying degrees of aeration were obtained by stirring the culture at different speeds. At oxygen levels of 2.5 p.p.m. and greater,

no reduction of nitrate occurred. Considering that the medium employed contained peptone as well as yeast extract, their results may be criticized on the basis that the unadapted cells, in the presence of a readily available source of reduced nitrogenous compounds, would preferentially use this form of nitrogen. At oxygen concentrations in excess of 5 p.p.m., complete oxidation of the substrate occurred before the population was able to reduce the nitrate. It would be of interest to determine what results would be obtained in a culture medium containing a high C/N ratio with nitrate as the sole source of nitrogen.

Lewis and Hinshelwood (18) investigated the growth of coliform bacteria in a simple medium containing nitrate and nitrite. They observed that the reduction of nitrate to nitrite by Bacterium lactis aerogenes was increased with lowered oxygen supply. Sudden aeration of an anaerobically growing culture caused a marked drop in the rate of reduction. In an explanation of these results, the authors assumed that some cell metabolite, denoted XH_2, is readily oxidized either by the oxygen of the air or by other suitable oxidizing agents, those present competing for the available hydrogen. A decrease in the concentration of a more powerful oxidizing agent would tend to allow an increase in the concentration of XH_2 to a point where the rate of reduction of others becomes appreciable. Thus, if the concentration of oxygen is reduced, the rate of reduction of nitrate may increase. The upward trend of the rate of nitrate reduction during growth may be explained if the rate of oxygen consumption exceeds the rate of supply to the medium by solution of the gas. There would then be a drop in the rate of hydrogen transfer with a consequent accumulation of oxidizable metabolites and an increase in nitrate reduction.

The count which has to be exceeded before this occurs will be higher the more efficient the degree of aeration. That the rate of nitrite reduction is not affected in this way by restricted aeration, may be inferred from the fact that the growth rate, which appears to be determined by the rate of nitrite reduction, was the same in all experiments.

Broadbent (19), after studying nitrogen losses in incubated sandy loam soils in an apparatus designed to vary the rate of aeration of the soil, concluded that a low oxygen concentration in the soil atmosphere is not a necessary condition for the denitrification process, and that the denitrification rate is affected more by the quantities of nitrate and oxidizable carbon in the soil than by partial pressure of oxygen.

In a similar series of experiments, Broadbent and Stojanovic (20) have presented evidence that denitrification of added nitrate is inversely related to partial pressure of oxygen, but is of appreciable magnitude even under fully aerobic conditions of soil incubation. Isotopic nitrogen was used to determine accurately the fate of added nitrate. The maximum recovery of added nitrate nitrogen was 75% under fully aerobic conditions. Data are presented suggesting that denitrification is not restricted to soils receiving recent additions of organic material. Isotope data show a negligible amount of the added nitrate was reduced to ammonia, but ammonification of organic nitrogen compounds did occur, except when glucose was added as a source of energy material. Their findings emphasize the advantages of ammonia fertilization from the standpoint of retention in the soil.

Janssen and Clark (21) have investigated the changes in organic, ammonium, and nitrate nitrogen occurring during the decomposition of oat

straw and alfalfa hay in the presence of added organic nitrogen under different conditions of incubation. It was found that vigorous bacterial activity, low fungi count, and an alkaline reaction were all necessary for any extensive denitrification. Their experiments demonstrate the toxicity of nitrite to bacteria under acid conditions and suggest that in the presence of abundant nitrate and with conditions suitable for biological denitrification, the denitrification process is self-inhibitory in acid substrates because of the nitrous acid toxicity which is developed. Mature oat straw constituted a less suitable substrate for denitrification than did alfalfa hay. Under standard conditions of incubation, and with moisture content at 2/3 saturation, there was no denitrification in oat straw mixtures. However if the water concentration was increased to saturation, or a more finely ground plant material was employed, or if sugar or peptone was added to the substrate, denitrification occurred. These modifications in incubation influence oxygen supply, and the authors suggest that the "biological oxygen demand" in such instances should not be overlooked.

In previous studies conducted by Bowers and MacVicar (22) in this laboratory, it was observed that in solution cultures of heterogeneous soil bacteria approximately 34% of the total nitrogen added as $KN^{15}O_3$ was lost during the first 24 hours under normal aerobic conditions of incubation.

In an extensive series of experiments, using isotopic nitrogen, Marshall, Dishburger, and MacVicar (23) followed nitrate reduction and denitrification by determining both the amount of reduced nitrogen appearing in the cells and the amount of nitrate lost from the mixture. Incubation of Pseudomonas fluorescens both in still and in vigorously aerated

cultures, resulted in a rapid decrease of nitrate from the culture. Similar results were obtained with a culture of Pseudomonas denitrificans obtained from Sacks and Barker (16), although this organism was more sensitive to low oxygen tensions. In view of these observations, it is suggested that nitrate disappearance and denitrification occur under aerobic as well as anaerobic conditions. The contrary results obtained by Sacks and Barker (16) were attributed to difficulties in manometric techniques, and to the measurement of nitrite accumulation as a criterion for exact measurement of nitrate reduction. It is possible that the time of formation and disappearance of such an intermediate may be so short that this method of detection is not applicable. The California workers did not measure residual nitrate in the media, but based their conclusions solely on nitrite determinations.

The Mechanism of Nitrate Reduction

The influence of ammonia and hydroxylamine in the processes of nitrate reduction and nitrogen fixation has been investigated extensively. It is possible that the assimilation of combined nitrogen as nitrate and molecular nitrogen are related by a common intermediate. The hypothesis generally accepted by earlier investigators gave ammonia the role as the key intermediate. Meyer and Schulze (24) challenged this theory as early as 1884, proposing hydroxylamine as a likely intermediate, since it could arise from ammonia and molecular nitrogen by oxidation, or from nitrate by reduction.

An examination of the recent literature reveals considerable controversy concerning the role of these two compounds in nitrogen fixation. One group of workers, headed by Virtanen (25), champions the hydroxylamine hypothesis, while Burris and Wilson (26) favor ammonia as the key intermediate.

Virtanen and Csaky (30) have examined the intermediate products in the fixation of nitrogen by Torula utilis suspended in nitrate solution under vigorous aeration. Free hydroxylamine and oximino-nitrogen were present within fifteen minutes, after which time there was a rapid diminution. These authors conclude that the cells evidently utilize oxime-nitrogen, but also point out that their findings do not give conclusive evidence of the significance of oxime formation, nor explain its mode of formation. Oxime-nitrogen formation from ammonium-nitrogen by the action of Nitrosomonas is reported by Lees (31). In experiments carried out on washed suspensions of Nitrosomonas, he found that while hydroxylamine is toxic to the nitrification of ammonia and is not itself nitrified at concentrations in excess of a few micrograms of nitrogen per milliliter, yet at concentrations below about 1.5 microgram nitrogen per milliliter, it is nitrified as rapidly as ammonia, and is thus a possible intermediate in the nitrification process. At a pH less than 8.4, hydroxylamine is nitrified a good deal more rapidly than ammonia. Despite its recognized toxicity, therefore, hydroxylamine may be an intermediate in the oxidation of ammonia, and hence could presumably function as an intermediate in the reductive process.

Rautanen (32), in a study of amino acid formation in green plants, has shown that nitrate is partly reduced to ammonia and is thus assimilated in the same way as ammonia. On the other hand, positive tests for oxime-nitrogen support the theory that hydroxylamine can act as an intermediate. These observations are in agreement with those of Burström (33) who stated that the reduction of nitrate in the green parts of plants proceeds only so far as hydroxylamine and this then reacts with compounds containing the carbonyl group, with subsequent amino acid and protein

synthesis. That this could take place in cultures of Pseudomonas fluorescens, assuming that hydroxylamine is formed, is given support by the recent report of Koepsell et al. (34). These investigators found that alpha-ketoglutarate is produced during glucose oxidation by this organism in rather significant amounts, 0.5-0.55 mole being formed for each mole of glucose consumed.

Virtanen and Järvinen (35) have also examined the formation of oxime-nitrogen by Azotobacter vinelandii in nutrient solutions containing as a sole source of nitrogen either ammonium or nitrate salts. Oxime-nitrogen was produced in every case, but the formation was much more rapid from nitrate-nitrogen and molecular nitrogen than from ammonium-nitrogen. Ammonium-nitrogen was assimilated by the cells as rapidly as, or more rapidly than nitrate-nitrogen or molecular nitrogen. They concluded that nitrate is not entirely reduced to ammonia, but that hydroxylamine preceding it forms oximes with carbonyl compounds.

