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Title: Enzymatic Versus Nonenzymatic Denitrification

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W. B. Bollen

Denitrification is classically defined as the microbial reduction of nitrate and nitrite with the liberation of molecular nitrogen and, in some instances, nitrous oxide. The sequence of reactions in which nitrogen is evolved as an end-product is essentially a respiratory mechanism in which nitrate and/or nitrite replaces molecular oxygen. The organisms which are capable of such activities are termed facultative aerobes. However, denitrification is not the only means by which microorganisms reduce nitrate and nitrite. Microorganisms also reduce these anions to the ammonium level for incorporation as cellular nitrogen.

The process of denitrification by organisms is a biological one and is supplemented in the soil by another series of reactions not involving biological mechanisms. This form of denitrification is nonbiologically mediated via nonenzymatic avenues of soil nitrogen loss. One of the end-products of this nonenzymatic process is different. The characteristic gaseous end-products are molecular

nitrogen and nitric oxide whereas the enzymatic route is characterized by the end-products nitrogen and nitrous oxide.

Studies were carried out in an attempt to determine the magnitude of nitrogen loss, mediated via biological (enzymatic) and nonbiological systems (nonenzymatic) to verify, using appropriate tracer techniques, the origin of the nitrogen gases evolved in each case, and to provide a further evaluation of the effect of pH as well as on other soil characteristics and environmental factors on nitrite and nitrate decomposition.

By using sterile soils amended with ^{15}N labeled sodium nitrite it was shown that nonbiological denitrification is significant in the nitrogen cycle. To compare this nonenzymatic process with enzymatic denitrification, several soil types as well as a bacterium isolated from marine sediment were used. This gram negative, polarly flagellated bacterium was found to be unique in that it degrades nitrite and nitrate, producing nitrogen gas. Gases evolved from nitrite under sterile conditions were nitrogen and nitric oxide, but no gas was evolved from nitrate. From nonsterile soil, nitrate yielded nitrogen and nitrous oxide. This suggests that nitrite, a critical intermediate in nitrification and denitrification, is degraded nonbiologically and that nitrate, unless reduced to nitrite, cannot be degraded nonbiologically. Hydrogen ion concentration alone cannot fully explain the instability of nitrite in a sterile soil system. It was found that cation

exchange capacity, water tension, organic matter, and clay fraction as well as pH are involved in both enzymatic and nonenzymatic denitrification. The reaction sequences of enzymatic and non-enzymatic denitrification appear to be different and unrelated.

It is concluded that nonbiological route(s) of soil nitrogen loss must be given equal emphasis in the classical denitrification pathway. Because nitrogen represents a major end-product of the non-enzyme mediated breakdown of nitrite-nitrogen, it becomes difficult to differentiate between the relative contribution of these routes of soil nitrogen loss. However, it does become clear that with nitrate and nitrite-nitrogen, greater field losses of nitrogen occur than had been previously considered possible, particularly since pH represents only one soil factor influencing the conversion of nitrate or nitrite salts to the gaseous state.

Enzymatic Versus Nonenzymatic Denitrification

by

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ENZYMATIC VERSUS NONENZYMATIC DENITRIFICATION

INTRODUCTION

Man, animals, and plants depend on microbiological processes for food production. It has been recognized for a long time the important role which microorganisms play in producing and maintaining fertile soils. Even though it is sometimes difficult to attribute crop production to soil fertility, it goes without saying that microbiological processes enable man to enjoy the comforts provided by food, shelter, and clothing.

There is a definite dependence by the higher forms of life on the microorganisms. Without them, the higher forms could not exist within the biosphere, as we recognize them today. Because of this extreme dependence on microorganisms, it should be borne in mind that the role played by the microorganisms, particularly in the soil, should not be overlooked. Microorganisms indigenous to the soil carry out a wide variety of physico-chemical reactions which in turn exert a very real control over soil fertility--that is, the availability of plant nutrients. Of the reactions mediated by the soil microflora and fauna such as nitrogen fixation, assimilation and dissimilation, hydrolytic, and oxidative-reductive, the most important biological mechanisms in rendering soil nitrogen deficient are

involved in denitrification. In turn, a deficient supply of nitrogen in the soil exerts a deleterious influence on crop production.

Denitrification is one means by which microorganisms reduce nitrate and nitrite, and is an important aspect of the over-all nitrogen cycle. In this cycle, a series of reactions follow from plant nitrogen to animal nitrogen or microbial nitrogen, thence to ammonium to nitrate and back to the plant.

Denitrification represents a sequence of reactions in which nitrate and nitrite are converted to nitrogen cases. The utilization of these ions is a metabolic feature of certain bacteria, facultative aerobes, which otherwise would not be able to grow under anaerobic conditions.

That this enzymatic process functions in soil systems, particularly those in which low-oxygen tensions are present, has been long recognized. However, that a nonenzymatic process occurs and is also responsible for producing nitrogen deficits in the soil has not been realized in the past. There is little question that both enzymatic and nonenzymatic processes are operative.

Of primary concern is the relative balance between so-called nonenzymatic routes and the classical biologically mediated, enzymatic, avenues of soil nitrogen loss. Of secondary concern is the nature of the nitrogen gases evolved via the aforementioned pathways.

It was with the above considerations in mind that experiments were designed to determine the magnitude of nitrogen loss, mediated via biological (enzymatic) and nonbiological systems (nonenzymatic), and to verify, using appropriate tracer techniques, the origin of the nitrogen gases evolved via the enzymatic versus nonenzymatic routes, and to provide a further evaluation of the effect of pH as well as other soil characteristics and environmental factors on nitrite and nitrate decomposition.

This investigator hopes that the data presented herein will help to clarify these particular questions and provide better insight into the understanding of denitrification so that further investigations can be more easily undertaken.

HISTORICAL REVIEW

The first recorded observation of denitrification was in 1841 by Davy (16, p. 117), who reported that nitrogen was evolved in the decomposition of organic matter. He also indicated that denitrification occurs in soils, although this was not his own interpretation. Others such as Goppelsroder (27) and Reiset (49) made observations which also indicated that denitrification occurred in soils. Goppelsroder demonstrated that the nitrogen formed was the result of the decomposition of nitrate and that this event was carried out by microorganisms present in the soil. Later Schloesing (54) noted that gaseous evolution from tobacco plant liquors occurred when nitrate was present.

In 1886, Gayon and Dupetit (25, p. 201-307) made extensive studies on the evolution of nitrogen and wrote a now classical monograph in which they observed certain microorganisms carrying out a step-wise oxidation of organic matter coupled with the reduction of nitrate to nitrogen. They reported in detail the physiology of two microorganisms, Bacterium denitrificans α and β. It was noted that strain β produced both nitrous oxide and nitrogen when grown on a synthetic medium, but that strain α produced only nitrogen; nitrate was not consumed. Products and effects of oxygen level, composition of the medium, temperature and growth

conditions were also studied. The first evidence that denitrifying microorganisms had a truly ubiquitous distribution over the earth's surface was provided by Giltay and Aberson (26, p. 341). Also, they worked with pure cultures of a bacterium called Bacillus denitrificans and verified that nitrogen and nitrous oxide appeared when this organism was grown in the presence of nitrate.

Numerous speculations as to the significance of denitrification resulted from these early experiments. Some scientists who were completely unaware of at least a partial balance provided by nitrogen fixation were alarmed at the idea of the world starving because of the depletion of the soils' fixed nitrogen resources. It took a while for the significance of Winogradsky's demonstration (61, p. 1385; 62, p. 353; 63, p. 297) of nitrogen fixation to be realized. This was later modified to produce a picture of nitrogen transformations in the nitrogen cycle. Most scientists then realized that this alarm was somewhat unfounded and denitrification no longer held the interests of many investigators even though it was widely recognized that denitrification is a common occurrence in soil. However, direct evidence that it took place in soil was lacking due to the extreme difficulties involved in obtaining experimental evidence. Probably due to Winogradsky's conclusions, many scientists became disinterested in the subject because they were convinced that denitrification could no longer cause appreciable and

significant economic losses. It was not until about 1946 that Adel (1, 2) regenerated interest in denitrification by publishing his observations that nitrous oxide is a constituent of the earth's surface and that the concentration of this gas is greater near the earth's surface than in the higher strata of the atmosphere. His viewpoint that the origin of the gas was soil nitrogen compounds which become degraded, along with Arnold's data (5) showing that nitrous oxide evolution from soil occurred both in the laboratory and in the field, convinced most soil scientists that more intensive studies of denitrification were necessary. Because an increase in the use of nitrogen fertilizers up to that time had been tremendous, many investigations concerning the role and mechanism of denitrification began to develop.

Importance and Scope of Denitrification in Soils

It should first be mentioned, as had already been suggested above, that denitrification is a property of certain microorganisms found in the soil. However, these organisms are not restricted to the soil alone but have been shown to exist in other environments such as sea water, sewage and fresh water reservoirs. Issatchenko (30, p. 53), Lloyd (37), and others have demonstrated their presence in sea water. Gawel (24, p. 74) isolated from ocean bay sediment a microorganism tentatively identified as an

Achromobacter, capable of reducing only nitrite but not nitrate to nitrogen. According to Youatt (70), there is an Achromobacter species capable of reducing nitrite to nitrogen gas while being incapable of reducing nitrate.

Delwiche (17, p. 233-256) has stated that there is a wide variety and large number of organisms which are capable of the dissimilatory reduction of nitrate. However, if one considers the strict definition of denitrification to be the reduction of nitrate and nitrite with the liberation of molecular nitrogen and, in some instances, nitrous oxide, only a small number of bacterial species can be demonstrated to bring about this transformation. According to Alexander (3, p. 299), the active denitrifying species are largely limited to members of the genera Pseudomonas, Achromobacter, Bacillus, and Micrococcus although Thiobacillus denitrificans and an occasional Chromobacterium, Mycoplana, Serratia, and Vibrio species will catalyze the reduction. The dominant denitrifiers in the soil are Achromobacter and Pseudomonas species.

Up to this point, only biological denitrification has been mentioned. But it should be brought to mind that nonbiological denitrification does occur and that it may also play an important role in nitrogen balance in the soil. As will be discussed more fully later in this thesis, nonenzymatic denitrification in soil systems can take place. Bulla, Gilmour, and Bollen (12) have demonstrated

for the first time that in sterile soil systems nonbiological reduction of nitrite yields nitrogen and nitric oxide. Dhar and Pant (18) were probably the first to obtain experimental results which showed that nonbiological denitrification is possible. Their conclusions were that not only does nonenzymatic denitrification occur, but that it is enhanced by strong light conditions. Wullstein (65, p. 44) has also suggested that nonbiological denitrification occurs and that the mechanism of such a process may be attributed to the reaction of nitrite with certain transition metals such as iron and manganese.

