STUDIES ON NITRATE REDUCTION

BY PSEUDOMONAS FLUORESCENS

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By

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PREFACE

Nitrogen losses from the soil due to conversion of nitrates to gaseous nitrogen by soil microorganisms has long been a problem of agronomic importance. In Oklahoma and the southwest the disposal of wheat straw in such a manner that will at the same time reduce the effect of wind and water erosion is a critical problem. During the conversion of material of a wide carbon: nitrogen ratio to the more stable soil organic matter, it has been suggested that denitrification may occur even under conditions of normal oxygen supply.

The experiments reported in this thesis were conducted with one of the most widely distributed nitrate-reducing organisms 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 large amounts of readily decomposable organic matter.

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INTRODUCTION

In 1862 Goppelsröder (9) observed that nitrate in the soil was reduced to nitrite. Shoenbein (25) in 1868 and Meusel (19) in 1875 recognized the bacterial nature of the process. Since Gayon and Dupetit (8) performed their classical experiments in 1882, a 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 the nitrogen cycle of nature and to its agronomic importance. Despite the large number of extensive investigations, the present-day state of knowledge of the chemical reactions underlying the process of denitrification and nitrate assimilation by bacteria is very incomplete.

The Influence of Oxygen on Nitrate and Nitrite Reduction.

The influence of oxygen on the process has been of considerable interest from the beginning. Weissenberg (29) 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 complete absence of oxygen. In the medium exposed to air, nitrate was reduced only as far as nitrite, while under anaerobic conditions complete denitrification occurred. Lloyd and Cranston (14) measured the gas exchange that occurred when denitrifying cultures were in air and 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 reduced aerobically, although some nitrogen was lost from the medium even under the most aerobic conditions. Seiser and Walz (24) observed a considerable nitrogen loss from nitrate containing cultures of Pseudomonas putida

exposed to air, though this was less than under anaerobic conditions.

Stickland (26) in 1931 determined the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by Escherichia coli. He observed that very small partial pressures of oxygen caused marked inhibition of the process. Increasing the nitrate concentration tenfold did not modify the results. It was concluded therefore, that the inhibition was non-competitive.

Meiklejohn (18) in 1940 described two unidentified species of Pseudomonas which reduced nitrate to nitrite and nitrogen gas in a simple medium containing adequate amounts of a suitable organic compound. She observed that both species would denitrify under aerobic or anaerobic conditions and that at C/N quotient = 10, better growth was obtained under aerobic than under anaerobic conditions. Her experiments were similar to those of Seiser and Walz (25) and the results obtained were in agreement with them. Similarly previous studies in this laboratory using N^{15} tracer techniques by Bowers, D. G. (1) have indicated that in solution cultures of heterogeneous 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.

Van Olden (27) in 1940 observed that the ability of washed bacteria to produce nitrogen from nitrate was dependent upon their previous history and concluded that "nitrate reductase" is an adaptive enzyme in the sense of Karstrom (10) which develops under conditions suitable for denitrification.

Korsakova (11) recently reported that when the organic carbon source supplied was 5 to 10 times in excess of that required to reduce the available nitrate, the reduction of nitrate by Achromobacter siccum and Pseudomonas aeruginosa was not affected by aeration or partial pressure of oxygen. It

was concluded that reduction of nitrate as a fermentation process does not require anaerobic conditions because the energy of oxidation of organic matter needed for respiration exceeds the energy required for denitrification. Under anaerobic conditions denitrification, he concluded, becomes a physiological necessity.

Lemoigne (13) in 1946 found that growth of Bacillus megatherium on media containing nitrate as the sole source of nitrogen, did not take place after innoculation if the organism was cultured in pure oxygen. When oxygen replaced air at the end of a six hour incubation period, the organism still failed to grow. After 24 hours incubation in air, however, the replacement of air by oxygen did not arrest growth. When the organism was cultivated on media with complex peptone in an oxygen atmosphere the oxygen had no effect. Thus it was concluded that oxygen arrests the mechanism involved in nitrate assimilation, a conclusion in agreement with the findings of Weissenberg (29).

Sacks and Barker (22), using an organism isolated from soil after enrichment in a succinate-nitrate medium and identified as Pseudomonas denitrificans, have recently reported, on the basis of quantitative data obtained from the use of manometric techniques, that oxygen affects nitrate reduction and denitrification in two ways: it suppresses nitrate and nitrite-reducing enzyme systems and when these systems are present it decreases the rate of the reduction processes by interferring with the enzymes. The formation of enzymes responsible for nitrite reduction are suppressed to a greater extent than those responsible for reduction of nitrate to nitrite. At oxygen tensions of about 5 per cent, nitrate was reduced only as far as nitrite, which accumulated in the medium. At lower oxygen tensions an abnormally large accumulation of nitrite also occurred but this was accompanied by denitrification. In this range of oxygen tensions, the authors conclude, both the accumulation

of nitrite and the rate of denitrification are greatly affected by relatively small changes in the oxygen level. On the basis of these data the experiments of Seiser and Walz (24), Meiklejohn (18), and Korsakova (11), are criticized. It is claimed that these investigators employed methods of aeration which are 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. In some cases the authors failed to describe their experimental conditions in sufficient detail to determine the degree of aeration. Inadequate aeration, they claim, is the reason for the contradictory results obtained by these other investigators who used different methods of aeration than themselves.

Lewis and Hinshelwood (15) investigated the growth of Coliform bacteria in media containing nitrate and nitrite. In one of their experiments it was observed that with lowered oxygen supply the reduction of nitrate by Bacterium lactis aerogenes increased. Sudden aeration of an anaerobically growing culture led to a catastropic drop in the rates of reduction of nitrate and nitrite. This, they postulated, may be due to a cell metabolite denoted XH_2, which is readily oxidized either by the oxygen of the air or by other suitable oxidizing agents present which compete for available hydrogen. A decrease in the concentration of the more powerful oxidizing agent could tend to allow an increase in the concentration of XH_2 to a point where the rate of reduction of less powerful oxidizing agents becomes appreciable. Thus, if the concentration of oxygen were reduced, the rate of reduction of nitrate may increase as was found. The upward trend of nitrate reduction during growth was explained by the count becoming so large that the rate of oxygen consumption exceeded the rate of supply to the medium. There would than be a decrease in

the rate of hydrogen transfer with consequent accumulation of XH_2, resulting in 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. The growth rate appeared to be determined by the rate of nitrite reduction and was the same in all experiments conducted on this phase of the work. It may be inferred, therefore, that the rate of nitrite reduction is not affected in the same way as nitrate reduction.

The Role of Ammonia in Nitrate and Nitrite Reduction.

The influence of ammonia on the process of nitrate reduction and assimilation and its possible role as an intermediate in the reduction process is of much interest. It is probable that the processes of assimilation of combined nitrogen as nitrate and the assimilation of molecular nitrogen are related by a common intermediate. Therefore, Clemo and Swan (5) point out in a recent report that for an understanding of the assimilation of nitrogen, one of the important questions is whether nitrate is reduced to ammonia, or whether ammonia is the central nitrogenous compound which is produced from all nitrogen sources, including molecular nitrogen, before amino acid synthesis. One observation against this thesis is the formation of oxime nitrogen during nitrate assimilation by Torula, demonstrated by Virtanen (28). Wood et. al. (30) recently demonstrated that Azotobacter readily uses oximinosuccinic and oximinoglutaric acids as sources of nitrogen in the absence of atmospheric nitrogen or other sources. This would lend convincing support to the theory offered by Burstrom (4) from studies on the nitrate nutrition of plants. However, Novak and Wilson (20) have recently reported that the nitrogen in the oximes of pyruvic, oxaloacetic and a- ketoglutaric acids is not available to Azotobacter vinelandii. The reason for this direct contradiction may have

been due to experimental conditions such as pH of the medium or to reasons not made apparent.