On the other hand, Novak and Wilson (36), in a series of carefully controlled trials, demonstrated that the nitrogen in the form of the oximes of pyruvic, oxalacetic and alpha-ketoglutaric acids was not available to Azotobacter vinelandii. Similarly, Burris and Wilson (37), in a study of aerated cultures of Azotobacter, suggested that ammonia is the key intermediate in nitrate assimilation and nitrogen fixation. When such cultures, grown on nitrogen gas, were supplied labelled ammonium ion, the culture immediately stopped fixing nitrogen and used ammonia as its exclusive source of nitrogen. When labelled nitrate was supplied, no isotope appeared in the cells for thirty minutes, in contrast to its detection in one minute when ammonia was supplied. From these studies it was concluded that ammonia is the first stable intermediate formed in

the fixation of nitrogen by Azotobacter vinelandii.

Segal and Wilson (18) have demonstrated that hydroxylamine is not utilized as a source of nitrogen by Azotobacter even in non-toxic concentrations of 1.0-2.0 microgram per milliliter, thus confirming the work of Novak and Wilson (36).

Lewis and Hinshelwood (18) observed that if ammonia was added to a rapidly growing culture of Bacterium lactis aerogenes, almost complete inhibition of nitrate and nitrite reduction occurred. Resumption of reduction began only after the concentration of ammonium ions was negligible. If the addition of ammonia was made before growth was started, little or no reduction occurred. Imperfect aeration also was found to reduce the inhibitory action of ammonia on the nitrate reduction processes and on the concentration of nitrite built up in the medium. The delayed recovery suggested that during growth in ammonia, some substance was possibly formed which inhibited the reduction of nitrate to nitrite. These authors concluded that the ratio of X/XH_2, oxidized and reduced form of the postulated intermediate previously discussed, was high and under these conditions nitrate and nitrite were not reduced.

In another report by these authors (39), a steady concentration of ammonia of a low order of magnitude, 1 milligram per milliliter, accumulated in cultures of Bacterium lactis aerogenes grown on nitrate. From a consideration of quantitative relations to growth rate, this order of magnitude was that to be expected if nitrate utilization proceeded via ammonia. Their results, however, were not precise enough to provide conclusive evidence for this view.

Zelitch et al. (40) have recently reported evidence in support of the theory that ammonia is a key intermediate in nitrogen fixation by

the anaerobe Clostridium pasteurianum. When such cells, actively fixing nitrogen, were supplied $N_2{}^{15}$ for a short time, free ammonia with an extremely high N^{15} concentration was isolated from the culture medium. Somewhat lower concentrations, but still considerably in excess of the average of either the intact cells or the supernatant medium, were found in the amide fraction. These data, these workers concluded, provide direct support for the view that ammonia is a key intermediate in biological nitrogen fixation.

The subsequent role of ammonia in cellular synthesis has been examined by Burris and Wilson (41) and Zelitch et al. (42). The former, in studies on the distribution of N^{15} from nitrogen or ammonia in Azotobacter, found that with either source of the isotope, the N^{15} accumulated in highest concentration in the glutamic acid of the cells. The latter investigators found similar results for Clostridium, indicating the marked similarity of nitrogen fixation mechanisms of aerobes and anaerobes. The investigations of Roine (43), Aqvist (44), and Virtanen et al.(45) on the synthesis of nitrogenous compounds by Torula utilis harmonize with the above findings.

Dishburger and MacVicar (46), using isotopic nitrogen, have clearly demonstrated that assimilation of nitrate nitrogen and its incorporation into cellular constituents were dramatically halted by the addition of ammonium-nitrogen in aerated cultures of Pseudomonas fluorescens and Pseudomonas denitrificans, although both nitrate and ammonium nitrogen continued to disappear at a rapid rate. When nitrate and ammonia were present simultaneously, ammonium-nitrogen was assimilated preferentially, and converted to cellular components. This was demonstrated by the small

amount of isotope appearing in the cells when labelled nitrate was supplied, and by the rapid incorporation of labelled ammonia into the cellular fraction when labelled ammonium-nitrogen was available. This supports the view that ammonia constitutes an intermediate in the reduction of nitrate to cellular constituents. Disappearance of nitrate from the medium and conversion to gaseous form was essentially unaffected by the presence of the ammonium ion. In cultures of Pseudomonas fluorescens supplied $N^{15}H_4^+$ and NO_3^- simultaneously, virtually complete incorporation of the label into cellular protein was obtained. If the nitrate was labelled, the loss of isotopic nitrogen through denitrification indicates that conversion of nitrate to cellular components and the denitrification processes proceed by different pathways. This suggests that denitrification does not proceed via ammonia as an intermediate, despite the report that nitrogen fixation does.

Allen and van Neil (47) and Allen and Najjar (48, 49) have proposed nitramide as a key intermediate in denitrification. They found that if nitramide was added to a growing culture of Pseudomonas stutzeri, rapid evolution of nitrogen occurred. Such results were not obtained with hyponitrite. These authors concluded that the formation of nitrogen from nitramide involves a simple reduction rather than an hydration followed by the elimination of peroxide. Application of the technique of simultaneous enzymatic adaptation (50) suggested that nitrous oxide is either an intermediate product in denitrification or is reversibly derived from an intermediate product. These workers have also reported the conversion of nitramide and of nitrite to nitrogen gas or nitrous oxide by cell-free extracts of Pseudomonas stutzeri and by lysozyme lysates of a thermophilicid denitrifying Bacillus subtilis strain (48, 49).

These results are open to serious criticism. The probability that nitramide is an intermediate in denitrification is not supported by their data, if one examines the stability of the compound. The authors state that optimum denitrification occurred at a pH of 8. As far as can be determined from the literature, however, no blank determinations were included in their experiments. Bronsted and Pederson (51) have studied the decomposition of nitramide. Its decomposition to yield one mole each of nitrous oxide and of water is of importance as being the first reaction for which general basic catalysis was demonstrated. These investigators found the reaction to be of the first order, uncatalyzed by acids, but strongly catalyzed by the hydroxyl ion. Apparently, under the conditions employed by Allen and co-workers, nitramide would decompose spontaneously to yield N_2O, which would then be reduced to N_2, if the results of their experiments employing the techniques of simultaneous enzymatic adaptation are valid. Clearly, then, it seems highly questionable whether the data presented supports the view that nitramide is a key intermediate in the process of denitrification.

EXPERIMENTAL

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, and a strain of Bacillus subtilis from the stock collection of the Department of Bacteriology, Oklahoma Agricultural and Mechanical College. These organisms were trained by long serial subculture 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 medium was brought to a pH of approximately 6.8 with KOH, using bromthymol blue as an indicator. A precipitate formed on autoclaving, but this did not interfere except to make turbidimetric measurements of growth impractical. Transfers were normally made every 48 hours.

In the experiments, one liter of nitrate medium was placed in a two-liter filter flask fitted with a fritted-glass gas dispersion tube. A stream of air was passed through a tightly packed cotton filter before it entered the medium. To minimize losses due to evaporation, the stream of air was bubbled through sterile water prior to entering 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 37-38° C. in a constant temperature water bath. Upon depletion of the original nitrate, detected by the brucine-sulphate method (23, 52), the culture was immediately sampled. Usually 25-50 mg. of N as $KN^{15}O_3$, containing approximately 36 atom % N^{15} excess, and 25-50 mg. of N as

$(NH_4)_2SO_4$ was added to the medium, and the culture again sampled. Subsequently, sampling was repeated at definite intervals over a period of 5-6 hours. Samples were obtained by withdrawing a 25 ml. aliquot of the culture and pipetting into 2 ml. of boiling 0.8 normal H_2SO_4. The sample was then brought to a boil, stoppered immediately, and reserved for analysis. At the completion of sampling, the samples were transferred to plastic centrifuge tubes and the cells were packed by centrifugation. (In Experiment I, the cells were harvested in a Sharple's Super Centrifuge.) The supernatant was carefully decanted into 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. 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.