The importance of nitrogen losses from soils cannot be overemphasized. Nitrogen is the one plant nutrient that may be considered the cornerstone of proteinaceous matter of living tissue. Of all the nutrients, except carbon, hydrogen, and oxygen, it is required in greatest quantity. Along with potassium and phosphorous, nitrogen is one of the major plant nutrients derived from the soil and is considered of prime economic importance. Available nitrogen is most often the limiting nutrient in soil fertility and deficient amounts will markedly reduce crop yield and quality. Thus any nitrogen transformations which may cause loss of this element from the soil are of great agricultural importance. Because denitrification, both nonenzymatic and enzymatic, does occur, these processes may have far-reaching significance in

future conservation practices to maintain soil nitrogen balances.

Biochemistry of Denitrification

Figure 1 shows a diagram of the nitrogen cycle depicting transformations of one form of nitrogen to another. Nitrogen is liberated from organic matter in an inorganic form by microbial processes of mineralization. In turn, by microbial assimilation this is immobilized and becomes unavailable. Numerous references in the literature are made to microbial oxidations and reductions of nitrogen compounds. The transformation of ammonium to an oxidized form is called nitrification whereas the sequence of steps from an oxidized form to a reduced form with subsequent gaseous loss is known as denitrification. The latter process has, for the most part, always been linked with the microbial reduction of nitrate and nitrite with the liberation of molecular nitrogen. In certain instances nitrous oxide is also found as an end-product of this reaction. As has already been mentioned, nonenzymatic denitrification also occurs but there is little mention of this in the literature. With the exception of Dhar and Pant (18) and Wullstein and Gilmour (67), Bulla et al. are the only investigators to describe nonenzymatic denitrification. The latter mentioned investigators have shown this process to be a real one by using appropriate tracer techniques, and, as will be described more fully in

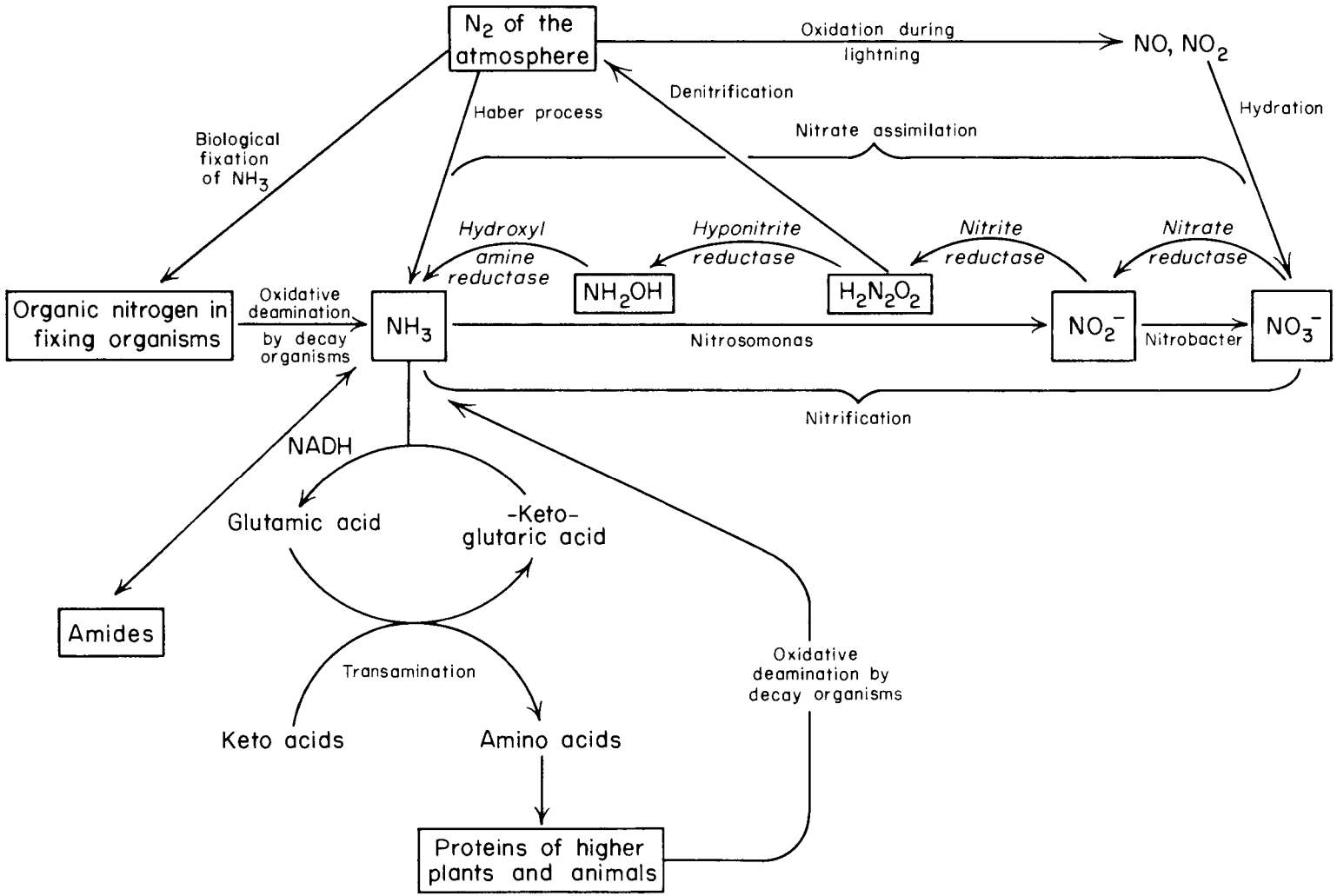


Figure 1. The nitrogen cycle

another section of this thesis, have determined the spectra of gases evolved via both enzymatic and nonenzymatic routes.

Denitrification is not the only means of reducing nitrate and nitrite. Microbes are also capable of reducing these two anions to ammonium, a form suitable for amino acid synthesis within the cell. Enzymatic denitrification is a biological process accomplished by facutatively aerobic bacteria capable of utilizing nitrate and/or nitrite in place of oxygen as a hydrogen acceptor. Thus denitrification is essentially a respiratory mechanism. This has also been referred to as nitrate respiration. Contrary to this, the utilization of nitrate as a nutrient source has been referred to as nitrate assimilation. Agronomically, as a result of nitrate assimilation, the nitrogen remains in the soil and is eventually released to become potentially available as a crop nutrient.

The oxidation of a carbohydrate such as glucose by facultatively anaerobic bacteria with oxygen present brings about the formation of carbon dioxide and water:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

If nitrate substitutes for oxygen, these same bacteria can carry out denitrification and utilize the nitrate-oxygen as the terminal hydrogen acceptor. This is expressed as the following:

$$C_6H_{12}O_6 + 4NO_3^- \longrightarrow 6CO_2 + 6H_2O + 2N_2$$

This type of respiration represents one of the energy-yielding mechanisms found in facultative aerobes.

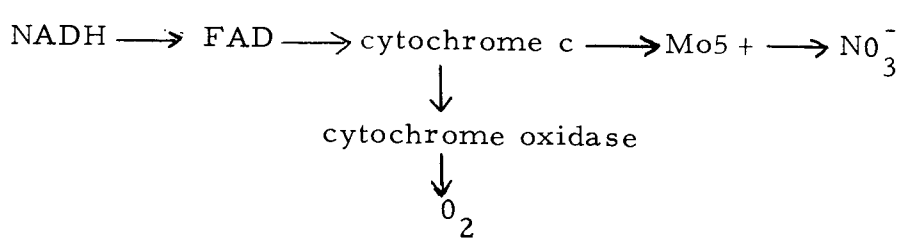
Another type of nitrate reduction is nitrate fermentation (58) which takes place in young cotyledons of bean seed embryos. These organs are anerobic and dissimilative and accumulate nitrite from nitrate. Egami et al. (20) discussed these aspects and showed that the enzyme involved in NADH-linked and apparently functions by means of substrate phosphorylation.

Because nitrate is commonly present in soil, plants and soil organisms have developed an ability to utilize this anion as a nitrogen source for their growth and development. As can be seen in Figure 1, the major route for the incorporation of inorganic nitrogen into organic nitrogen is the reaction catalyzed by glutamic dehydrogenase. The first step in this over-all incorporation mechanism is the reduction of nitrate to nitrite, which is catalyzed by nitrate reductase:

$$NO_3^- + NADH + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O$$

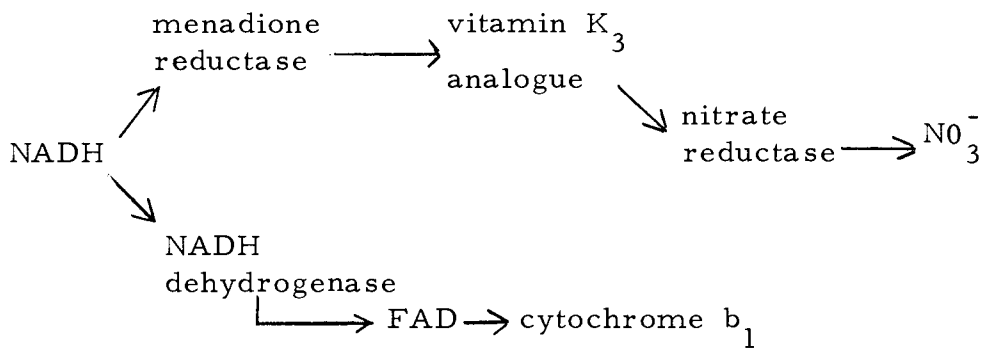
Using a cell-free preparation of Bacterium coli, Yamagata (68) showed nitrate reduction to occur. Later, Nason and Evans

(42) purified for the first time the enzyme nitrate reductase from Neurospora. Sadana and McElroy (52) by reversing PCMB inhibition through the addition of cysteine or glutathione demonstrated the adaptive nature of the enzyme and showed that it contains a sulfhydryl component. Nicholas, Nason, and McElroy (44) first showed that nitrate reductase requires molybdenum and that there is a correlation between its specific activity and its molybdenum content (45). Fewson and Nicholas (22) demonstrated a dependency on both molybdenum and iron by this enzyme isolated from Pseudomonas aeruginosa. This established a requirement for both the cytochromes and molybdenum components. In addition, it was found that nitrate was reduced with reduced NAD but not with reduced NADP, and that increased oxygen tension decreased the nitrate reduction process. This oxygen effect was found to be lessened by the addition of cobalt to the enzyme preparation. Further (23) these same individuals found the enzyme to be NADH dependent and that it contains FAD, cytochrome c, and molybdenum as functional components. They postulated the sequence of actions in the electron transport system:



It was also noted that cytochrome c content and nitrate reductase activity decreased with increased oxygen tension. Thus, the inducibility of the enzyme by anerobic conditions was noted. In addition, a valence change of the molybdenum during nitrate reduction was shown to occur.