Csaky (6) recently studied the effect of pH and certain inhibitors on ammonia and nitrate assimilation by Azotobacter. He observed that growth on ammonia was greatly reduced at pH values above and below 6.8 while that on nitrate was nearly independent of pH (5 - 7.2). It was concluded that assimilation of nitrate does not necessarily involve reduction to ammonia. This appears to contradict the report of Korsakova (12) that nitrate reduction by several species of faculative and obligatory anaerobes studied was influenced by pH and composition of the medium.

In recent years the technique of applying the heavy, stable isotope of nitrogen, N^{15}, to label physiologically important compounds in metabolic studies has clearly demonstrated its usefulness in attacking problems which have heretofore failed to lend themselves to classical methods of investigation. Burris and Wilson (3) in 1946 supplied NH_4^+ labeled with N^{15} to an aerated culture of Azotobacter vinelandii grown on N_2; the culture immediately stopped fixing nitrogen and used NH_4^+ as its sole source of nitrogen. When NO_3^- labeled with N^{15} was supplied under the same conditions, no N^{15} was found in the cells for thirty minutes in contrast to one minute when $N^{15}H_4^+$ was added. The uptake of ammonia was at a constant rate, whereas the rate of NO_3^- assimilation increased with time. In further experiments aerated cultures of Azotobacter vinelandii previously grown on media with N_2 or with NO_3^- as a source of nitrogen assimilated NH_4^+ in preference to NO_3^- when supplied $N^{15}H_4NO_3$. If $N^{15}H_4NO_3$ was added at the time of innoculation to cultures previously grown on NO_3^- the growing organisms used NH_4^+ preferentially. Upon exhaustion of NH_4^+ utilization of NO_3^- was resumed. Addition of $N^{15}H_4NO_3$ to heavy aerated cultures

fixing molecular nitrogen resulted in an immediate uptake of NH_4^+, but NO_3^- was not utilized until the NH_4^+ approached exhaustion. Heavy aerated cultures utilizing NO_3^- as a sole source of nitrogen immediately shifted to utilization of NH_4^+ after addition of $N^{15}H_4NO_3$. Resumption of NO_3^- utilization occurred while approximately two-fifths of the added NH_4^+ still remained but the rate of assimilation appeared to be slower than that of NH_4^+. It is apparent from these data that Azotobacter vinelandii grown either on N_2 or NO_3^- has preformed enzyme systems capable of utilizing NH_4^+ immediately and to the exclusion of other nitrogenous compounds. To use NO_3^- the cells must first adapt themselves to it, which demonstrates the adaptive nature of "nitrate reductase".

Lewis and Hinshelwood (15) added ammonium sulfate to an actively growing culture of Bacterium lactis aerogenes in both nitrate and nitrite, and observed a rapid fall in the rate of nitrate and nitrite reduction to a point of near complete inhibition. The reduction process did not restart until the amount of ammonia remaining was negligible. If addition was made before growth was started, little or no reduction of nitrate or nitrite occurred. Imperfect aeration reduced the inhibitory action of ammonia on the nitrate reduction process and the concentration of nitrite built up in the medium. If both nitrite and nitrate were present when all of the ammonia was used up, growth on nitrite restarted almost immediately, but a lag occurred before nitrate reduction again returned to its optimum rate. The delayed recovery suggested that during growth in ammonia, some substance was formed which inhibited the reduction of nitrate to nitrite which was removed during subsequent growth. It was suggested that this inhibitory substance used for growth on both nitrate and ammonia was a normal cell metabolite since it was removed very rapidly during growth on nitrate when added in the form of filtrate from a culture

grown on ammonia. It was inferred from the inhibition of growth by ammonia that the ratio of X/XH_2, the oxidized and reduced forms of the metabolite previously discussed, was high and under these conditions nitrate and nitrite were not reduced. As the concentration of ammonia became negligible and dehydrogenation mechanisms continued to function; the O-R potential of the medium became more reducing, allowing reduction of nitrate and nitrite to become possible once more. In these terms, the authors concluded, the inhibition and recovery process described were generally understandable. This was borne out in another experiment (17) in which the reducing power of the organism was measured by means of methylene blue. It was shown that when growth in nitrate medium was arrested by ammonia, the reducing power decreased.

In another report by these same authors (16) a steady concentration of ammonia of a low order of magnitude (1 mg./liter) accumulated in cultures of Bacterium lactis aerogenes on nitrate nutrition. From quantitative relations to growth rates the authors stated this is of the order to be expected if nitrate utilization is by way of ammonia. However, it was stated that the results were not numerically precise enough to provide positive evidence for this view.

The evidence summarized above strongly indicates that ammonia inhibits the processes of nitrate and nitrite reduction and favors the postulate that nitrate is reduced to ammonia before amino acid synthesis.

EXPERIMENTAL

The organism used in these studies was a strain of Pseudomonas fluorescens from the stock collection of the Department of Agricultural Bacteriology, University of Wisconsin.[1] It had previously been cultured on nutrient agar slants. Due to its prolonged culture on complex peptone it was necessary to train the organisms to growth in a simple medium having the following composition in grams per liter: KH_2PO_4, 3.96; $NaNO_3$, 1.0; glucose, 20.0; $MgSO_4 \cdot 7\ H_2O$, 0.4; $FeSO_4$, 0.02; Na Cl, 0.02; $MnSO_4$, 0.08. In addition to the above components 0.1 mg. of biotin was added, and the solution brought to a pH of approximately 6.8 with NaOH using bromthymol blue as an indicator. A rather heavy precipitate formed upon autoclaving. This did not interfere with the experiments other than to make turbidimetric measurements of growth impractical. Training was accomplished by serial subculture in the nitrate medium. The organism was cultured in 50 ml. Erhlenmeyer flasks containing 20 ml. of the medium, 1 ml. of the cell suspension being used as an innoculum in each case. Transfers were normally made every 48 hours except when a large culture was to be innoculated. When this was the case, the organism was subcultured every 24 hours for three or four days prior to the experiment. Ten ml. of cell suspensions obtained in this way per liter of media was used as innoculum in all experiments.

In the experiments in which the culture was aerated, 1 liter of the nitrate medium was placed in a 2 liter filter flask fitted with a fritted

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[1] The culture was obtained through the courtesy of Dr. C. M. Gilmour of the Department of Bacteriology, Oklahoma A. and M. College.

glass gas dispersion tube. The air supply was obtained from an ordinary air compressor and passed through a tightly packed cotton filter before it entered the medium. A stream of air sufficient to churn the medium as well as disperse minute bubbles throughout was passed through the medium during incubation at room temperature. Disappearance of nitrate was followed semi-quantitatively from the time of innoculation by withdrawing 1 ml. samples, and observing the yellow color developed upon addition of brucine sulfate. This method of nitrate determination will be described in detail later in this paper. When little or no color development occurred, indicating that the original nitrate in the medium was virtually exhausted, the culture was immediately sampled. Usually 50 mg. of N was then added in the form of $N^{15}H_4NO_3$ containing 34.0 atom $\%N^{15}$ excess and the culture again sampled. This was designated as the sample for zero time and 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 0.8 N 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 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 residue of packed cells was transferred with a stream of distilled water from a wash bottle to semimicro Kjeldahl flasks, made distintly basis (pH 8-9) with N/14 NaOH and boiled for at least five minutes to remove occluded ammonia. Cellular nitrogen was then determined

by a semi-micro modification of the Kjeldahl procedure.