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 the iron-sulphuric-acid-reduction procedure as developed by Pucher et al. (53). The Kjeldahl titration mixtures from these analyses were prepared for conversion as suggested by Schoenheimer (54). 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 Mr. Glenn Hallmark of the Department of Electrical Engineering, Agricultural and Mechanical College of Texas.

Analysis for ammonia was made on suitable (0.1-0.3 mg. N) aliquots of the supernatant. Samples were usually rendered strongly alkaline and aerated into 5 ml. of N/7 H_2SO_4 for one hour at elevated temperatures.

In Experiment V, ammonia was determined according to the method of Pucher et al. (55). The distillates from the aeration apparatus were each 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 (23) of the brucine-sulphate method developed by Gad (52). 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 within 5% by checking it against the aeration procedure using Devarda's alloy to reduce nitrate to ammonia.

Analysis for free hydroxylamine and oximino-nitrogen was according to Endre's modification taken from Novak and Wilson (36).

Paper chromatograms were prepared using the method of Berry et al. (56). Solvents employed were phenol saturated with a buffered water solution, and a solution of Lutidine in water. Both one and two dimensional chromatograms were made.

RESULTS AND DISCUSSION

Previous investigations of Dishburger and MacVicar (46) on the assimilation of ammonia and nitrate nitrogen by Pseudomonas fluorescens and Pseudomonas denitrificans suggested the presence in the medium of a form of nitrogen, neither nitrate, nitrite, nor ammonia, capable of contributing to protein synthesis. In a culture of Pseudomonas denitrificans supplied with labelled nitrate, a high concentration of isotopic nitrogen remained at the termination of the sampling period. Characterization of this unidentified form of residual nitrogen would possibly elucidate the pathway of nitrate reduction. Accordingly, Experiment I was designed to make the isolation of these intermediary forms of nitrogen possible, if they were found to be present.

Six liters of nitrate media were prepared and innoculated with 25 ml. of a 24-hour subculture of Pseudomonas fluorescens. Incubation was allowed to proceed under vigorous aeration at room temperature for 57 hours when all the original nitrate was exhausted. At this time, a 25 ml. aliquot of the culture was taken for analysis, and 300 mg. of N as $KN^{15}O_3$, containing 36.0 atom % N^{15} excess, and 300 mg. of N as $(NH_4)_2SO_4$ were added. Subsequently, 25 ml. samples of the culture were taken at definite intervals. Analysis was made for cellular nitrogen, total medium nitrogen, ammonia nitrogen, and nitrate nitrogen. The Kjeldahl titration mixtures were prepared for N^{15} analysis. Due to incomplete reduction of the nitrate by the iron-sulfuric acid procedure employed in this experiment, the values of total medium nitrogen and medium isotope concentration prior to 120 minutes are unreliable and are not

presented on Figure I. After this time, however, the nitrate supply was virtually exhausted and the data shown in Figure I are thought to be reliable.

Examination of the data presented in Figure I shows that several important changes occurred during the course of growth of the organism. Prompt and nearly complete disappearance of NO_3-N takes place despite the fact that NH_4-N was present in somewhat higher concentration throughout the course of the experiment, and despite vigorous aeration of the culture medium. Decrease in medium nitrogen amounted to nearly 9.5 mg. nitrogen per 100 ml. of medium, while increase in cellular nitrogen amounted to 5.0 mg. per 100 ml. Correcting for the NH_4-N and NO_3-N remaining at the termination of the experiment and for the increase in cellular nitrogen leaves a net deficit of 4.8 mg. nitrogen per 100 ml. Assuming that all this loss was derived from NO_3-N, the amount denitrified is equal to 96%. Confirmation of this is seen by inspection of the isotope data. This reveals that incorporation of N^{15} into the cells was small, accounting for a total of only 0.53 mg. of N^{15}. Residual N^{15} in the medium at the end of 300 minutes amounted to 0.20 mg. There was, therefore, a deficit of 108.3 mg., or 99%. Thus, virtually complete denitrification must have occurred. Such a substantial deficit can hardly be explained on any basis save conversion to some gaseous form that was swept from the culture by the aeration stream.

At the 300 minute sampling period, the culture medium was analyzed for a-amino nitrogen by means of a van Slyke apparatus. The analysis revealed that 2.25 mg. nitrogen per 100 ml. was present in this form. Amide nitrogen, determined by the method of Pucher *et al.* (55) amounted to 2.0 mg. nitrogen per 100 ml. From an inspection of the isotope data,

it is impossible to tell whether these forms of nitrogen were due to the breakdown of cells prior and after the addition of the labelled nitrate.

The negligible level of N^{15} in the medium at the termination of the sampling period made it desirable to study a smaller culture, since previous studies in this laboratory were made with one liter cultures. It was thought that growth under the conditions described in experiment I may have prevented the formation of the compound or compounds having a high isotope concentration. In experiment II (Figure II) one liter of nitrate medium was prepared and innoculated with 10 ml. of a 24-hour subculture of Pseudomonas fluorescens. Incubation was allowed to proceed under vigorous aeration at 37-38° C., until the original nitrate supply was exhausted, 44 hours after innoculation. After a 25 ml. aliquot was taken for analysis, 25 mg. of N as $KN^{15}O_3$, containing 36.0 atom percent N^{15} excess, and 25 mg. of N as $(NH_4)_2SO_4$ were added simultaneously to the culture. During the ensuing period, samples were withdrawn and analyzed for the following components: Cellular nitrogen, ammonia nitrogen, nitrate nitrogen, and total medium nitrogen. Cellular and medium nitrogen Kjeldahl titration mixtures were analyzed for N^{15} concentration, the former throughout the sampling period and the latter for the last three sampling periods.

Inspection of Figure II reveals results both similar to and different from that presented in Figure I. Prompt and nearly complete disappearance of nitrate is again observed, despite vigorous aeration and the presence of ammonia nitrogen. Decrease in medium nitrogen amounted to nearly 4.9 mg. per 100 ml., while increase in cellular nitrogen amounted to 2.3 mg. per 100 ml. Correcting for the NH_4-N and NO_3-N remaining at the termination

FIGURE I

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

5.0 mg. $N^{15}O_3$-N and 5.0 mg. NH_4-N Added per 100 ml. at Zero Time

(Six Liter Culture)