Nitrate reductase systems from other bacteria have been purified and studied. For the most part, all of these systems are quite similar in nature, differing only in certain coenzyme requirements. One such system studied was that from Escherichia coli in which Taniguchi and Itataki (59) demonstrated the enzyme to be of a particular type containing no flavin. It was reasoned that this system transfers its electrons from reduced NAD or formate via cytochrome b_1. Other nitrate reductases isolated from various bacteria have been shown not to be associated with flavin. In particular, the reductase isolated from a different strain of E. coli by Heredia and Medina (29) was shown to reduce nitrate to nitrite under both aerobic and anaerobic conditions. Under aerobic conditions vitamin K_3 served as the electron carrier and NADH as the electron donor. The following diagram depicts such a mechanism:



In the series of reactions involving nitrate reduction the next step after nitrate reduction is the reduction of nitrite. Again, this reductive step is catalyzed by a nitrite reductase:

$$NO_2^- + NDAPH + H^+ \longrightarrow N_2O_2^{2-} + NADP^+ + H_2O$$

The intermediate $N_2O_2^{2-}$ thus formed is converted to hydroxylamine which in turn is converted to ammonium to be incorporated in the cell. The first of these reactions is catalyzed by hyponitrite reductase and the second reaction by hydroxylamine reductase. However, a secondary pathway is available to some denitrifying organisms in which nitrous oxide and nitrogen are formed. Nitrogen gas has been shown to be evolved as the result of either nitrite or nitrate being reduced anaerobically by a cell-free extract of Pseudomonas aeruginosa (69).

Chung and Najjar (14) showed that enzyme preparations from P. stutzeri can convert nitrite to nitric oxide and the latter to nitrogen gas. Both of these steps were shown to require copper

and iron as well as pyridine and flavin nucleotides for full activity. These investigators believed the flavin nucleotides to be obligatory carriers in the electron transport from reduced pyridine nucleotides to nitrite. However, they were not able to show an absolute dependence on the flavin, as was possible with the pyridine nucleotides. Of the metal ions tested, Cu^{++}, Fe^{++}, and Fe^{+++} were shown to stimulate nitrite reductase activity.

One point that should be brought out at this juncture is that nitric oxide was detected in those experiments by Chung and Najjar. Further, they reported the isolation of nitric oxide reductase from P. stutzeri (15) and the dependence on this enzyme on the same cofactors reported for nitrite reductase. No role for cytochrome in the extracts was shown to occur. The following diagram is a depiction of their postulated electron transfer mechanism:

$$H^+ + \begin{matrix}\text{NADPH} \\ \text{or} \\ \text{NADH}\end{matrix} \longrightarrow \text{FAD} \rightarrow Cu^{++} \text{ or (and) } Fe^{+++} \text{ (cytochrome)} \rightarrow NO$$

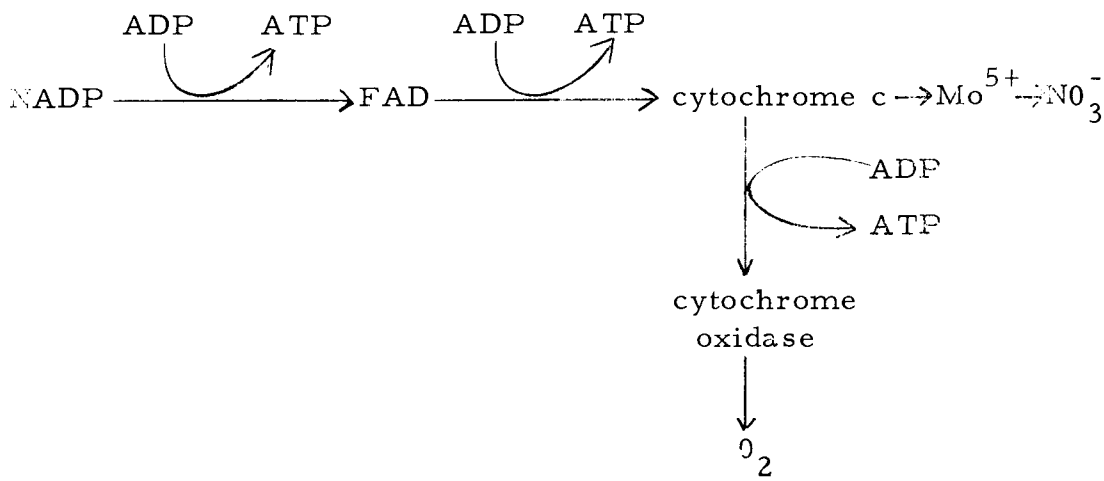
Nitric oxide is shown here to be a key intermediate in the denitrification scheme. However, as will be discussed in another section of this dissertation, data has been gleaned which do not substantiate these findings.

Another enzyme which occurs in the over-all denitrification process is hydroxylamine reductase. Those organisms capable of

forming ammonia contain this enzyme. Hydroxylamine reductase has been demonstrated in a species of Micrococcus (35), but not in Micrococcus denitrificans or P. stutzeri.

The energy production of denitrifying bacteria has been under investigation recently by Hadjipetroa and Stouthamer (28). The results of their studies showed that oxidative phosphorylation occurs in whole-cell preparations of Aerobacter aerogenes and that three moles of ATP are produced per each mole of nitrate reduced. The nitrate reductase involved in the reactions of A. aerogenes was shown to be associated with the conversion of glucose to acetate via the glycolytic pathway. In these reactions, nitrate serves as a terminal hydrogen acceptor in place of oxygen. The nitrate reductase was shown to be involved in the oxidation of formate.

Elliot (21, p. 50-58), using growing cell systems, determined the P/O ratios for both aerobic and anaerobic respiration in P. stutzeri. He showed that P/O ratios of 2:1 existed for anaerobic respiration and 3:1 for aerobic respiration. The method employed was that of equating oxygen uptake with cell numbers under aerobic conditions and nitrate-oxygen uptake with cell numbers under anaerobic conditions. The following is a proposed scheme which, according to Elliot's interpretation of Fewson and Nicholas' data (23), shows the sites of oxidative phosphorylation:



In order to summarize the aforementioned data which have, for the most part, made easier an attempt to fit together the pieces of the puzzle concerning nitrate reduction processes, the biochemical pathway of nitrate reduction and denitrification is presented in Figure 2.

It can be seen from Figure 2 that a shift of eight electrons occurs in that the reduction of nitrate to ammonia proceeds by a sequence of two-electron changes from the +5 oxidation state of nitrate to the -3 of ammonia. Those enzymes involved in this series of reactions are the nitrate, nitrite, hyponitrite, and hydroxylamine reductases (Figure 3).

As has already been mentioned, molybdenum is required for nitrate reductase. Thus, an explanation exists for the poor growth of bacteria in those media deficient in molybdenum and whose sole source of nitrogen is nitrate. Nicholas (43) has shown the

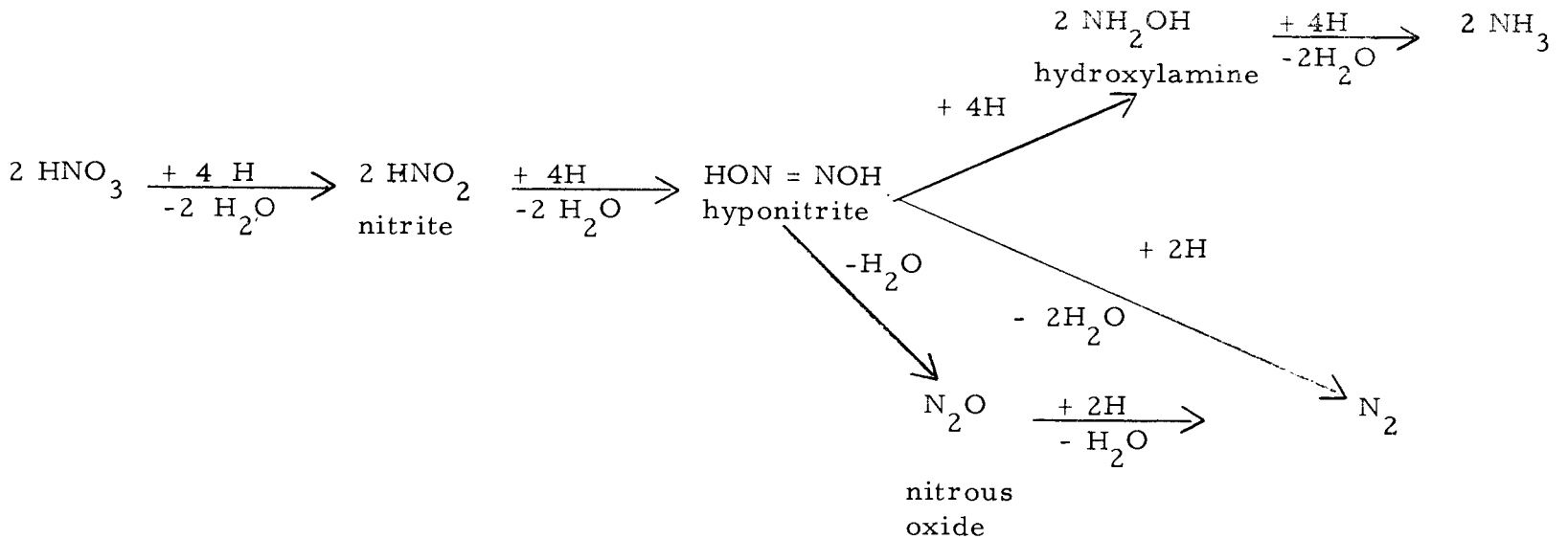


Figure 2. Biochemical pathway of nitrate reduction and denitrification.

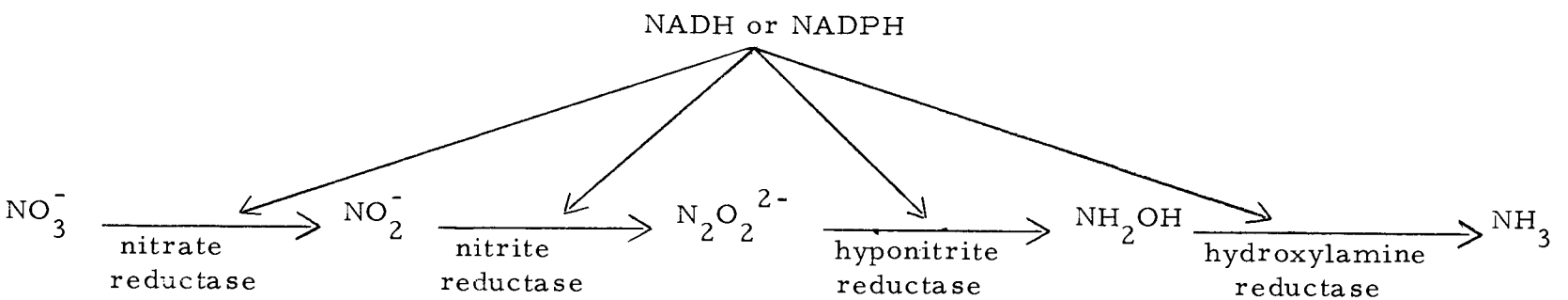


Figure 3. Enzymes of nitrate reduction.

mangenous ion to be the cofactor for hydroxylamine reductase and copper and iron to be functional for nitrite and hyponitrite reductases. Currently, not much is known about those enzymes catalyzing the final reactions of denitrification.