Analysis for ammonia was made on 50 ml. aliquots of the supernatant after dilution to 250 ml. The sample was rendered strongly alkaline and aerated into 5 ml. 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. The intensity of the color developed was read on an Evelyn photo-electric colorimeter employing a 440 mu filter.

Analysis for nitrate was made by a modification of the method developed by Gad (7). This method was decided upon due to the desirability of a rapid colorimetric method for nitrate determination in a study such as this. Colorimetric methods for the analysis of nitrates in the presence of carbohydrate material are generally unsatisfactory due to the charring which occurs in the presence of the concentrated H_2SO_4 required in the determination. Several methods were tried but none proved satisfactory in the presence of the rather high concentration of glucose present in the samples. The method employed here can be applied with reasonable accuracy in the presence of 1 mg. per ml. of glucose. NO_2^- and N_2O_3 interfere but for the results desired in this study this was deemed irrelevant. Recoveries on known samples have been obtained consistently with an accuracy of about 5%. The procedure for the analyses was as follows: 10 ml. of a solution containing 64% H_2SO_4 and 1% HCl was added to 2 ml. aliquots of the cell-free supernatant. The excess HCl is to prevent interference of chlorides in the sample. One ml. of a solution containing 1 gram of brucine sulfate dissolved in 100 ml. glacial acetic acid was then added, and the tubes placed in a boiling water bath for 20 minutes. At the end of this period, the tubes were

cooled immediately and the intensity of yellow color developed was determined photoelectrically at 440 mu using the Evelyn instrument. Standard solutions were developed periodically with the samples, but deviations from the original standard curve prepared were found to be rare.

To further test on the accuracy of the method, it was checked by the aeration procedure previously described using Devarda's Alloy to reduce the nitrates to ammonia. A comparison of the results obtained in one trial are presented in Table I.

TABLE I

Comparison of Values Obtained for
Analysis of Nitrate by the Brucine
Method and by the Use of Devarda's Alloy

NO_3 N Brucine Method	NO_3 N Devarda Method
26.5	26.0
25.8	22.6
20.4	18.4
21.8	18.4
21.2	16.4
17.3	14.0
11.9	11.0
1.9	3.0
0.4	0.4

Values given in micrograms N per ml.

N added as $N^{15}H_4NO_3$

Values obtained by Devarda's reduction were generally lower than those obtained by the brucine method. The maximum variation obtained was about 10%. This would appear to be considerable but consideration of the small quantities of nitrogen being determined would justify the values obtained

by Devarda reduction being low due to the possibilities of incomplete reduction and mechanical losses of ammonia in the distillation apparatus employed.

Non-aerated cultures were grown by innoculating the bacteria into 1 liter of the nitrate medium contained in a 4 liter Erhlenmeyer flask. The flask was thoroughly agitated prior to sampling to break up clumps of cells and facilitate the removal of a homogeneous sample as possible. Samples were obtained and given the same treatment as previously described for aerated cultures.

Total nitrogen was run on the cell-free supernatant in experiment 5 by the semi-micro Kjeldahl procedure. The nitrate present was reduced by a semi-micro modification of the iron procedure as developed by Pucher and Vickery et al. (21) prior to digestion.

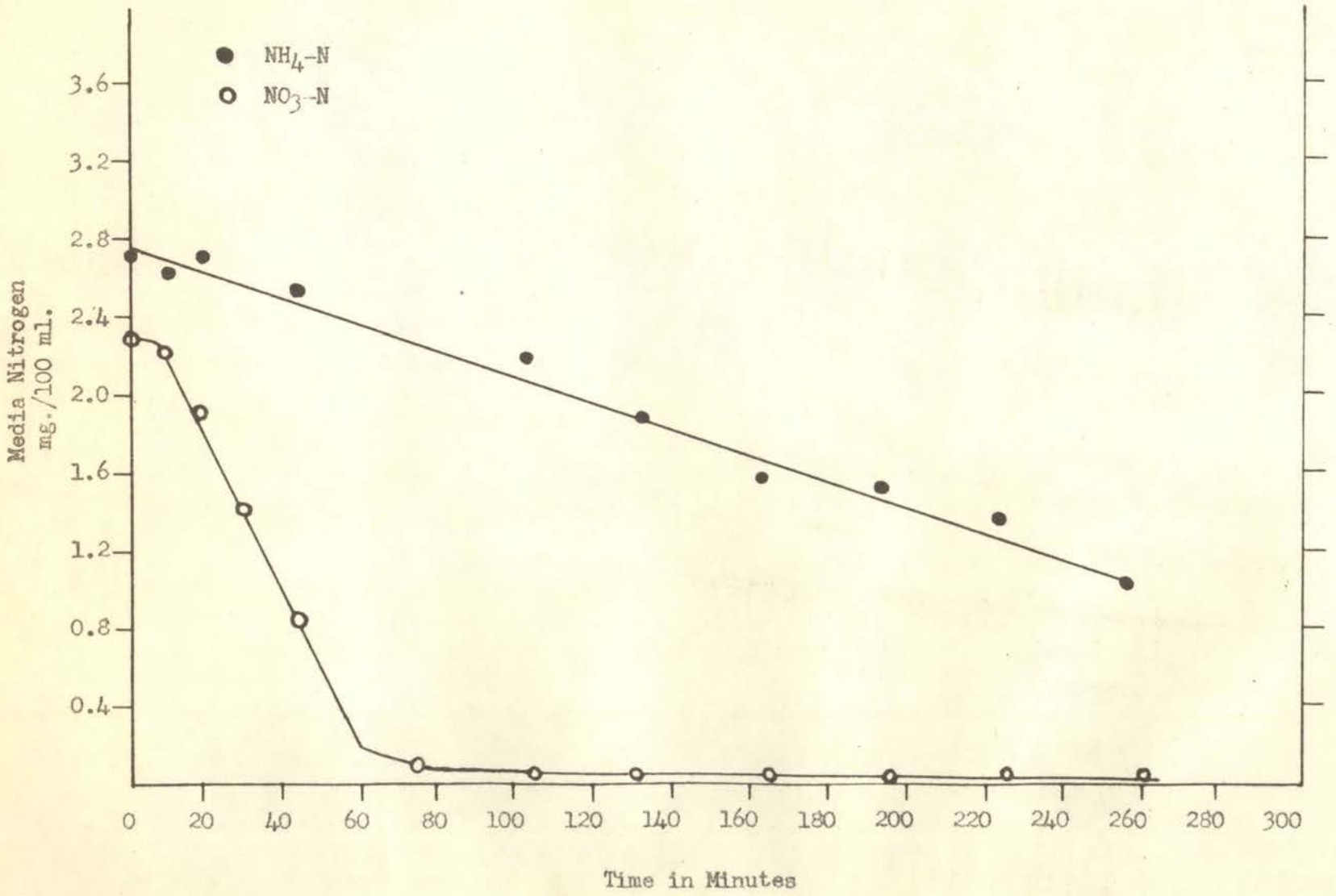
Kjeldahl titration mixtures from the cellular nitrogen analysis were prepared for conversion as suggested by Schoenheimer (23) and the conversion apparatus for N^{15} used was similar to that described by him. N^{15} analyses were made on a Westinghouse mass spectrometer under the supervision of Mr. Freeman A. Tatum of the Department of Electrical Engineering, A. and M. College of Texas.