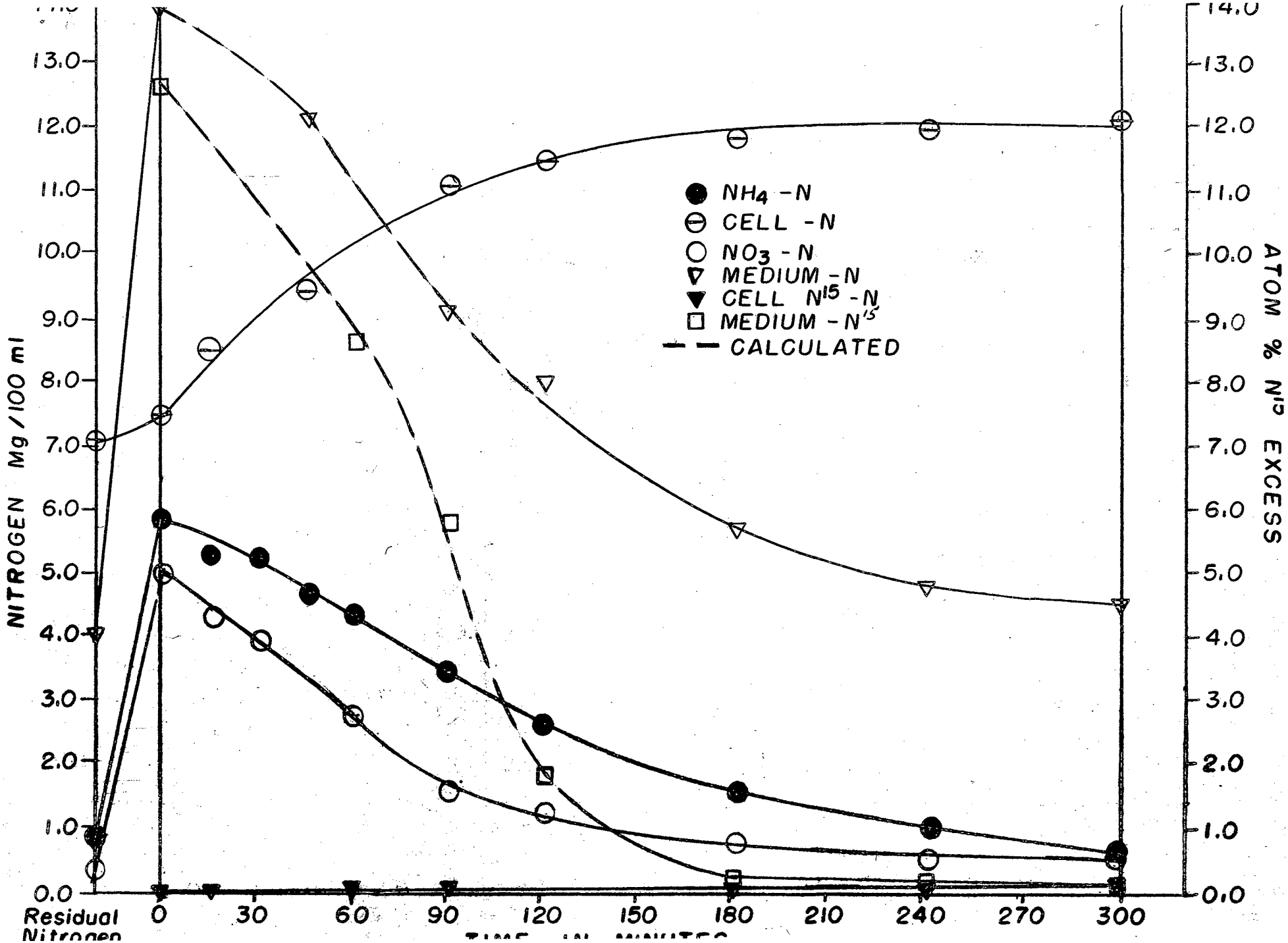
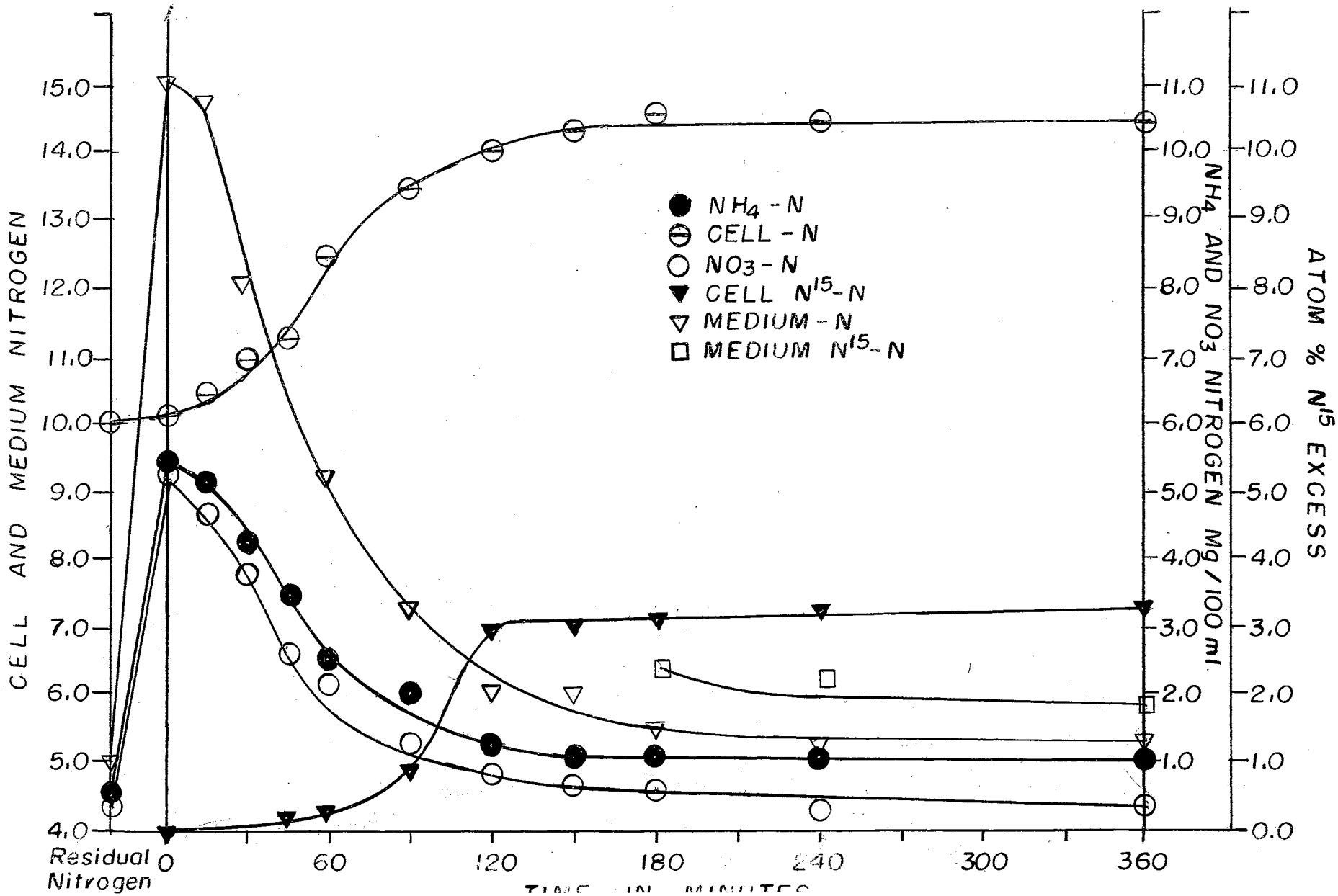

NITROGEN Mg/100 ml
ATOM % N15 EXCESS
NH4 -N
CELL -N
NO3 -N
MEDIUM -N
CELL N15 -N
MEDIUM -N15
CALCULATED
Residual Nitrogen
0
30
60
90
120
150
180
210
240
270
300
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0

FIGURE II

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

2.5 mg. $N^{15}O_3$-N and 2.5 mg. NH_4-N Added per 100 ml. at Zero Time


PSEUDOMONAS FLUORESCENS AERATED CULTURE
2.5 Mg NH_4-N And 2.5 Mg $N^{15}O_3$-N Per 100 ml
CELL AND MEDIUM NITROGEN
NH_4 AND NO_3 NITROGEN Mg/100 ml
ATOM % N^{15} EXCESS
NH_4-N
CELL-N
NO_3-N
CELL N^{15}-N
MEDIUM-N
MEDIUM N^{15}-N
Residual Nitrogen
TIME IN MINUTES

of the experiment leaves a net deficit of 2.0 mg. per 100 ml. Assuming that all this loss was derived from the NO_3-N, the percentage denitrified during this period was 80%. Incorporation of N^{15} into the cells was substantially larger than in the previous experiment, accounting for a total of 1.8 mg. N^{15}. Residual N^{15} in the medium was 0.56 mg. at the end of 360 minutes. This gives a deficit of 5.86 mg. or 72%.

Comparison of Figures I and II shows that in experiment I the ammonia concentration was at all times considerably higher than the nitrate concentration. Though this is also seen in Figure II, the difference is not as great. Although a strict comparison of the two experiments is not justified, because of non-similar conditions of growth, it seems that preferential utilization of ammonia nitrogen for cellular synthesis prevents the assimilation of nitrate nitrogen, although denitrification proceeds rapidly.

As in experiment I, a very low concentration of N^{15} was found in the medium at the termination of the sampling period. In view of this, only labelled KNO_3 was supplied to an aerated culture of Pseudomonas fluorescens in experiment II. Dishburger and MacVicar (46) had observed a high N^{15} concentration in the medium at the termination of 300 minutes in a culture of Pseudomonas denitrificans supplied only labelled nitrate, but it was impossible to tell from their experiments whether this high concentration was due to incomplete utilization of nitrate, to cellular breakdown, or to some soluble form of nitrogen formed from nitrate and excreted into the medium.

One liter of vigorously aerated nitrate medium was innoculated with 10 ml. of a 48-hour subculture of Pseudomonas fluorescens. Upon

exhaustion of the original nitrate, 36 hours after innoculation, 50 mg. N as $KN^{15}O_3$, containing 36.5 atom percent N^{15} excess, was added. The sampling procedure of the previous experiments was followed. Analysis was made for nitrate nitrogen, total medium nitrogen, cellular nitrogen, and for N^{15} in the cellular and total medium nitrogen.