Figures 2 and 3 depict the enzymatic processes involved in denitrification. As for the nonenzymatic production of nitrogen and its oxides, reference is made to Wullstein's review article (66) in which he shows a hypothetical model of transition metal and inorganic nitrogen interactions. Refer to Figure 4.

Factors Affecting Denitrification

One of the most confusing terms which has been used in association with denitrification is the term aerobic denitrification. As has already been mentioned, denitrification per se is defined with regard to anaerobic conditions in which nitrate or nitrite substitutes for oxygen as the terminal hydrogen acceptor. The probable explanation for the promiscuous use of aerobic and anaerobic denitrification is in the different ways in which these terms are used and defined. In its strictest sense, aerobic denitrification cannot exist. Only under anaerobic conditions does denitrification occur, as was defined earlier. Why then is there the existing discrepancy or terminology as found in the literature? Again, the answer is actually one of semantics. No real contradiction actually exists in these apparent conflicting findings. Before becoming too

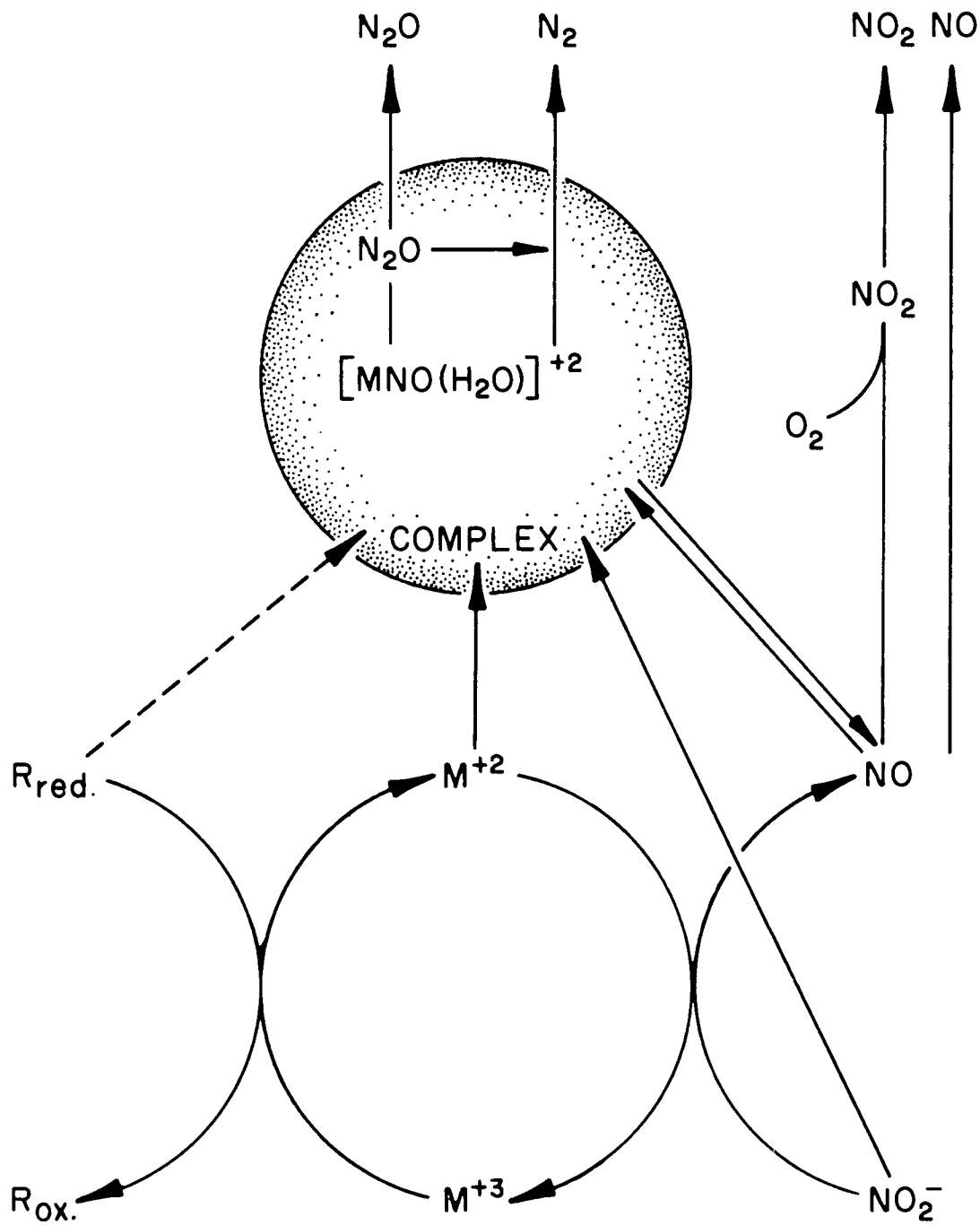


Figure 4. Hypothetical model of transition metal and inorganic nitrogen interactions

involved in this argument, a review of some of the literature is in order so that one may understand this author's concern for the use of both these terms.

Early investigators found that denitrification is most likely to occur in water-logged soils. Of course, this is in full agreement with more recent investigations of denitrifying bacteria. These studies have also shown these organisms to be facultative aerobes which utilize, and in fact prefer free oxygen in the culture media. It is when the oxygen supply is depleted that these organisms utilize any available nitrate as a hydrogen acceptor. Skerman, Lack, and Millis (56), and Skerman and MacRae (57) have performed experiments in which they polarographically measured dissolved oxygen concentrations to determine the concentration which inhibits the reduction of nitrate. This oxygen concentration threshold was found to be quite low. In contrast to these results, reports have also appeared in the literature in which denitrification has been observed under aerobic conditions. That denitrification can occur with an adequate oxygen supply has been proposed by Meiklejohn (39). This investigator described two species of Pseudomonas capable of denitrification in aerated cultures. Others, including Marshall et al. (38), Korsakova (36), and Kefauver and Allison (34) have reported observations similar in nature to Meiklejohn's. Conflicting reports have been published by Sacks and Barker (51)

and by Jones (32) in which they found no evidence of denitrification under aerobic conditions whereas Broadbent (10, p. 344-359), Broadbent and Stojanovic (11), and Allison, Carter, and Sterling (4) reported to have observed denitrification occurring in controlled atmospheres containing some oxygen as well as in aerated soils.

Thus, from just a brief review of some of the recent literature, one can see the problem existing with regard to aerobic denitrification. The probable explanation for the observed denitrification under aerobic conditions is that an anaerobic micro-environment is produced by those microorganisms effecting a rapid decomposition of decomposable substrate. This micro-environment created by the bacteria in consuming all available free oxygen is truly an anaerobic one. Therefore, even though the soil or medium is well aerated, denitrification can occur in these micro-environments. In this manner it is quite obvious that bacteria are capable of utilizing two alternative respiratory pathways even though they are separate and distinct. Furthermore, it is quite clear that with such anaerobic micro-environments existing, true aerobic denitrification does not occur. This term has been applied to define denitrification as occurring in well aerated systems. Aerobic denitrification in this investigator's opinion, should not be used in any context.

With regard to the reduction of nitrite under aerobic conditions, not much can be said of its relationship to the over-all denitrification scheme. That it is of considerable importance may be proven in the near future. It is conceivable to ascertain that nitrite reduction is critical under aerobic conditions since nitrite may be formed in soils either from reduction of nitrate when oxygen is lacking or from oxidation of ammonium when oxygen is present. Kefauver and Allison (34) and Skerman, Carey, and MacRae (55) have shown that certain bacteria are capable of reducing nitrite in the presence of oxygen whereas others reduce nitrite independent of the oxygen concentration.

From the discussion presented above, one might justifiably conclude that oxygen tension is one factor which affects denitrification. In those soils which are fine-textured, denitrification under well aerated conditions can and will occur more readily than in sandy soils. Nommik (46) studied denitrification with different sizes of soil aggregates and observed that denitrification decreased with increasing size of aggregates. When the aggregates reached a certain minimum size, the rate of denitrification increased. This was attributed to the longer time required for oxygen to diffuse to the center of the large aggregates.

The partial pressure of oxygen does not seem to be as critical to denitrification in the field as are other environmental factors.

However, the rate of denitrification by microorganisms is quite dependent upon the partial pressure of oxygen. Denitrification is an important agronomical and economic factor because it occurs in virtually all soils and may be especially rapid under temporary anaerobic conditions which can occur after irrigation of crop lands or after rainfall.

Bremner and Shaw (8) have shown that high water content indirectly affects denitrification by inhibiting the diffusion of oxygen, but, at the same time, directly exerts a very pronounced effect. They observed increased losses of nitrogen as a function of moisture content up to 45 percent of water-holding capacity. At less than 60 percent of the water-holding capacity, little loss of nitrogen occurred even when all other factors were very favorable for denitrification. Jansson and Clark (31) and Nommick (46) have made similar observations.

Temperature is another parameter which has a bearing on denitrification taking place in soils. Nommick (46) and Bremner and Shaw (8) have reported the optimum temperature range for biological denitrification to be approximately 60-65 degrees C. They observed that concentrations of nitrogen oxides present in the gaseous atmosphere of experimental denitrifying systems varied with temperature. Nitrous oxide predominated at lower temperatures whereas elemental nitrogen was predominant at

higher temperatures. As yet there is no evidence for definite temperature coefficients applicable to production of these two gases but it does appear that there is a higher coefficient for nitrous oxide reduction than for the other steps in denitrification. McGarity (40) states that freezing and drying of soil enhances denitrification. Because no mention was made of the exact amount of increased denitrification under these conditions, McGarity's claim must be accepted with reservation. For the most part, high moisture content favors denitrification while low moisture content does not. This could have been due, at least in part, to nonenzymatic rather than enzymatic denitrification. This may be an example in which nonbiological reactions were confusing the results of experiments designed to study biological denitrification.

Little work has been done on the influence of oxidation-reduction potential on denitrification in soil. Probably the main reason for this is that accurate measurement of soil Eh is extremely difficult. Among those data which seem to be the most sound are those presented by Pearsall and Mortimer (48) and Kefauver and Allison (34). These reports are conflicting. Pearsall and Mortimer used a series of soil samples adjusted to different oxidation-reduction potentials. They concluded that nitrates accumulate above 350 millivolts and disappear below 320 millivolts. Thus they inadvertently suggested an optimum

oxidation-reduction potential range for active denitrification. This conclusion was confirmed by Patrick (47, p. 494-500) who attested that the maximum nitrate instability occurs at a value of 338 millivolts. Contrary to these findings, Kefauver and Allison (34) found no such optimum Eh values and further concluded that oxidation-reduction potential plays no part in limiting nitrate reduction.