RESULTS AND DISCUSSION

Preliminary studies on the utilization of nitrate as a sole source of nitrogen by cultures of Pseudomonas fluorescens grown in shallow layers of medium indicated that nitrate nitrogen disappeared from the medium at a very rapid rate between 24 and 36 hours. In all cultures tested the concentration of NO_3^- remaining at the end of 36 hours was insignificant. If NH_4^+ and NO_3^- were supplied to an actively growing culture on nitrate nutrition at the time when the original supply of NO_3^- was depleted, the organism should preferentially use NH_4^+ if ammonia is an intermediate in the process of nitrate assimilation. Nitrate reduction should be halted until the level of ammonia reached the steady state attained in its role as an intermediate. To test this postulate, 1000 ml. of nitrate medium was innoculated with a 36 hour culture of Pseudomonas fluorescens which had been serial subcultured on nitrate media for a period of four months. At the end of 36 hours the original nitrate in the medium was exhausted and 50 mg. of N as NH_4NO_3 was added. The culture was sampled over a period of six hours and analyses for NH_4^+ and NO_3^- in the medium at various periods of time were made. Figure I shows that NO_3^- continued to disappear from the medium at a very rapid rate. NH_4^+ began to disappear immediately but at a much slower rate. This experiment was repeated several times and in each case the results were similar. The difference between the initial concentrations of NH_4-N and NO_3-N can be partially accounted for on the basis of residual NH_4-N in the medium prior to addition of the NH_4NO_3. If this is considered, the initial concentration of both ions were essentially equal to the theoretical amounts added. The slight lag in the nitrate curve apparently was due to the time of addition of the NH_4NO_3. In experiments in which the NH_4NO_3 was added within 30

Figure I

Typical Media Nitrogen Changes
in a Non-Aerated Culture
of Pseudomonas fluorescens



minutes following exhaustion of the original nitrogen in the medium this lag was not observed. Previous evidence from the literature strongly indicates that both NH_4^+ and oxygen arrest nitrate reduction. However, the results obtained in Experiment 1 strongly indicated that in cultures of Pseudomonas fluorescens in shallow layers of medium exposed to air and in the presence of NH_4^+ continue to remove nitrate at a rapid rate.

In the absence of data relating the disappearance of NH_4^+ and NO_3^- to other nitrogen changes in the culture it would be difficult to postulate the significance of the results in Experiment 1. Therefore, it was decided to employ N^{15} tracer techniques to obtain information concerning which N source was exclusively used for synthesis or if both were being utilized at the same time. Even though the organism was grown in shallow layers of medium exposed to air in Experiment 1, the existence of partially anaerobic conditions in parts of the medium due to the slow rate of diffusion of oxygen was deemed possible. If this were the case, the nitrate disappearance might be partially explained on the basis of nitrate being required as a source of oxygen for growth on ammonia. To eliminate this possibility, Experiment 2 was conducted under identical conditions except that the culture was vigorously aerated. The culture was incubated at room temperature until the original NO_3-N was exhausted. The time required for exhaustion of the nitrate dropped markedly, reaching a very low level at the end of 21 hours. At this time 50 mg. of N as $N^{15}H_4NO_3$ containing 34 atom % N^{15} excess was added. Aliquots of 25 ml. were taken at definite time intervals as previously described. Analysis was made for NH_4^+ and NO_3^- in the cell-free medium and cellular nitrogen was determined by semi-micro Kjeldahl procedure. The Kjeldahl titration mixtures were prepared for N^{15} analysis but

due to operational failure of the mass spectrometer normally used for analysis of the samples, a complete set of N^{15} data is not available at this time. The results of Experiment 2, (Figure II), show that under conditions of vigorous aeration NO_3-N disappeared from the medium at approximately the same rate as in the non-aerated culture, reaching an insignificant level in about 60 minutes from the time of addition of the $N^{15}H_4NO_3$. The rate of disappearance of NH_4-N, however, was enhanced considerably and nearly paralleled the increase in cellular nitrogen. The ratio of NH_4-N disappearance to cellular nitrogen increase was so near unity that one might assume that all of the cellular nitrogen was being derived from NH_4-N. At this point, the availability of the N^{15} data which is forthcoming would be of great value in facilitating an explanation. The one sample on which an anlysis for N^{15} was obtained was taken at 250 minutes. This sample contained 9.3 atom % N^{15} excess. At this time the values for cellular nitrogen was 5.6 mg. which represents an increase of 2.5 mg. over the value for zero time. During the same time interval 2.52 mg. of added NH_4-N containing 34 atom % N^{15} excess disappeared from the medium. This indicates that roughly 27% of the nitrogen present in the cells at 250 minutes or 60% of the cellular nitrogen increase over this period was derived from added NH_4-N. On the basis of the increase in cellular nitrogen over this period of time, these calculations suggest that NH_4-N was being used to a greater extent than NO_3-N, but not exclusively as the ratio of NH_4-N disappearance to cellular nitrogen increase would lead one to assume. It must be pointed out, however, that in the absence of further isotope data this cannot be offered as a reliable conclusion.

In Experiment 3, $KN^{15}O_3$ containing 31.4 atom % N^{15} excess equivalent to 15 mg. of nitrogen and 25 mg. of N as $(NH_4)_2SO_4$ were added under conditions similar to those described in Experiment 2. Figure III shows that nitrate

Figure II

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Pseudomonas fluoresens
5 mg./100 ml. of N as $N^{15}H_4NO_3$ Added at Zero Time