Examination of Figure III (as well as Figures I and II) shows that there was a substantial amount of nitrogen present in the medium at the time of the addition of the nitrate. Since these cultures had been grown on nitrate as the sole nitrogen source, this had been derived from reduction of nitrate. Whether this nitrogen represents some metabolic product similar to the ammonia shown by Zelitch et al. (40) to be elaborated by cultures of Clostridium pasteurianum fixing atmospheric nitrogen, or whether it merely represents the soluble nitrogenous compounds resulting from the decay of earlier generations, was not known. Examination of the data in Figure III helps resolve this problem.

Rapid diminution of nitrate content of the medium, declining from 5.6 to 0.6 mg. per 100 ml., occurred in a 360 minute incubation period. During this same period the cellular nitrogen increased by 2.0 mg. per 100 ml. This leaves a total of 3.0 mg. nitrogen unaccounted for. A portion of this evidently remained in the medium at the termination of the experiment, there being present 4.5 mg. of medium nitrogen per 100 ml., containing an isotope concentration of 7.2 atom percent N^{15} excess. Prior to the addition of the nitrate, medium nitrogen totaled 3.7 mg. per 100 ml. giving an increase of 0.8 mg. nitrogen per 100 ml. during the 360 minute incubation period. This form of nitrogen is not nitrate since the nitrate in the medium was depleted within 180 minutes. Distillation, after rendering the medium samples strongly basic, gave a negative

FIGURE III

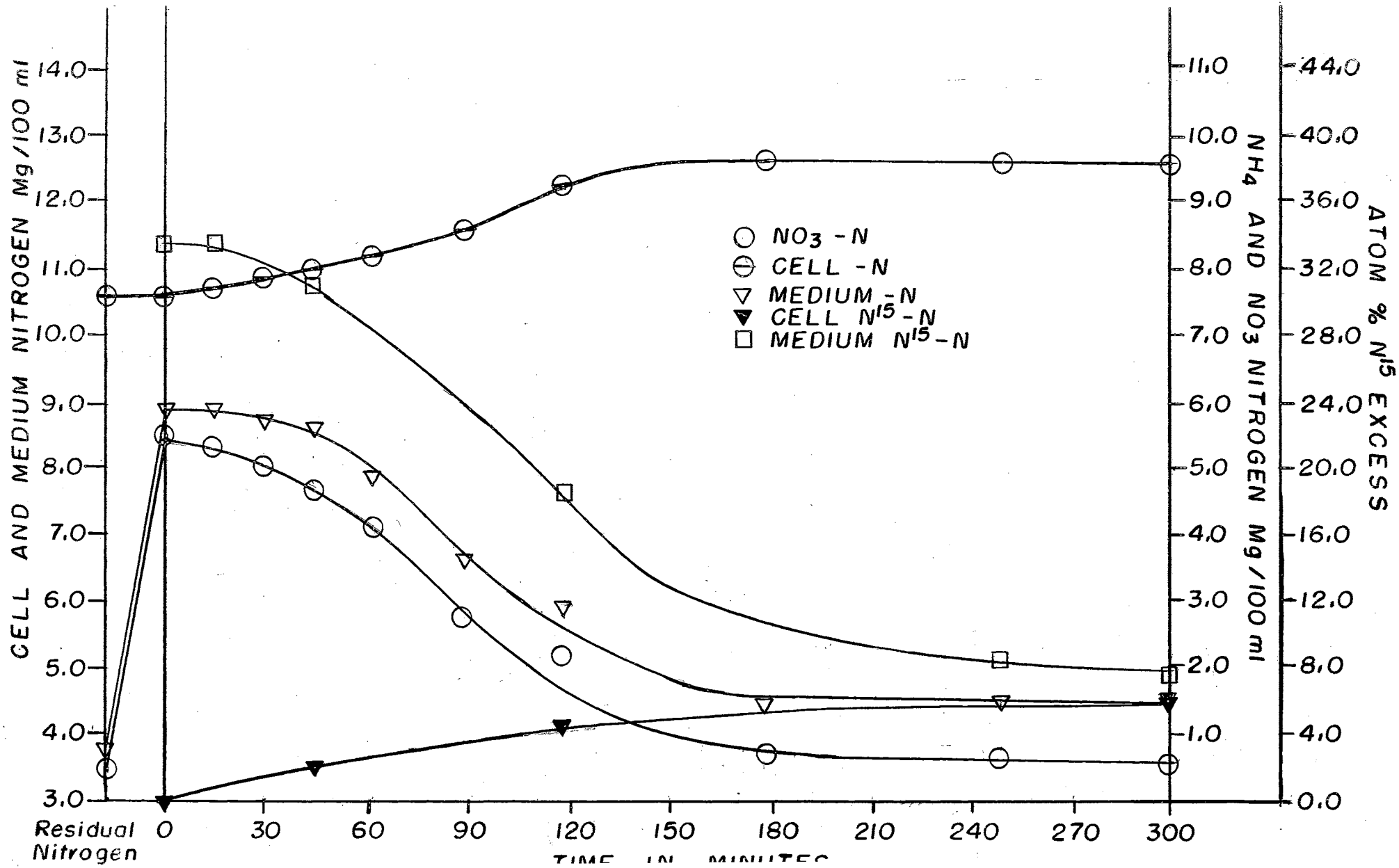
Relation of Cellular Nitrogen to Nitrate

Disappearance in an Aerated Culture

of Pseudomonas fluorescens

5.0 mg. $N^{15}O_3$-N Added per 100 ml. at Zero Time

PSEUDOMONAS FLUORESCENS AERATED CULTURE
5.0 Mg $N^{15}O_3$ - N Per 100 ml



test for ammonia. The assumption that this increase came from the breakdown of cells after 180 minutes (when the N^{15} content of the cells had reached a maximum) would account for 0.48 mg. N^{15} excess per 100 ml., giving the medium nitrogen an atom percent N^{15} excess equal to only 1.3%. It is apparent then, that the high medium isotope concentration at the end of 360 minutes did not arise from cellular breakdown. Further investigation of the nature of this excretion product was not undertaken at this time due to exhaustion of media with which to work.

As can be seen both from the nitrogen balance data of experiments I and II, and from the fact that medium nitrogen at the termination of these experiments contained only a small amount of isotope, this excretion apparently does not occur when ammonia, as well as nitrate, is provided. Presumably, therefore, the presence of ammonia blocks the conversion of nitrate to whatever compound is excreted into the medium. Further study on the nature of this compound, and of the role of ammonia in blocking its formation, should prove of great interest.