The role of hydrogen ions in the conversion of nitrate to nitrogen and its oxides has been the concern of many investigators. From the many reports found in the literature, only those which appear to exhibit a fairly high degree of sophistication are considered. First impressions are that soil pH has a pronounced effect on the rate of denitrification, it being quite rapid in alkaline soils and somewhat sluggish in acid soils. At a pH of approximately 6.0, denitrification rates appear to be fairly constant regardless of other factors except for moisture content. This point has been agreed upon by both Delwiche (17, p. 233-256) and Nommick (46). Jansson and Clark (31) state that alkaline conditions are required for extensive denitrification and that nitrite toxicity with acid substrates inhibits denitrification. Wijler and Delwiche (60) have noted pH dependence for the relative proportions of nitrous oxide and nitrogen produced. They found that nitrous oxide reduction was strongly inhibited below pH 7. With

acid soils, Cady and Bartholomew (13) observed a nearly complete reduction of nitrous oxide to nitrogen. Karlsen (33, p. 1-79), one of the first investigators to study the influence of pH on denitrification, found the optimum pH value for denitrification by P. aeruginosa to be between 7 and 8 with a minimum pH of 5.8 to 6 and a maximum of 9 to 9.2. Among the interesting results he obtained was that toward pH extremes, nitrite accumulated in the medium but disappeared as the pH increased. Within the optimum pH range, no nitrite accumulated. On the other hand, denitrification did not occur upon addition of nitrite to a medium adjusted below neutrality. It would appear that nitrite exerted an inhibitory effect in both the acid and alkaline media. With an accumulation of nitrite there was a drop in pH, thus probably intensifying the toxic effect of nitrite. With no accumulation of nitrite, the denitrification rate was determined by the pH alone. However, if additional quantities of nitrite were added to the neutral medium, the poisonous effects of nitrite were apparent and the optimum range for denitrification seemed to narrow and approach a value close to pH 8.

From these investigations it might be concluded that pH alone cannot be responsible for decreased denitrifying activity but that its effect is closely linked with the presence of certain anions such as nitrite, and is also probably related to the oxidation-reduction

potential of the medium.

Broadbent and Clark (10, p. 344-359) point out that the effect of organic matter on denitrification is dual in nature. First, the free energy change in nitrate reduction to nitrous oxide and nitrogen is positive. Therefore, an oxidizable substrate is required in order to furnish an adequate supply of energy for growth of the denitrifying microorganisms and to serve as an electron donor for the process of denitrification. Second, organic matter decomposition rates are definitely responsible for marked influences on the oxygen demand. Bremner and Shaw (7, 8) have compared the effect of several types of organic materials on denitrification with respect to their susceptibility to microbial breakdown. Those substrates which seemed to induce rapid denitrification were sodium citrate, glucose, sucrose, and mannitol. On the other hand, cellulose, sawdust, and lignin produced variable results with the least rapid rate of denitrification occurring with lignin- and sawdust-amended soils.

The effect of the rhizosphere on denitrification has also been investigated. Woldendrop (64) studied the quantitative influence of the rhizosphere and concluded that the effects of living roots on nitrogen losses by denitrification are caused by the consumption of oxygen by rhizosphere microorganisms during decomposition

of root excretions. This microbial activity lowers the immediate soil oxygen tension and may produce materials capable of serving as electron donors in denitrification.

MATERIALS AND METHODS

Preparation and Collection of Soil Samples

Soil samples used for the denitrification studies were collected at several depths so as to obtain representative samples from the various horizons. Depths from which these samples were obtained were 8, 16, 24, 32, and 48 inches. The following is a list of the soils used:

1. Amity silty clay loam
2. Dayton silty clay loam
3. Woodburn silty clay loam
4. Concord silty clay loam
5. Willamette silty clay loam
6. Gooch silty clay loam
7. Olympic silty clay loam
8. Walla Walla silt loam
9. Cloquato silt loam
10. Cove clay loam
11. Astoria silt loam
12. Chehalis silty clay loam
13. Corvallis sandy loam
14. Marine sediment (Norwegian coast)

These samples represent a group of soils which have been subjected to different environmental stresses. The Amity, Dayton, Woodburn, Concord, and Willamette soils were selected from a well established bench marked Catina, a soil chain in the Willamette Valley. The Olympic silty clay loam sample was obtained from the Olympic National Park in extreme northwestern Washington and is considered a virgin soil. The marine sediment was

collected about 20 miles off the coast of Norway by the use of a two-inch Phlager sampler which had been sterilized by chemical means prior to sampling. The sediment was transferred promptly to an incubator adjusted to five degrees C. and kept this way until used. The remaining soils were obtained from locations which had not been subjected to continuous cultivation or commercial fertilizers for the past four to five years. All samples were air-dried, passed through a 10-mesh sieve, and then stored at four degrees C. in plastic-coated containers.

Determination of Water-holding Capacity

Ten gram soil samples were placed in gooch crucibles and left standing in a tray of water until completely saturated by absorption of the water from beneath. Whatman No. 1 filter paper was cut and fitted into the bottom of the crucibles so as to prevent leakage of the soil. These were then transferred to a moisture-saturated atmosphere and allowed to stand until they reached a constant weight. The samples were then carefully weighed, oven-dried at 105 degrees C., and again weighed. The water-holding capacity was calculated from the amount of water held against gravity.

Determination of Substrate pH

The hydrogen ion concentration of all media, solutions, and soil samples were measured with a model G Beckman pH meter. Soil pH was determined on 1:3 (weight:volume) soil suspensions in distilled water after shaking for 30 minutes and maintaining the soil in a suspended state during measurement of the pH by means of a magnetic stirrer.

Soil Extraction

The soluble nitrate and nitrite ions are easily removed from soil by water. Therefore, distilled water was added to the soil samples at a ratio of 3:1 (volume:weight) and the suspensions were shaken for 60 minutes and then filtered through Whatman No. 3 filter paper. The clear filtrate was stored by freezing until nitrate and nitrite analyses were performed.

Cell Growth

All measurements of standard cell suspensions and color intensity were made with a Bausch and Lomb "Spectronic 20."

Analytical Methods

Mineral Nitrogen

Nitrate-nitrogen determination was carried out according to the procedure of Eastoe and Pollard (19). Interference by nitrite was eliminated by the addition of sulfamic acid according to the method of Bremner and Shaw (7). Saltzman's (53) modified technique using N-(1-naphthyl)-ethylenediamine dihydrochloride was employed for nitrite-nitrogen determination. Standard curves for these analyses were made (Figures 5 and 6).

Total Carbon

The method of Allison, Bollen and Moodie (4) was used in determining total organic carbon of the soils. In this procedure, all organic carbon present in the soil is completely oxidized to carbon dioxide by a stream of commercially compressed oxygen which has been scrubbed free of impurities by passing through various trains of sulfuric acid, Ascarite, and anhydrous magnesium perchlorate. After being oxidized the stream of gas leaves the furnace, passes through a dust trap, and an activated manganese dioxide trap which filters out nitrogen oxides, sulfur oxides, and halogen gases. Any water which remains is removed by successive passage through concentrated sulfuric acid and anhydrous magnesium perchlorate.