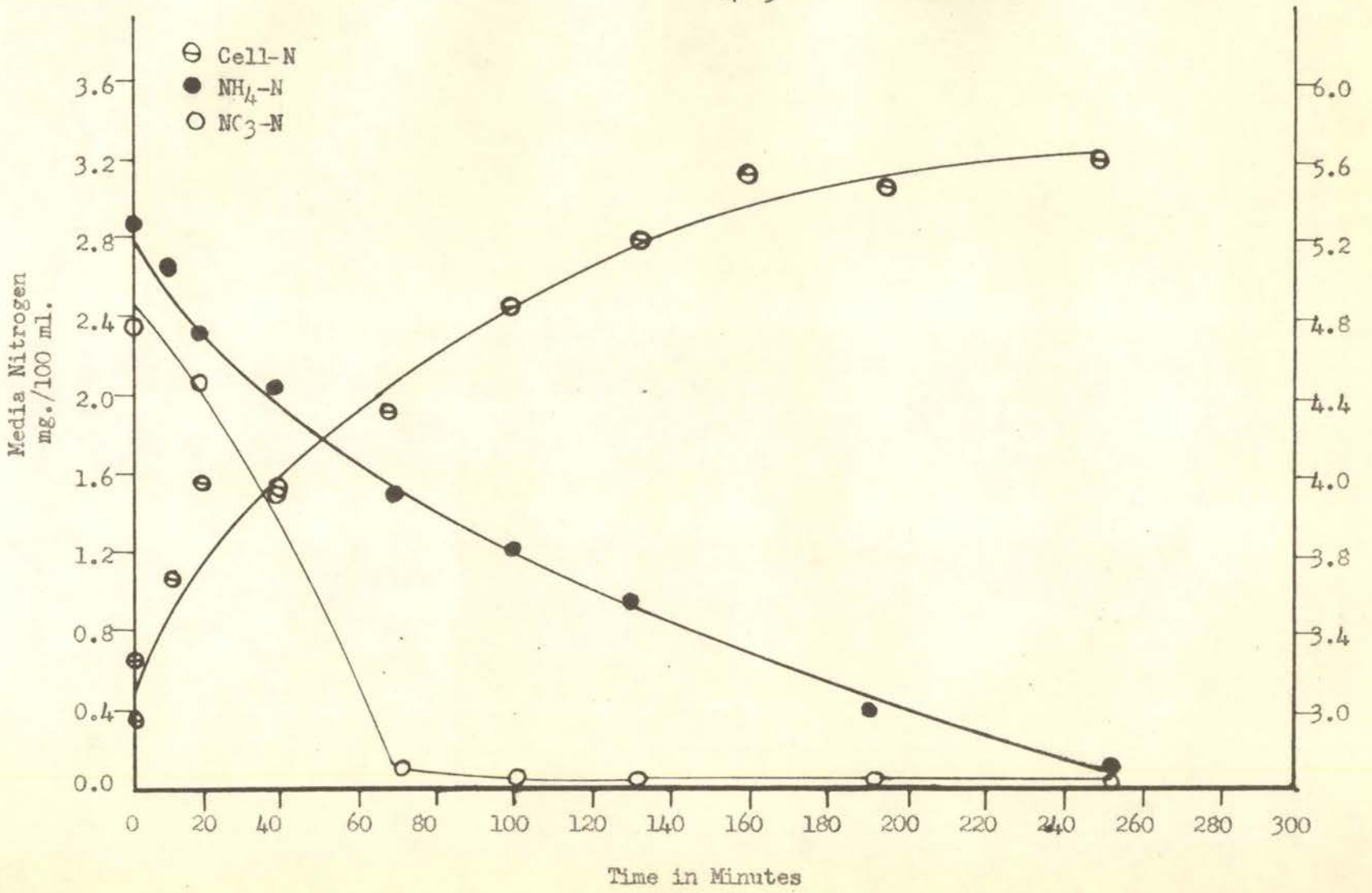
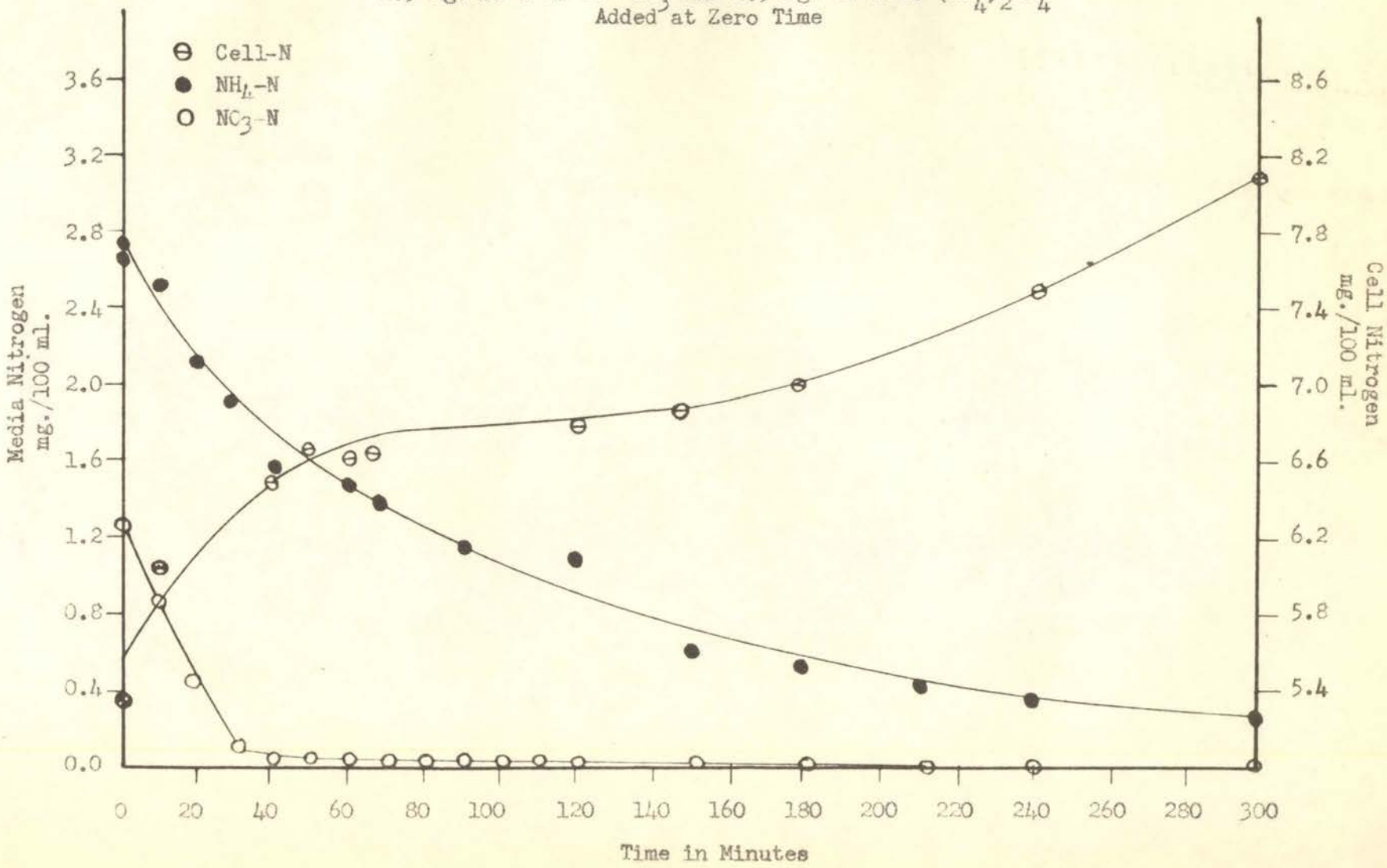


Figure III

Relation of Cellular Nitrogen to Nitrate and Ammonia Disappearance in an Aerated Culture of Pseudomonas fluorescens 1.5 mg. of N as $KN^{15}O_3$ and 2.5 mg. of N as $(NH_4)_2SO_4$ Added at Zero Time