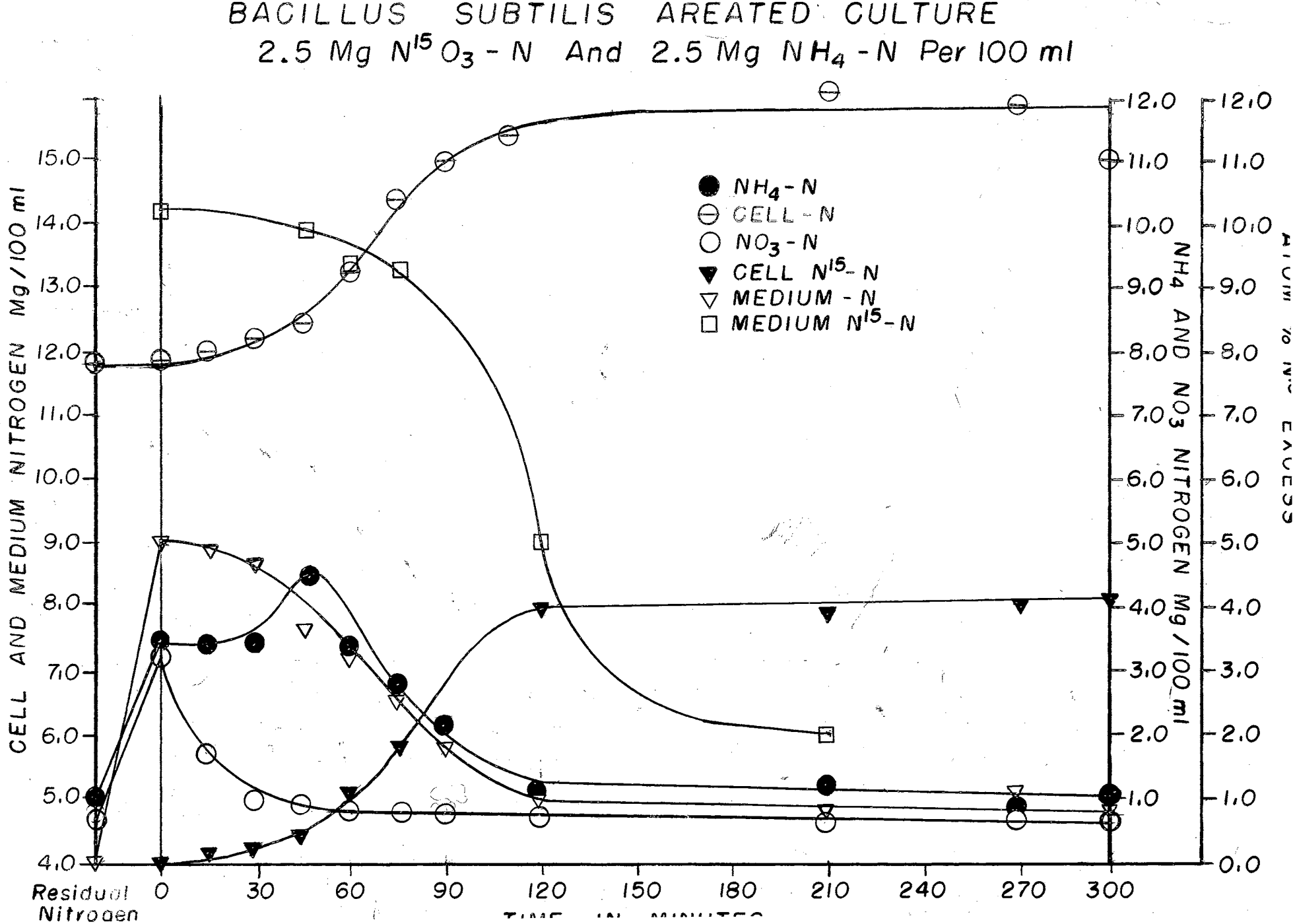
To extend the experiments to other bacterial species, a nitrogen balance study of the strict aerobe Bacillus subtilis was undertaken in experiments IV and V. Comparison of the rates of disappearance of nitrate and ammonia in a denitrifying and non-denitrifying organism would, it was hoped, give additional information concerning the fate of nitrate utilized for cellular synthesis. Examples of such experiments are presented in Figures IV and V.

In experiment IV, one liter of vigorously aerated nitrate medium was innoculated with 10 ml. of a 48-hour subculture of Bacillus subtilis adapted to nitrate nutrition by long serial subculture. When the original nitrate was depleted, 25 mg. of N as $KN^{15}O_3$ containing 36.5 atom

FIGURE IV

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Bacillus subtilis

2.5 mg. $N^{15}O_3$-N and 2.5 mg. NH_4-N Added per 100 ml. at Zero Time


BACILLUS SUBTILIS AREATED CULTURE
2.5 Mg $N^{15}O_3$ - N And 2.5 Mg NH_4 - N Per 100 ml
NH_4 - N
CELL - N
NO_3 - N
CELL N^{15} - N
MEDIUM - N
MEDIUM N^{15} - N
CELL AND MEDIUM NITROGEN Mg/100 ml
NH_4 AND NO_3 NITROGEN Mg/100 ml
Residual Nitrogen
TIME IN MINUTES
0
30
60
90
120
150
180
210
240
270
300
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0

percent N^{15} excess, and 25 mg. N as $(NH_4)_2SO_4$ were added. Aliquots of 25 ml. were withdrawn as previously described, and analyzed for nitrate nitrogen, ammonium nitrogen, cellular nitrogen, and total medium nitrogen. N^{15} data was obtained for both medium and cellular nitrogen.

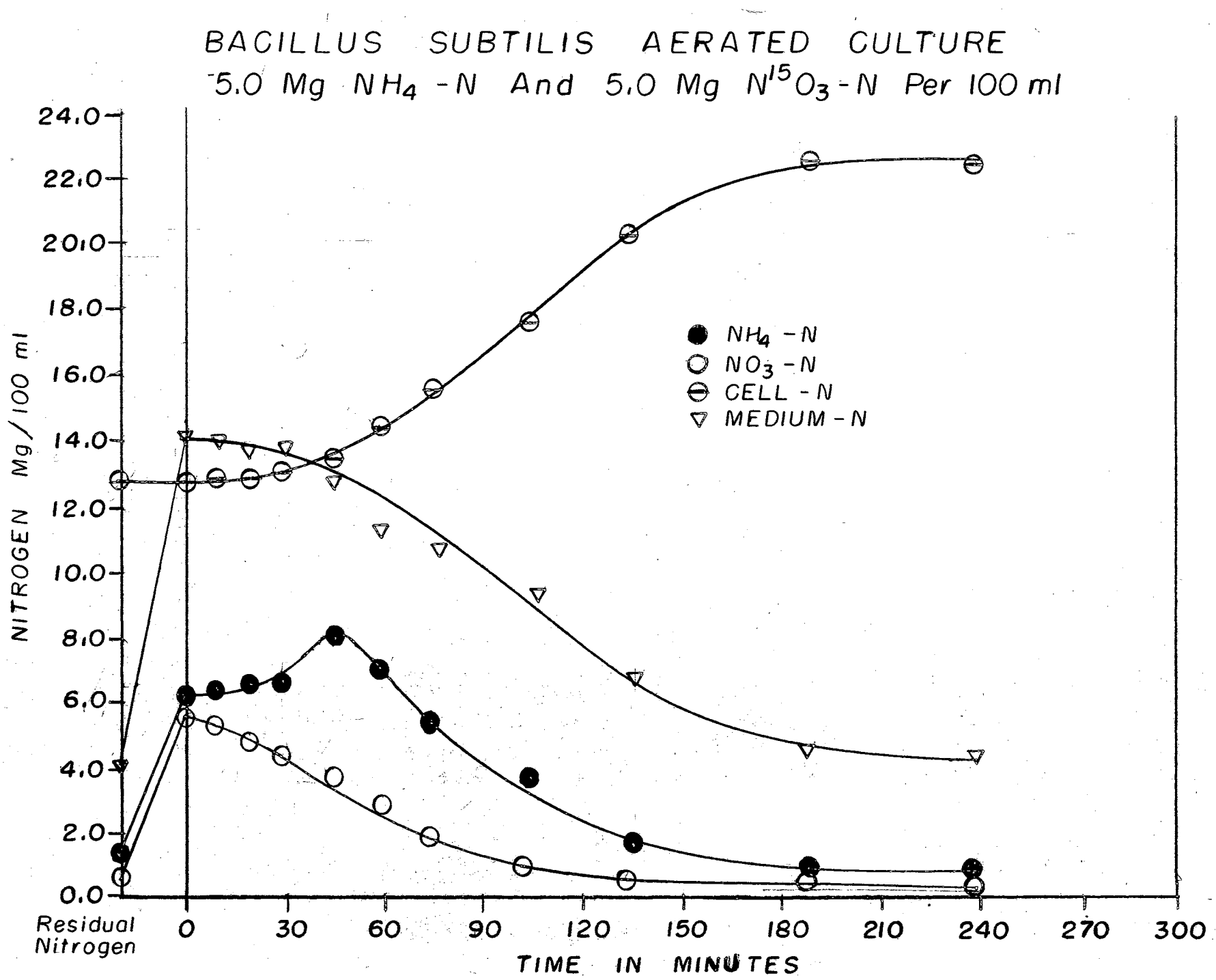
Inspection of Figure IV reveals that striking changes occurred during the 300-minute incubation period. Complete and extremely rapid disappearance of added nitrate occurred within the first 45 minutes. An abrupt increase in the ammonium nitrogen took place within the same period of time. Isotope data show that the ammonia, obtained by strongly basic distillation of this medium sample, contained 12.85 atom percent N^{15} excess. This phenomenally high isotope concentration gives convincing, direct evidence that ammonia is an intermediate in the reduction of nitrate for use in cellular synthesis. An inspection of the ammonia and nitrate curves, as well as the medium N^{15}-N curve prior to 30 minutes, suggests that nitrate is first converted to some other soluble nitrogenous compound which is then further reduced to ammonia.