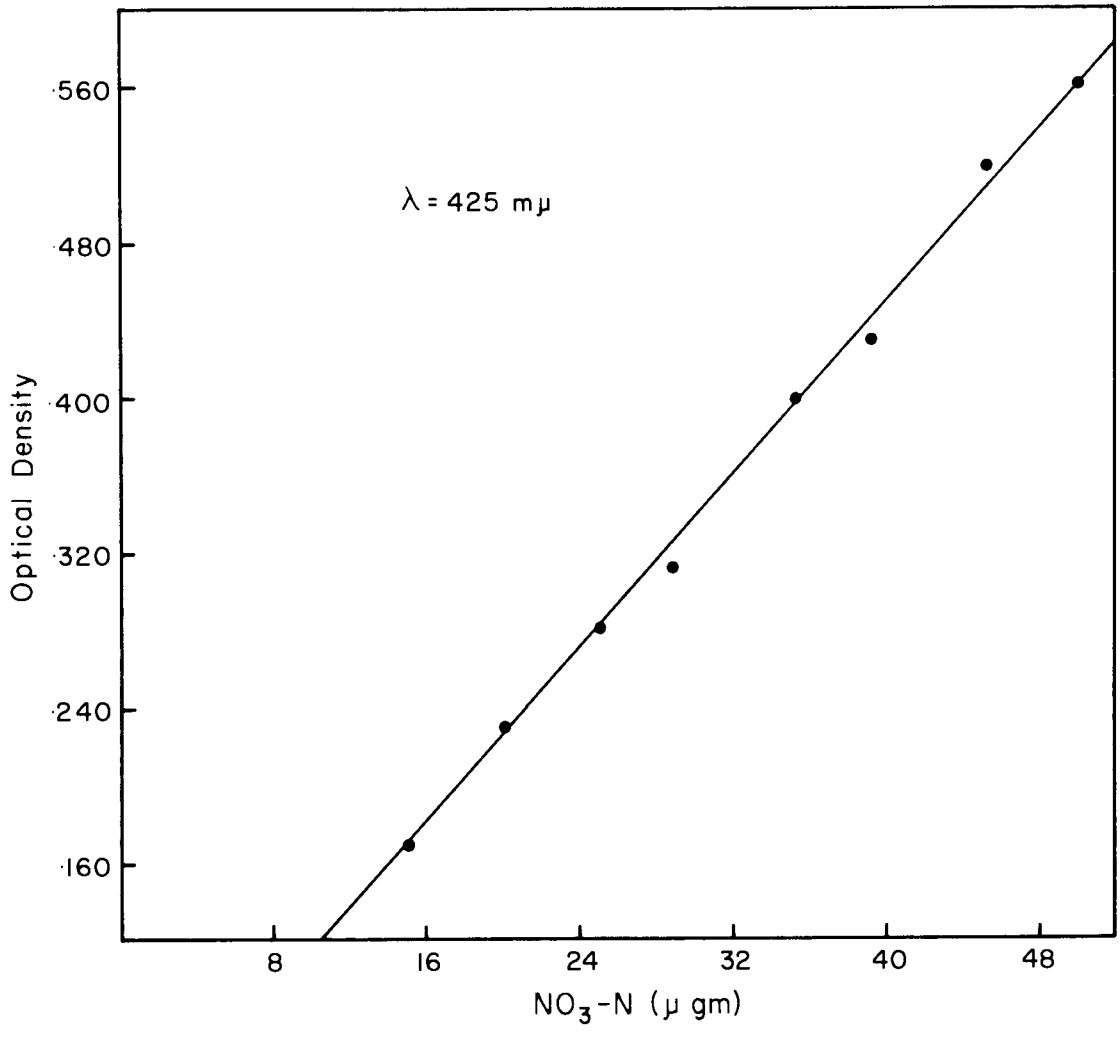


Figure 5. Calibration curve for nitrate-nitrogen

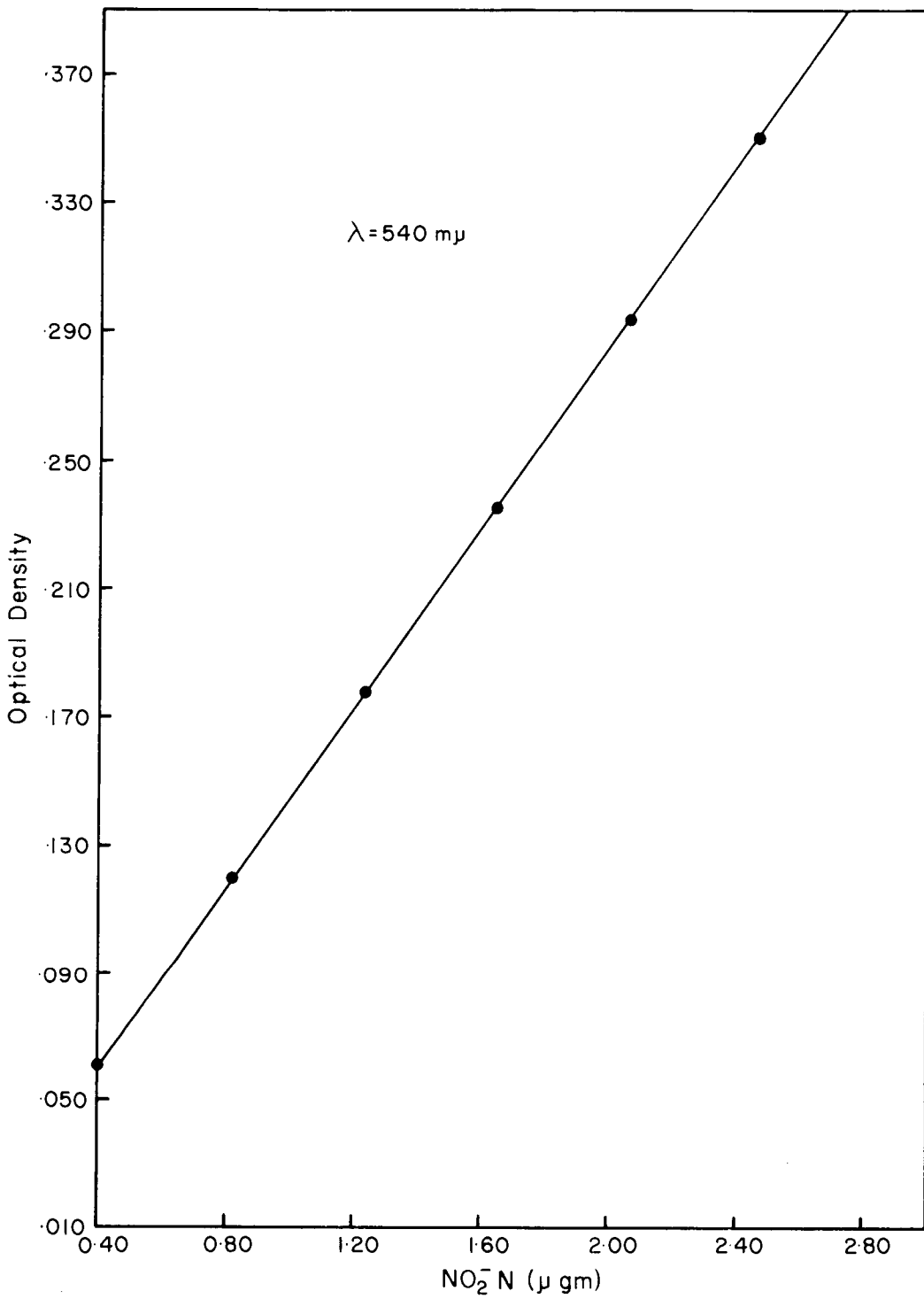


Figure 6. Calibration curve for nitrite-nitrogen

The carbon dioxide is absorbed in an Ascarite tower and calculated by weight increase.

Total Nitrogen

Total nitrogen was determined by the Kjeldahl method described in the Official Methods of Analysis of the Association of Official Agricultural Chemists, Ninth Edition, 1960.

Chemical and Physical Analyses

Various physical and chemical analyses were carried out on each of the soil samples by the Soil Testing Laboratory of the Soils Department at Oregon State University. These included particle size distribution, moisture tension, cation exchange capacity, organic matter content, and exchangeable potassium, calcium, magnesium, and sodium ions.

Water

Ten gram samples of the air-dried soils was determined by obtaining a weight difference after heating them at 105 degrees C. for 24 hours.

Ash

Ash was determined by ignition of 10g samples at 600 degrees C. and weighing the residue.

Design of Denitrification Experiments

Respiration Flask

In order to maintain absolute anaerobic conditions of the soils and media in the denitrification experiments, respirometer flasks of the type shown in Figure 7 were used.

This flask is unique in that it maintains anaerobic conditions and can be easily adapted for manometric measurement of its gaseous atmosphere. The flask was designed to facilitate quantitative measurement of the respective gases present within its atmosphere. The apparatus consists of a 300 ml Pyrex flask with a 29/26 standard taper joint to which a head assembly can be attached. This makes possible a system which is completely air-tight. The head assembly is pictured in Figure 8. This design was only recently completed and has the select advantage over that of Roa (50, p. 19) in that it can be disassembled and easily cleaned. Also, when attached to the Erlenmeyer flask, the center of gravity of the apparatus does not allow it to tip over easily as was true with the older design. The entire respirometer flask is much