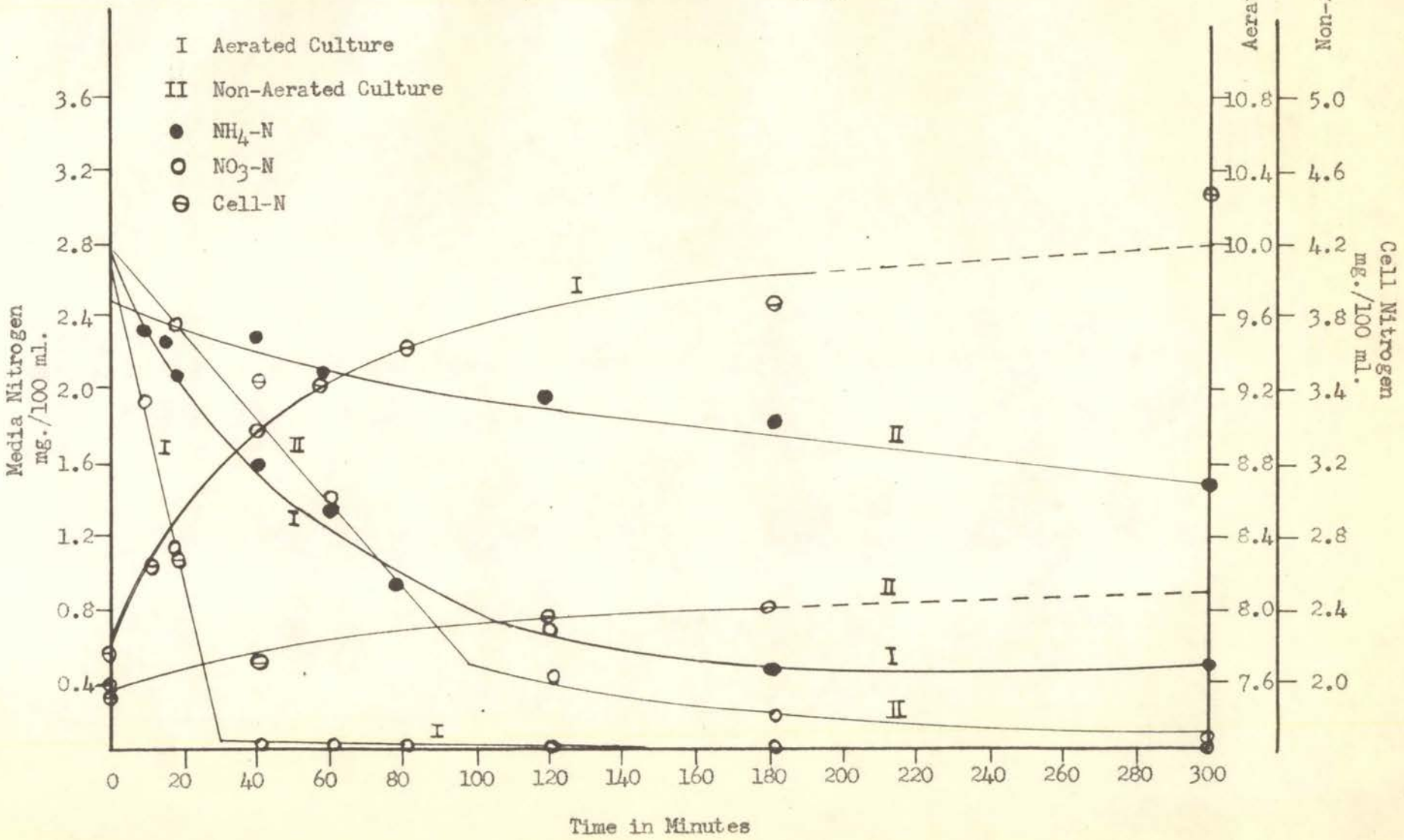


reduction continued in the presence of a large excess of NH_4^+ under conditions of vigorous aeration. The leveling off of the cell nitrogen curve for a period of time following disappearance of the NO_3-N in the medium could indicate that during the early stages of growth following addition of the NH_4^+ and NO_3^-, NO_3-N was being used preferentially for synthesis of cellular constituents. This would explain the results obtained from calculations of cell nitrogen derived from added NH_4-N in Experiment 2. Another implication is that the enzymes responsible for assimilation of NH_4-N are in low concentration following prolonged culture of the organism on a medium containing NO_3^- as a sole source of nitrogen. If this is true one might postulate that the assimilation of nitrate by Pseudomonas fluorescens grown under these conditions does not occur exclusively through ammonia. Here again isotope data, when it becomes available, should prove to be of great interest.

Experiment 4 was designed to compare the rates of disappearance of NO_3-N and NH_4-N to the rate of cellular synthesis in aerated and nonaerated cultures since it had been observed in previous experiments that growth was enhanced by aeration. Both cultures were of 1000 ml. Culture I was continuously aerated in a 2 liter filter flask. Culture II was contained in a 4 liter Erhlenmeyer flask. The cultures were innoculated in parallel from the same parent culture of Pseudomonas fluorescens previously maintained by serial subculture in the same medium and incubated at room temperature. Figure IV shows that growth in the aerated culture was roughly 4 times as great as in the non-aerated culture. Rates, approximated from linear portions of the curves, indicate that the NO_3-N disappearing per mg. of cellular nitrogen present during unit time was greater in the non-aerated culture. This indicates that the amount of molecular oxygen present had a definite effect on the rate of reduction of NO_3-N per cell. For example

Figure IV

Nitrogen Changes in Aerated and Non-Aerated
Cultures of Pseudomonas fluorescens inoculated in Parallel
5 mg. N/100 ml. added as $N^{15}H_4NO_3$ at Zero Time



during the period 0-20 minutes approximately 0.28 mg. of NO_3-N disappeared per mg. of cellular nitrogen present in the non-aerated culture. During the same period of time 0.19 mg. of NO_3-N disappeared per mg. of cellular nitrogen present in the aerated culture. The rate of ammonia disappearance on the other hand was enhanced somewhat in the aerated culture. The increased rate of reduction of nitrate in the non-aerated culture is readily understandable. On the other hand the increased utilization of NH_4^+ in the aerated culture could be explained on two bases: 1. Aeration stimulates the oxidative mechanisms involved in the utilization of carbohydrate material which supplies the energy necessary for synthesis of cellular protein, thus more NH_4-N is required to meet the demand. 2. A primary oxidative step occurs in the mechanism of NH_4-N assimilation. This second explanation is not attractive since the amino groups in the protein molecule are fully reduced and eventually oxidized compounds must be reduced to this status. Furthermore, experimental evidence for this pathway from the literature is meager (2).

Examination of the results from the experiments previously discussed showed the need for data relating the total nitrogen content of the cell-free medium to NO_3^- and NH_4^+ disappearance and the increase in cellular nitrogen. Previous results clearly showed that all of the N disappearing as NH_4^+ and NO_3^- was not appearing in the cells. Table 2 shows the relation of total nitrogen as determined by macro-Kjeldahl to the sum of the values obtained for NH_4-N, NO_3-N, and cellular -N in Experiment 4, Culture II (non-aerated). Consideration of this data shows that considerable nitrogen loss occurred from the time of innoculation. On the basis of total nitrogen added, including that originally present, the theoretical value for total nitrogen

was 21.6 mg./100 ml. This indicates that roughly half of the nitrogen available was lost through denitrification over a period of 50 hours, since the culture was 48 hours old at the time the concentration of NO_3^- in the medium became insignificant. It is known that all the nitrate is not measured by the Kjeldahl procedure unless first reduced. However, consideration of this would not account for the low value obtained for total nitrogen by the Kjeldahl procedure unless denitrification had occurred.

TABLE 2

The Relation of Total Nitrogen to NO_3-N, NH_4-N, and Cellular Nitrogen in a Non-aerated Culture of Pseudomonas fluorescens in mg./100 ml.