In order to confirm the results of experiment IV, and to gain knowledge of these possible intermediary compounds, experiment V, was set up. Several modifications were made in sampling and analytical procedure in this experiment, but conditions of incubation were identical with those in the preceding experiment. Upon exhaustion of the original nitrate, 50 mg. of $KN^{15}O_3$-N, containing 36.5 atom percent N^{15} excess, and 50 mg. of $(NH_4)_2SO_4$-N were added to the culture. Aliquots of 25 ml. were withdrawn from the culture for the residual and initial samples. Thereafter, for the following 75 minutes, 100 ml. samples were withdrawn. Subsequently 25 ml. samples were taken. The frequency of sampling during the initial period of incubation was also increased (see Figure V). The

FIGURE V

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Bacillus subtilis

5.0 mg. $N^{15}O_3$-N and 5.0 mg. NH_4-N Added per 100 ml. at Zero Time


BACILLUS SUBTILIS AERATED CULTURE
5.0 Mg NH_4 - N And 5.0 Mg $N^{15}O_3$ - N Per 100 ml
NITROGEN Mg / 100 ml
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Residual Nitrogen
0
30
60
90
120
150
180
210
240
270
300
TIME IN MINUTES
NH_4 - N
NO_3 - N
CELL - N
MEDIUM - N

organisms were killed in the usual manner. Instead of autoclaving the medium samples after harvesting the cells, as in experiment IV, the samples were frozen until the analysis was completed. The analysis for ammonia was carried out according to the method of Pucher, et al. (55), since it seemed possible that strongly basic conditions in the previous experiment had decomposed some intermediate to yield ammonia. Ammonia nitrogen was also determined by the same method, following autoclaving the medium samples for one hour at 18 pounds pressure. The pH of these samples was between 2 and 3. Results for ammonia obtained were identical, indicating that if unknown nitrogenous compounds were present, drastic treatment with acid would not liberate ammonia. This treatment also excludes amides as possible intermediates, since these compounds are unstable to such treatment. Nitrate nitrogen was determined both by the brucine-sulphate method, and by reduction with Devarda's alloy after distillation of ammonia. Results were identical, indicating that the intermediates were stable to strong reducing conditions. After the aeration of ammonia in a buffered system, the samples were transferred to semi-micro Kjeldahl flasks, rendered strongly basic, and distilled for one hour at elevated temperatures. The distillate gave an atypical color with Nessler's reagent. The intensity of color varied, showing no general trend throughout the course of the experiment. The significance of this is not known.

Examination of Figure V shows that the results obtained are very similar to those obtained in experiment IV. The rate of nitrate disappearance is not as rapid as in experiment IV, but a larger increase in the ammonia concentration is evident, being nearly double that

obtained in the previous experiment. This was to be expected, since the quantity of nitrate and ammonia supplied was greater by a factor of two.

From the above data, ammonia appears to be excreted into the medium as such. These data show striking similarity to those obtained by Zelitch et al. (40) in experiments in which Clostridium fixed stmospheric nitrogen.

The medium was also analyzed for free hydroxylamine and oximino nitrogen by Endre's modification, as given by Novak and Wilson (36). Absolutely no indication of these forms of nitrogen was obtained. According to Csaky (57) hyponitrous acid and nitrohydroxamic acid also give the typical color reaction with sulphanilic acid and alpha-naphthylamine without prior oxidation to nitrite. No nitrite was found present in the medium, although the method is sensitive to 0.1 mg. per ml.

Paper chromatagrams of the medium samples for the presence of amino-nitrogen, using the technique of Berry et al. (56), showed that only glutamic acid was detectable, and this was present in low concentration (approximately 25 micrograms per ml.). No increase in glutamic acid concentration was observed during the duration of incubation. Presumably, the formation of amino acids is secondary to the formation of ammonia, as discussed earlier, and these compounds do not arise from the reduction of oxynitrogen compounds.

GENERAL DISCUSSION

The primary formation of nitrite from nitrate is, and on satisfactory ground, generally considered the first step in the reduction of nitrate. The mechanism of further reduction of nitrite poses an intergoing biochemical problem, since the possible intermediate compounds in a reduction chain from nitrite to amino are very limited. Despite this fact, no satisfactory evidence at present exists for the actual participation of such compounds as nitroxyl, its dimer hyponitrous acid, the hypothetical compound dioxyammonia, or hydroxamic acid. The evidence presented in these studies is highly suggestive that such compounds are not intermediates in the reduction chain. However, it should be noted that the rates of formation and of disappearance of these possible intermediates may be such that their detection is not possible by the methods employed in these experiments.

Zelitch et al (40), in commenting upon the isolation of ammonia as the key intermediate in nitrogen fixation by Clostridium, state that "it should not go unnoticed that the attainment of this long-sought-for goal of the research on the mechanism of biological nitrogen fixation depended to a great extent on the fortunate circumstance that the organism possesses a metabolic eccentricity which causes it to eliminate free ammonia while growing actively. The recovery of this ammonia with a phenomenally high concentration of labelled nitrogen, constituting as it does the most direct and substantial support offered to date for the hypothesis that ammonia is a key intermediate in biological nitrogen fixation, was made possible by taking advantage of the capacity of the

organism itself to effect the first and most critical step in the chemical separation." Identical circumstances made the isolation of ammonia derived from nitrate reduction possible in these studies with Bacillus subtilis. It is evident then, that the pathways of nitrogen fixation by Clostridium, and nitrate assimilation by Bacillus subtilis, involve ammonia as a common intermediate. Strictly speaking, the highly specific data from these studies, indicating ammonia as the key intermediate in biological nitrate reduction, should be restricted to the aerobe Bacillus subtilis. However, comparison of the comparative biochemistry of biological nitrate reduction, particularly as indicated by N^{15} studies conducted in this laboratory and elsewhere, strongly suggests that the conclusion may be generalized to include many other agents.

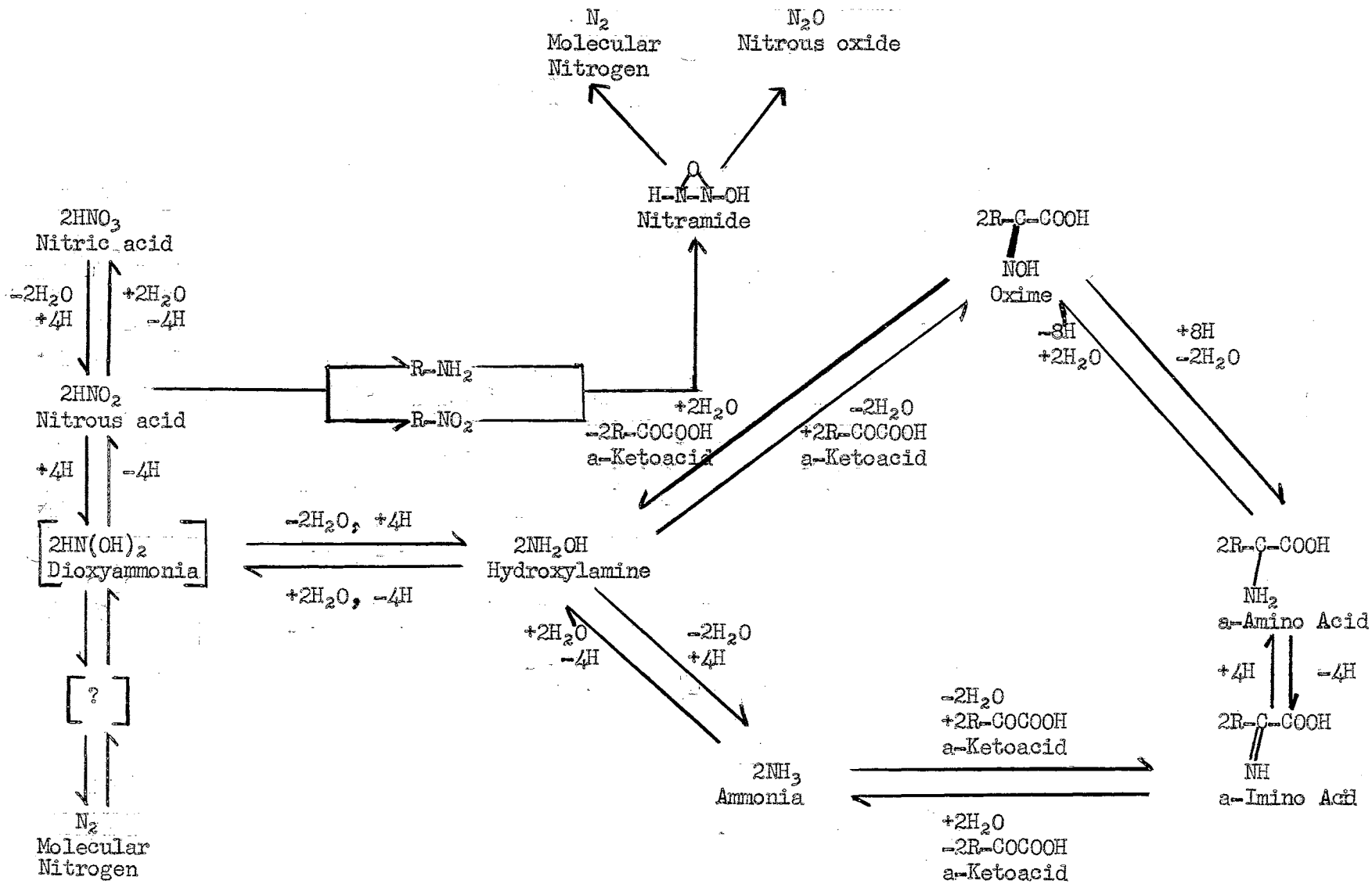
The chemical reactions underlying the processes of nitrate assimilation and denitrification has been the subject of frequent speculation. Two mechanisms have been proposed for the fixation of molucular nitrogen and for the assimilation of nitrate: One favoring hydroxylamine as the key intermediate and the other favoring ammonia. A proposed mechanism for these processes is presented in Figure VI.