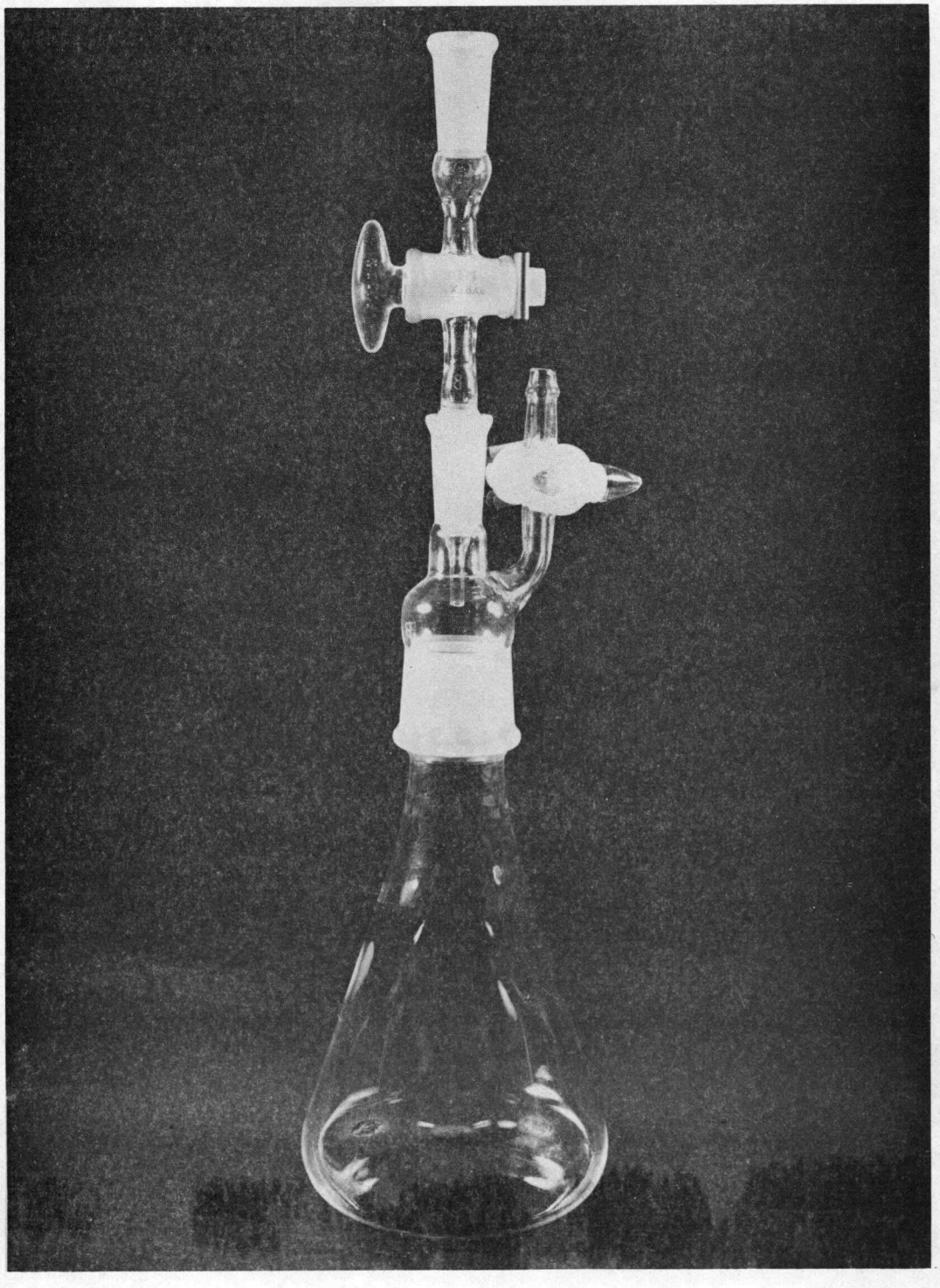


Figure 7. Respiration flask

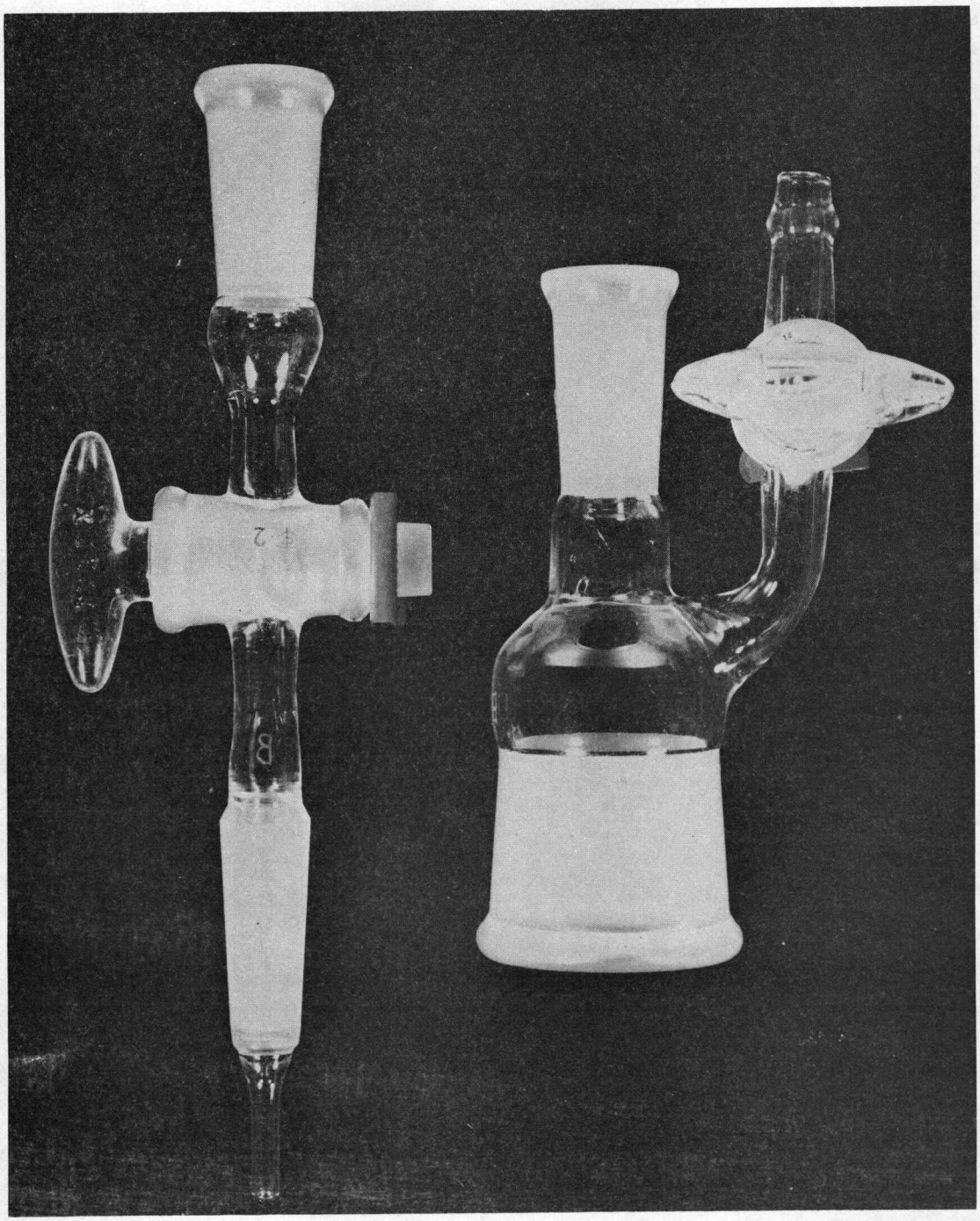


Figure 8. Head assembly of respiration flask

more suitable for experimental manipulation. It can be easily transferred from an electrolytic respirometer such as described by McGarity et al. (41) to a gas chromatograph unit for manometric measurements. Over-all, this respirometer flask makes easy the replacement of its gaseous atmosphere and subsequent sampling.

Aside from the features outlined above, some of the flasks were equipped with a center well which served for the deposit of a cup in which could be placed an absorbant for certain gases, and with a side-arm opening sealed with a serum cap to enable sampling with a hypodermic needle.

Replacement of the air within the flask with an inert gas such as helium is possible by connection of a gas supply to the respirometer flask through A. With stop-cocks B and D open, the gas can be flushed through the system for a desired period of time (approximately 60 minutes). While flushing, the outlet joint E is attached to a rubber tube and the end immersed in water. The water trap serves to prevent leakage of air back into the system and to gage the rate of gas flow. After complete flushing, stop-cock Y of the manifold system is closed first, followed by the closing of stop-cock A (Figure 9). Stop-cock D is closed after a few bubbles are seen to escape from the water trap. This is a precautionary step to ensure slight positive pressure within the system so as to not allow leakage back into the flask.

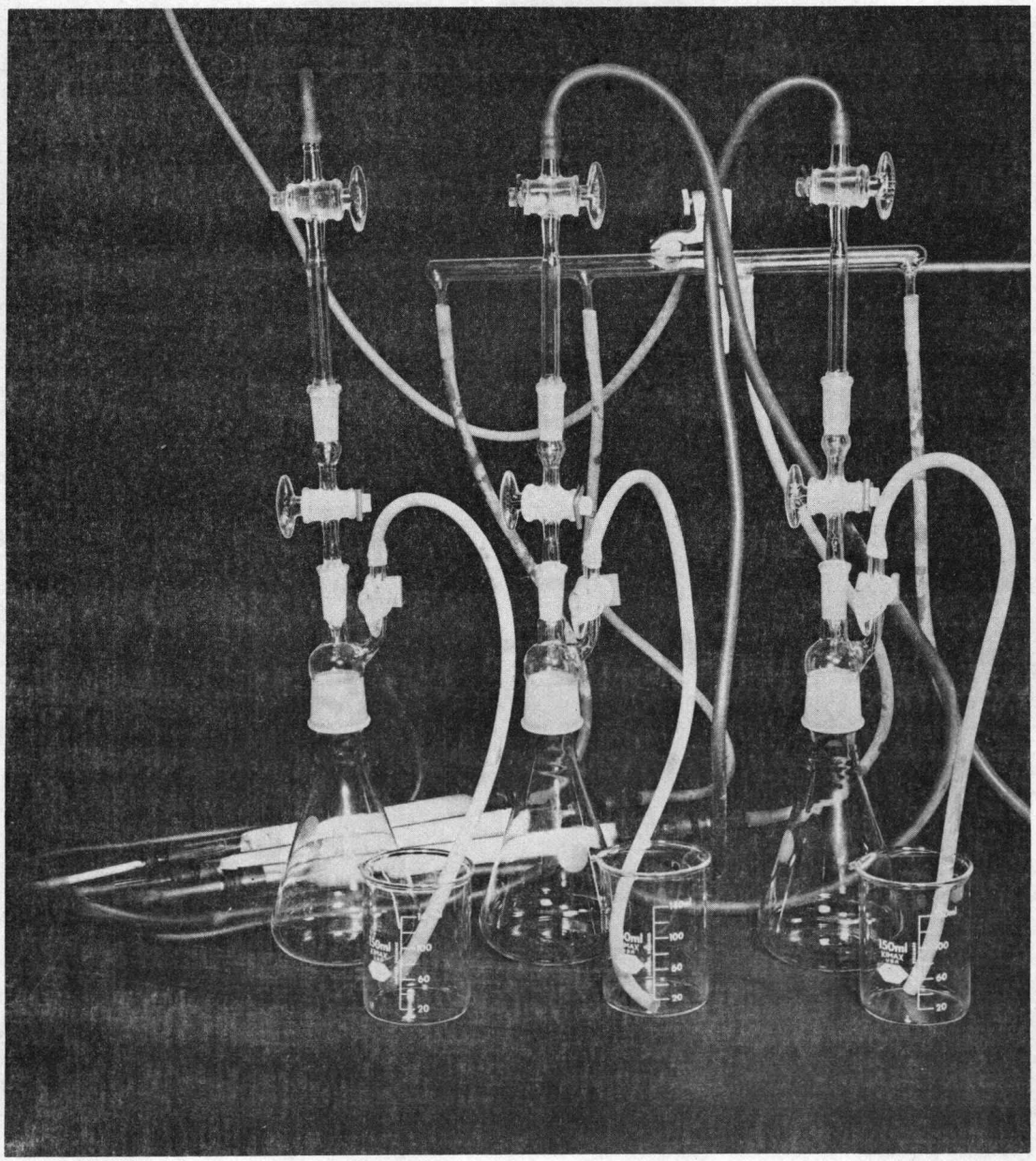


Figure 9. Manifold system and respiration flasks

Soil Treatment

Fifty gram soil samples were placed in the electrolytic respirometer flasks, plugged with cotton, and heat-sterilized for 12 hours at 121 degrees C. After sterilization the flasks were brought to 60 percent of the moisture-holding capacity, and 15 ml of 10 percent potassium hydroxide solution were added to the center well cups. Inorganic salts, sodium nitrate and nitrite, were added along with the water as well as glucose when an additional hydrogen acceptor was desired. All amendments were sterilized by either autoclaving or filtering before being added to the soil samples. The respirometer flask tops were then put in place and the flasks were flushed with helium as described above for approximately 60 minutes to remove all traces of nitrogen from the flask atmospheres. With all stop-cocks closed, the flasks were incubated for 14 days at 25 degrees C.

Both sterile and nonsterile soils were used. The nonsterile soils were amended in the same manner as the sterile soils. One series of experiments included the incorporation of the bacterium isolated from marine sediment, designated as A-62, and a strain of Pseudomonas stutzeri isolated from soil by Roa (50, p. 56). The soil samples to which this organism was added were presterilized as described above. Five ml of a heavy slurry of cells

(approximately 30 mgm/ml) washed in a buffer adjusted to pH 7, were aseptically added to the soil samples. These were also incubated for 14 days at 25 degrees C.

Gas Calibration and Identification

Nitrogenous gases evolved as a result of enzymatic and non-enzymatic denitrification were determined by a Beckman GC-2 gas chromatograph coupled with a Bristol Dynomaster Recorder (6, p. 1-71). The Beckman GC-2 was equipped with a Beckman 23800 two-column valve so that the gas chromatograph could be used with either of two columns. Two commercially available columns were used for gas identification. These were 13X Molecular sieve (6 ft.) for determination of oxygen, nitrogen, and nitric oxide, and a Charcoal-C22 Firebrick uncoated (1.5 ft.) column for the detection of carbon dioxide and nitrous oxide.

To sample the flask atmospheres, a gas-introducing device which allowed for quantitative measurement of the respective gases was coupled to the respirometer flasks. The inlet end of the gas sampling valve on the gas chromatograph was attached to a thick glass tube extension provided with three stop-cocks connected to a vacuum pump, a mercury manometer, and a gas sampling device. The gas sampling device contained a 12/30 standard taper joint which provided an air-tight connection with the respirometer flask.

Air leakage as well as the amount of gas sampled was determined by the mercury manometer.

It should be pointed out at this time that calibration of the gases detected by both of these columns is a critical step for the accurate determination of the gases evolved in the experimental systems. The sensitivity of the chromatograph detector is a function of the voltage sensitivity of the recorder, the volume of the gas sample, and the column conditions. Therefore, the operating conditions must be kept constant for the duration of the gas calibration and identification. For both the molecular sieve and charcoal columns, the carrier gas used was helium with a flow rate of 100 cc per minute at a temperature of 40 degrees C. and a filament current of 260 milliamperes. The recorder chart speed was adjusted to one cm per minute.

Elution times and relative peak areas for those gases detected are shown in Figures 10 and 11. Calibration curves for pure samples of nitrogen, nitric oxide, and nitrous oxide are shown in Figures 12, 13, and 14. Straight line functions were obtained because the peak weight increased in constant units with each unit increase in sample size. The calibration gases were obtained from the Matheson Gas Company.