1	2	3	4	5	6
Time in min.	NO_3-N	NH_4-N	Cell-N	Sum 2, 3, 4	Total-N in Culture
0	2.80	2.44	1.92	7.16	8.0
20	2.35	2.24	2.05*	6.64	7.6
40	2.20	2.30	2.15	6.65	7.8
60	1.40	2.16	2.20*	5.76	7.4
120	0.47	2.06	2.31	4.84	7.0

* Read from curve.

It is also of interest to note that the sum of NO_3-N, NH_4-N, and cell nitrogen in the culture declined after the addition of NH_4NO_3 about twice as fast as did total nitrogen. This suggests that NO_3-N or NH_4-N or both are converted to some soluble form which remains in the medium after removal of the cells. This suggestion is further supported by the

observation that growth continued in nearly all experiments after the concentration of NO_3-N and NH_4-N became insignificant.

Experiment 5 was conducted on a 2000 ml. aerated culture in a 4 liter filter flask. Innoculation was made with 20 ml. of a 24 hour nitrate adapted culture of Pseudomonas fluorescens. One hundred mg. of N as $N^{15}H_4NO_3$ containing 34.0 atom % N^{15} excess was added 32 hours after innoculation and 50 ml. samples were withdrawn over a six hour period. In addition to the usual analyses total nitrogen on the cell-free medium was run by semimicro Kjeldahl procedure following iron reduction. The results are illustrated graphically in Figure V. However, Table 3 better illustrates the significance of the data. The results were essentially the same as those represented in Table 2 for a non-aerated culture. Denitrification occurred to almost the same degree as in the non-aerated culture. This may have been due to insufficient aeration of the larger volume of medium since growth was not as great as had been obtained in other aerated cultures. This however, is not believed to be relevant to the conclusions to be drawn from the data. Total nitrogen in the medium declined rapidly during the first 80 minutes and then began to level off. During this period of time NH_4-N and NO_3-N were being rapidly removed and cellular synthesis was occurring at its most rapid rate. At 120 minutes both the NO_3-N and NH_4-N had reached insignificant levels. The increase in cell nitrogen had began to decline but had not stopped. The level of nitrogen in the cell-free medium however, was still roughly one-third the value at zero time. Cellular nitrogen during this time showed an increase of 1.99 mg. which accounts for approximately 39% of the nitrogen in the NH_4-N and NO_3-N present at zero time. This indicates

Figure V

Relation of Total Nitrogen Content of the Cell-Free Media to Other Nitrogen Changes in an Aerated Culture of *Pseudomonas fluorescens*
5 mg./100 ml. of N as $N^{15}H_4NO_3$ added at Zero Time

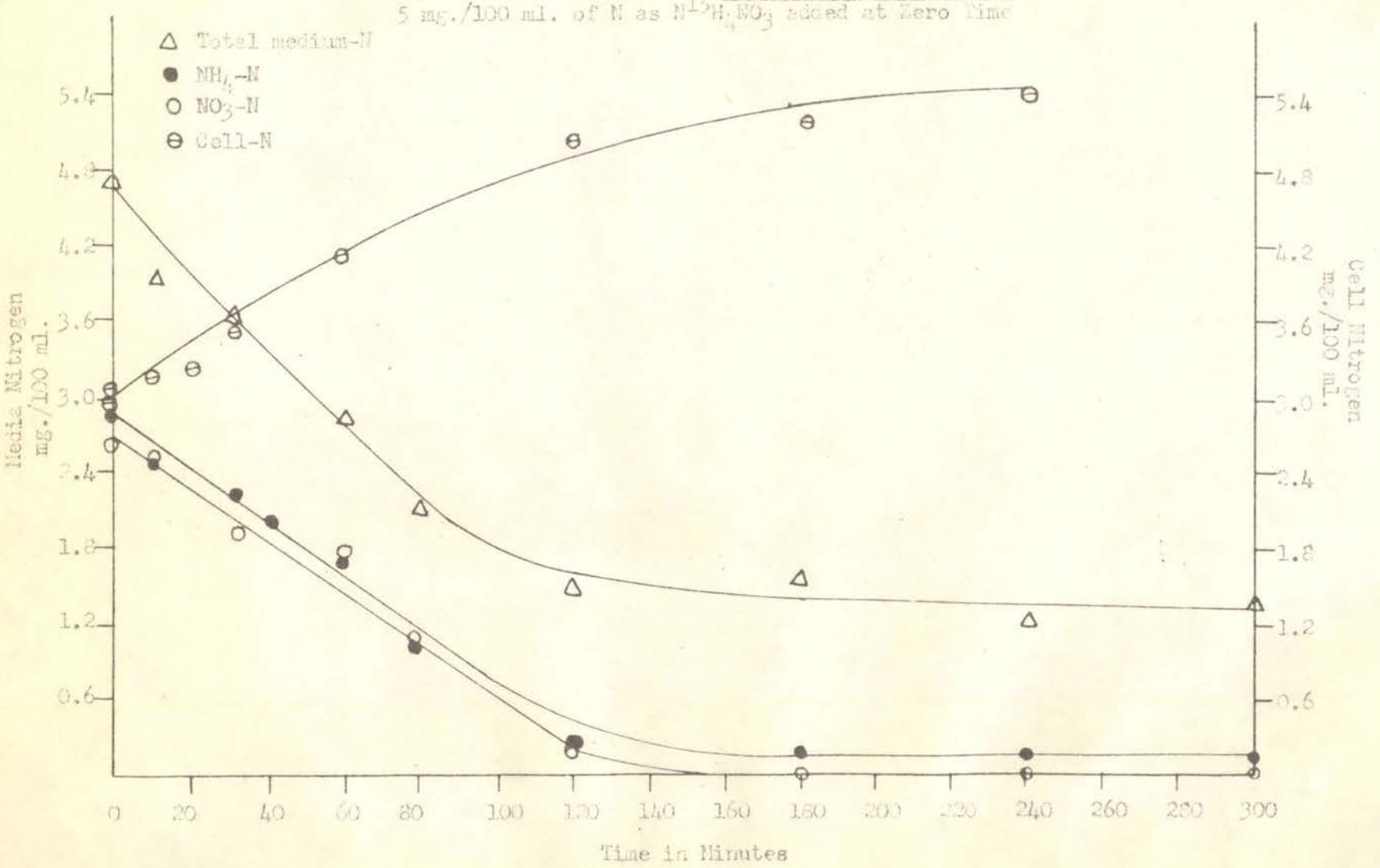


TABLE 3

The Relation of Total Nitrogen to NO_3-N, NH_4-N and Cellular- N in an Aerated Culture of Pseudomonas fluorescens in mg/100 ml.