A study of Figure VI 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 as the acceptor of fixed nitrogen. Hydroxylamine is separated from ammonia by a reductive step. Since it is most probable that the reduction and assimilation of nitrate 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 an a-ketoacid. Amino

FIGURE VI

Proposed Pathways of Nitrate

Assimilation and Denitrification


N_2
Molecular
Nitrogen
N_2O
Nitrous oxide
H-N-N-OH
Nitramide
$2HNO_3$
Nitric acid
$-2H_2O$
$+4H$
$+2H_2O$
$-4H$
$2HNO_2$
Nitrous acid
$R-NH_2$
$R-NO_2$
$+2H_2O$
$-2R-COCOOH$
a-Ketoacid
$-2H_2O$
$+2R-COCOOH$
a-Ketoacid
2R-C-COOH
NOH
Oxime
$-8H$
$+2H_2O$
$+8H$
$-2H_2O$
$+4H$
$-4H$
$2HN(OH)_2$
Dioxyammonia
$-2H_2O$, $+4H$
$+2H_2O$, $-4H$
$2NH_2OH$
Hydroxylamine
$+2H_2O$
$-4H$
$-2H_2O$
$+4H$
?
$2NH_3$
Ammonia
$-2H_2O$
$+2R-COCOOH$
a-Ketoacid
$+2H_2O$
$-2R-COCOOH$
a-Ketoacid
2R-C-COOH
NH_2
a-Amino Acid
$+4H$
$-4H$
2R-C-COOH
NH
a-Imino Acid
N_2
Molecular
Nitrogen

acid formation either from hydroxylamine *via* the oxime, or from ammonia *via* the a-imino acid, appears possible and there is no reason to believe the two mechanisms are mutually exclusive. Under particular conditions contributing to the formation of large quantities of oxaloacetic acid, the fixed nitrogen may be predominately arrested at the hydroxylamine stage with the formation of oximinosuccinic acid which on reduction yields aspartic acid (Wood *et al.* (58)). On the other hand, in the absence of appreciable quantities of oxaloacetic acid, the reduction may largely proceed to ammonia which, after combination with a-ketoglutaric acid, 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 hydroxylamine hypothesis. On the other hand, the studies with isotopic nitrogen by Burris and Wilson, and the studies presented here, are strictly compatible with the ammonia hypothesis.

Evidence to date concerning the pathway of denitrification is extremely limited. The highly questionable data of Allen and Van Neil (47) implicating nitramide as an intermediate has been discussed previously. Marshall, Dishburger and MacVicar (23) in studies with *Pseudomonas fluorescens* and *Pseudomonas denitrificans* have shown that ammonia is apparently not an intermediate in the denitrification process. This observation is also supported by the data presented in this thesis.

SUMMARY

Nitrogen changes in Pseudomonas fluorescens and Bacillus subtilis adopted to nitrate nutrition have been followed by quantitative procedures and by utilization of the stable isotope of nitrogen, N^{15}.

In aerated culture of Pseudomonas fluorescens containing nitrate as the sole source of nitrogen, nitrate disappeared rapidly from the culture despite vigorous aeration. Nitrogen balance studies, using both quantitative and isotopic techniques, indicate that the organism, adapted to nitrate nutrition by long serial subculture in nitrate medium, reduced nitrate to a gaseous form of nitrogen and to some uncharacterized substance, neither nitrite nor ammonia, which was excreted into the medium. When $KN^{15}O_3$ and $(NH_4)_2SO_4$ were added simultaneously 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 ammonia decreased at a somewhat slower rate. Despite vigorous aeration, and despite the presence of ammonia, nearly complete denitrification of the added nitrate occurred. The presence of ammonia, however, prevented the excretion of the uncharacterized substance(s) into the medium, and blocked the utilization of nitrate for synthesis of cellular constituents.

In aerated cultures of Bacillus subtilis 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 nitrate occurred. Quantitative measurements and isotopic techniques show that nitrate was reduced to free ammonia which was then utilized for cellular synthesis. No evidence

was obtained for the occurrance of hydroxylamine, oximino-nitrogen, hydroxamic acids, or hyponitrous acid in the reduction pathway. Paper chromatograms of the medium showed the presence of small amounts of glutamic acid throughout the sampling period (6 hours). No increase in the glutamic acid concentration was observed

These studies support the view that (1) denitrification is not exclusively an anaerobic process; (2) amino acid synthesis from nitrate and denitrification can occur simultaneously; (3) ammonia is assimilated in preference to nitrate for the synthesis of cellular constituents; (4) ammonia blocks the utilization of nitrate for synthesis of cellular constituents (in Pseudomonas fluorescens); (5) nitrate reduction and denitrification proceed via two alternative mechanisms which are not mutually exclusive, and only one of which involves ammonia; (6) ammonia is a key intermediate in biological nitrate reduction.

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THESIS TITLE: THE BIOCHEMICAL MECHANISM OF NITRATE REDUCTION IN BACTERIA

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Title of Study: The Biochemical Mechanism of Nitrate Reduction in Bacteria.

Number of Pages in Study: 43 Candidate for What Degree: Master of Science

Under Direction of What Department: Agricultural Chemistry Research

Scope of Study: Since the observation that nitrate in the soil is reduced to nitrite, and since the recognition of the bacterial nature of this process, a large number of investigators have studied the various aspects of the process of biological nitrate reduction. This interest is doubtless due to its importance in the nitrogen cycle of nature and to its agronomic import. Despite the large number of extensive investigations, knowledge of the chemical reactions underlying the process of denitrification and nitrate assimilation by bacteria is very incomplete. This study was undertaken to elucidate the chemical reactions underlying these processes.

Findings and Conclusions: The studies conducted support the view that (1) denitrification is not exclusively an anaerobic process; (2) amino acid synthesis from nitrate and denitrification can occur simultaneously; (3) ammonia is assimilated in preference to nitrate for the synthesis of cellular constituents; (4) ammonia blocks the utilization of nitrate for synthesis of cellular constituents (in Pseudomonas fluorescens); (5) nitrate reduction and denitrification proceed via two alternative mechanisms which are not mutually exclusive, and only one of which involves ammonia; (6) ammonia is a key intermediate in biological nitrate reduction.

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