1	2	3	4	5	6	7
Time in min.	NO_3-N	NH_4-N	Cell-N	Total-N in Medium	Sum NO_3-N NH_4-N	Total N in Medium Cell-N
0	2.65	2.82	3.01	4.70	5.47	7.71
10	2.58	2.50	3.15	4.35*	5.08	7.50
30	2.18	2.20	3.55	3.62	4.38	7.17
40	2.12	1.94	3.75	3.40*	4.06	7.15
60	1.73	1.64	4.15	2.80	3.37	6.95
80	1.19	1.04	4.40*	2.05	2.23	6.45
120	0.19	0.20	5.00	1.50	0.39	6.50
180	0.04	0.20	5.15	1.62	0.24	6.77
240	0.04	0.20	5.30	1.30	0.24	6.65
300	0.04	0.16	5.40*	1.42	0.20	6.82

* values obtained from curve

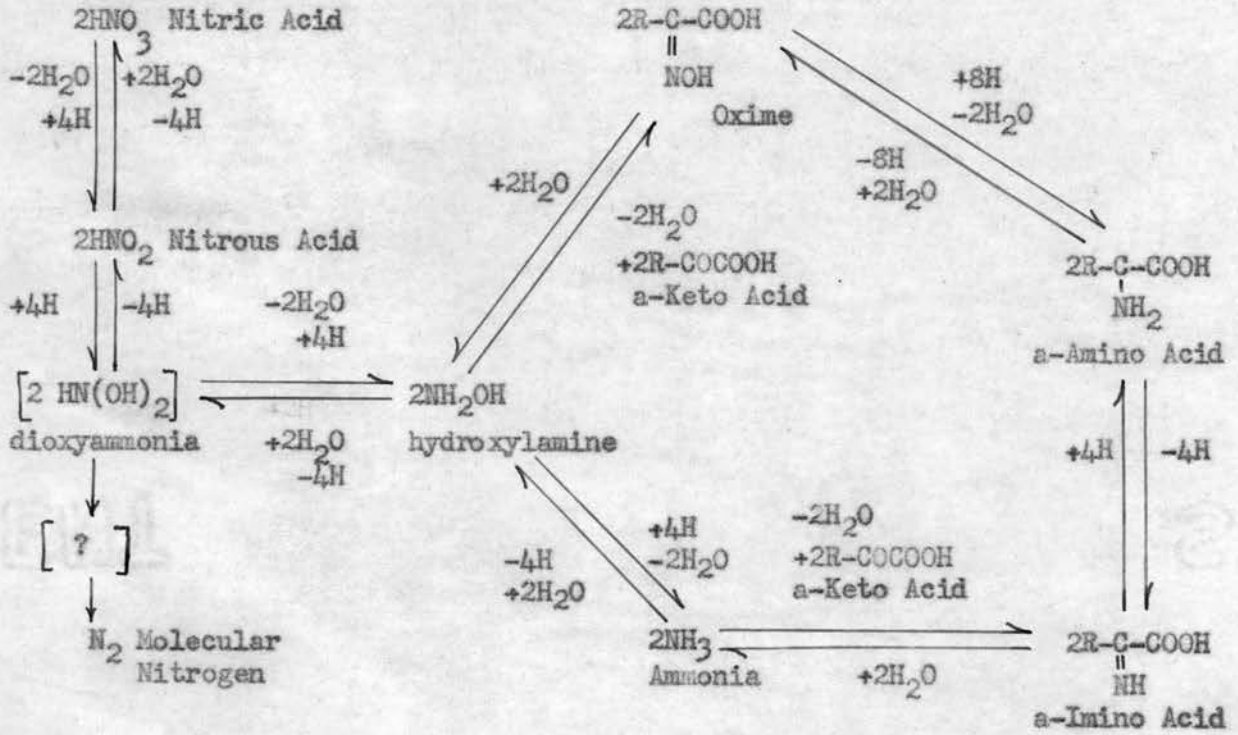
that about one-third of the nitrogen added at zero time was lost by denitrification during 120 minutes. If the nitrogen lost by denitrification came from nitrate, as it presumably would, it would represent nearly 60% of the NO_3-N added at zero time leaving approximately 40% for cellular synthesis. This corresponds well to the value obtained when the percent of cellular nitrogen derived from nitrate was calculated from the isotope analysis in Experiment 2 since growth was approximately equal in both experiments 2 and 5.

GENERAL DISCUSSION

From the data presented above it appears that in the early stages of growth Pseudomonas fluorescens, adapted to nitrate nutrition by long serial subculture in a nitrate medium, reduces nitrate under aerobic conditions to molecular nitrogen and to some other substance utilizable for cellular synthesis. This substance, a fraction of which appears to accumulate in the medium is neither nitrite nor ammonia since ammonia appears to be used simultaneously and nitrite was not observed in the medium in detectable quantities. This supports the view that amino acid synthesis from nitrate may occur by two alternative mechanisms which are not mutually exclusive. A proposed mechanism for the process is presented below.

Figure VI

Proposed Pathways of Nitrate
Assimilation and Denitrification



This mechanism was presented as a part of the theoretical pathway for nitrogen fixation in a recent review by Burris and Wilson (2) though it is not supported by them. The evidence for this pathway presented in this paper is far from conclusive but does strongly suggest that such a mechanism exists in Pseudomonas fluorescens grown as described in these experiments.

SUMMARY

Nitrogen changes in cultures of Pseudomonas fluorescens adapted to growth on NO_3^- as the sole source of nitrogen by prolonged serial subculture in a simple nitrate medium have been traced by quantitative procedures and by employing the heavy, stable isotope of nitrogen, N^{15}. Cellular nitrogen, NH_4-N, and NO_3-N were determined on all cultures periodically during growth. In some experiments total nitrogen in the culture or in the cell-free medium was determined.

In cultures of Pseudomonas fluorescens grown in shallow layers of medium exposed to air and containing NO_3^- as the sole source of nitrogen, NO_3^- disappeared from the medium at a rapid rate between 24 and 36 hours. When NH_4NO_3 was added to the cultures at the time the concentration of NO_3^- became insignificant, NO_3^- continued to disappear from the medium at a rapid rate. NH_4^+ began to disappear immediately but at a much slower rate.

In cultures vigorously aerated to eliminate the possibility of partially anaerobic conditions in some parts of the medium due to the slow rate of diffusion of oxygen, NO_3^- disappeared from the medium at approximately the same rate as in non-aerated cultures upon addition of $N^{15}H_4NO_3$. The rate of disappearance of NH_4^+ was enhanced considerably by aeration. On the basis of a single isotope analysis on a sample taken at 250 minutes following addition of $N^{15}H_4NO_3$ roughly 60% of the cellular nitrogen increase over this period came from NH_4^+. This implies that NH_4-N was being used preferentially but not exclusively to NO_3-N for cellular synthesis even though the ratio of NH_4-N decrease to cellular nitrogen increase is near unity.

In another experiment in which $KN^{15}O_3$ was added along with a large excess of NH_4^+ to a heavy aerated culture, there was some evidence that during the early stages of growth following the addition of NH_4^+ and NO_3^-, NO_3-N was being used preferentially for synthesis of cellular constituents. The results of Experiments 1 and 2 suggest that the enzymes responsible for the assimilation of NH_4-N were in low concentration following prolonged subculture of the organism on a medium containing NO_3^- as the sole source of nitrogen.

Rates of disappearance of NH_4-N and NO_3-N approximated from data obtained on aerated and non-aerated cultures innoculated in parallel from the same parent culture of Pseudomonas fluorescens showed that NO_3-N disappeared more rapidly from non-aerated cultures than from aerated cultures but that the reverse was true of NH_4-N. This was probably due to increased demand for nitrogen in vigorously aerated cultures due to stimulation of oxidative mechanisms involved in carbohydrate metabolism which supply the energy for protein synthesis, a view which was supported by the enhanced growth rate observed.

Nitrogen balance studies of both aerated and non-aerated cultures showed that roughly half of the nitrogen added during incubation periods of 36-48 hours was lost through denitrification.

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