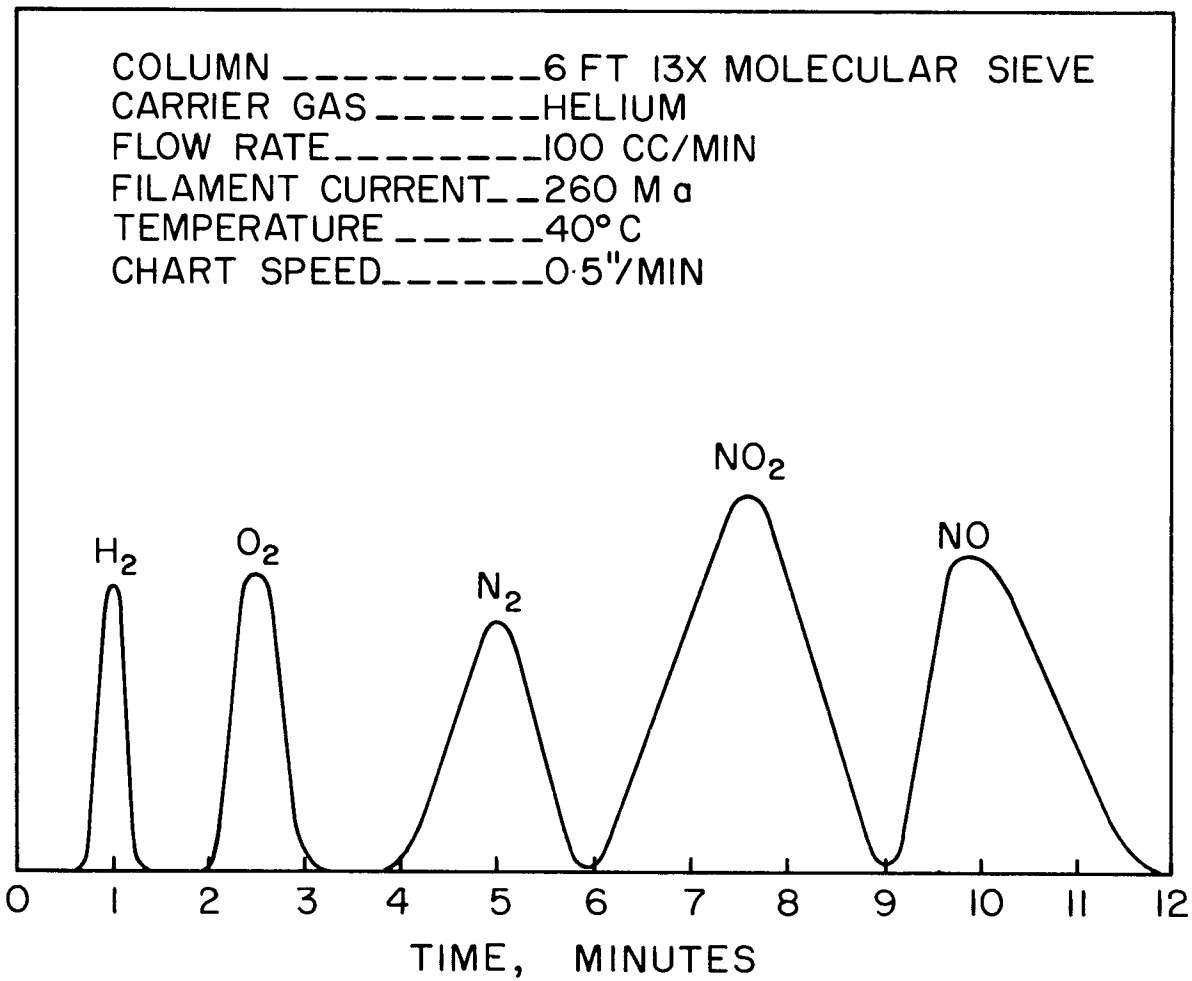


Figure 10. Elution times and relative peak area of gases evolved with a molecular sieve column

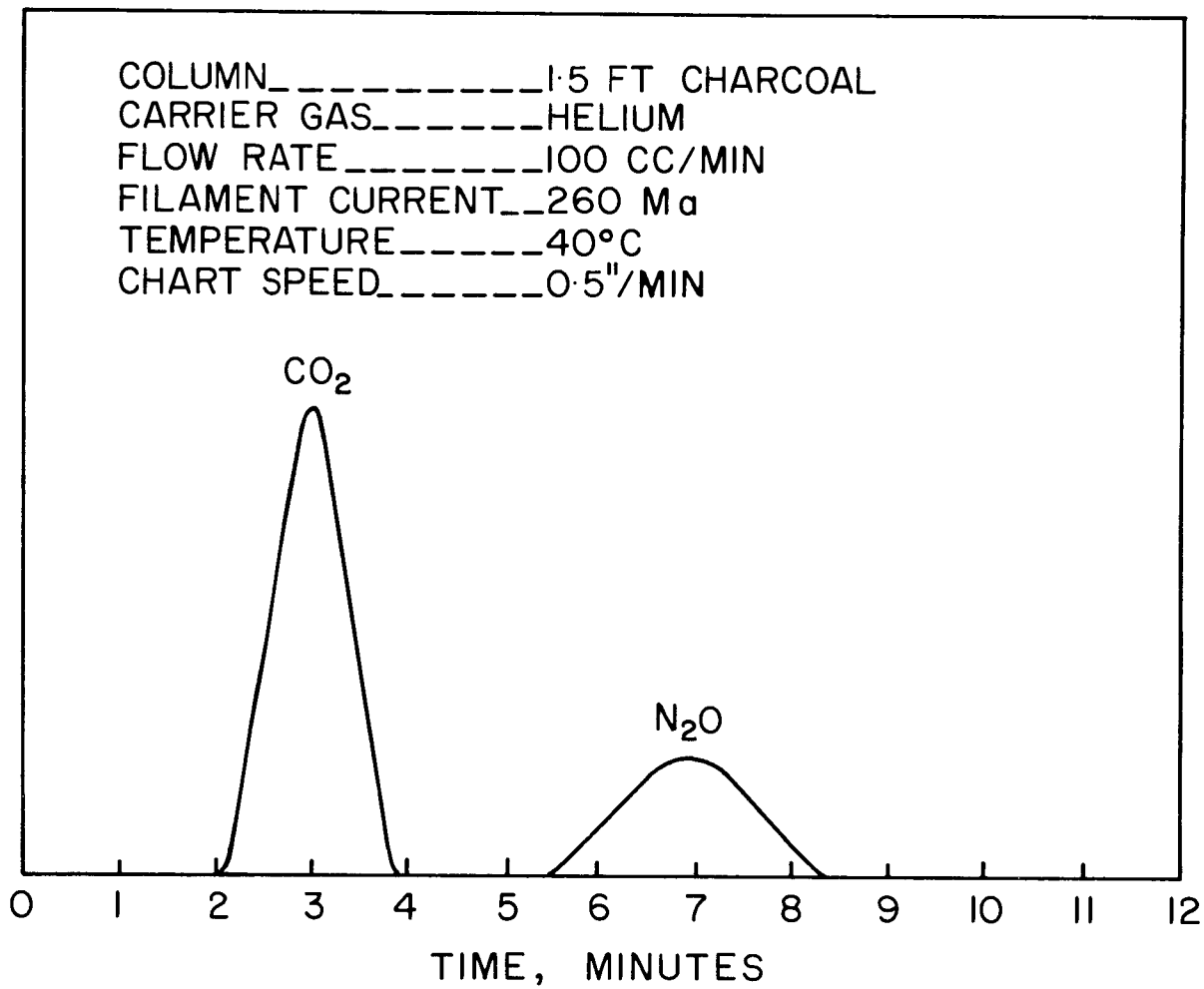


Figure 11. Elution times and relative peak area of gases evolved with a charcoal column

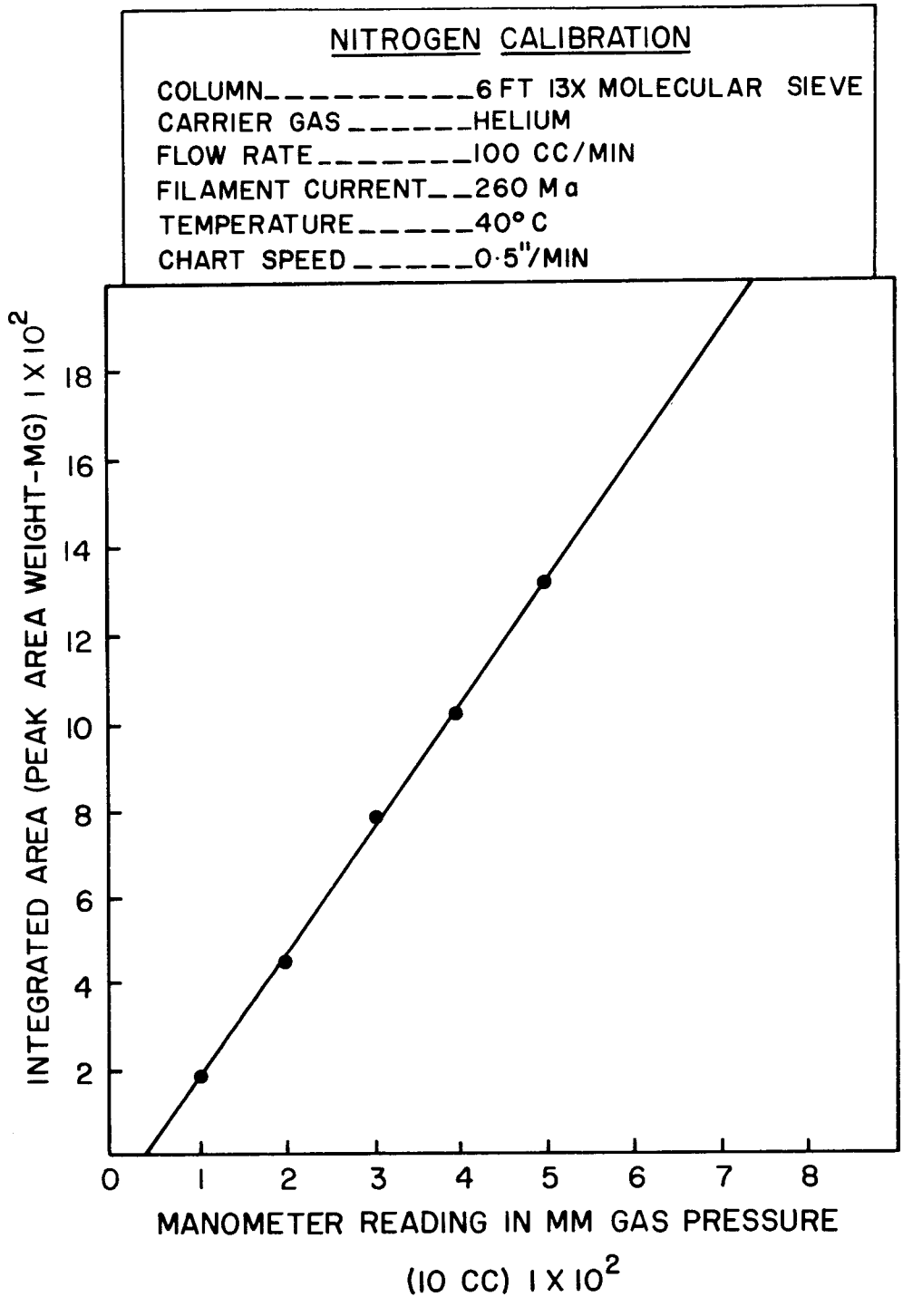


Figure 12. Nitrogen calibration curve

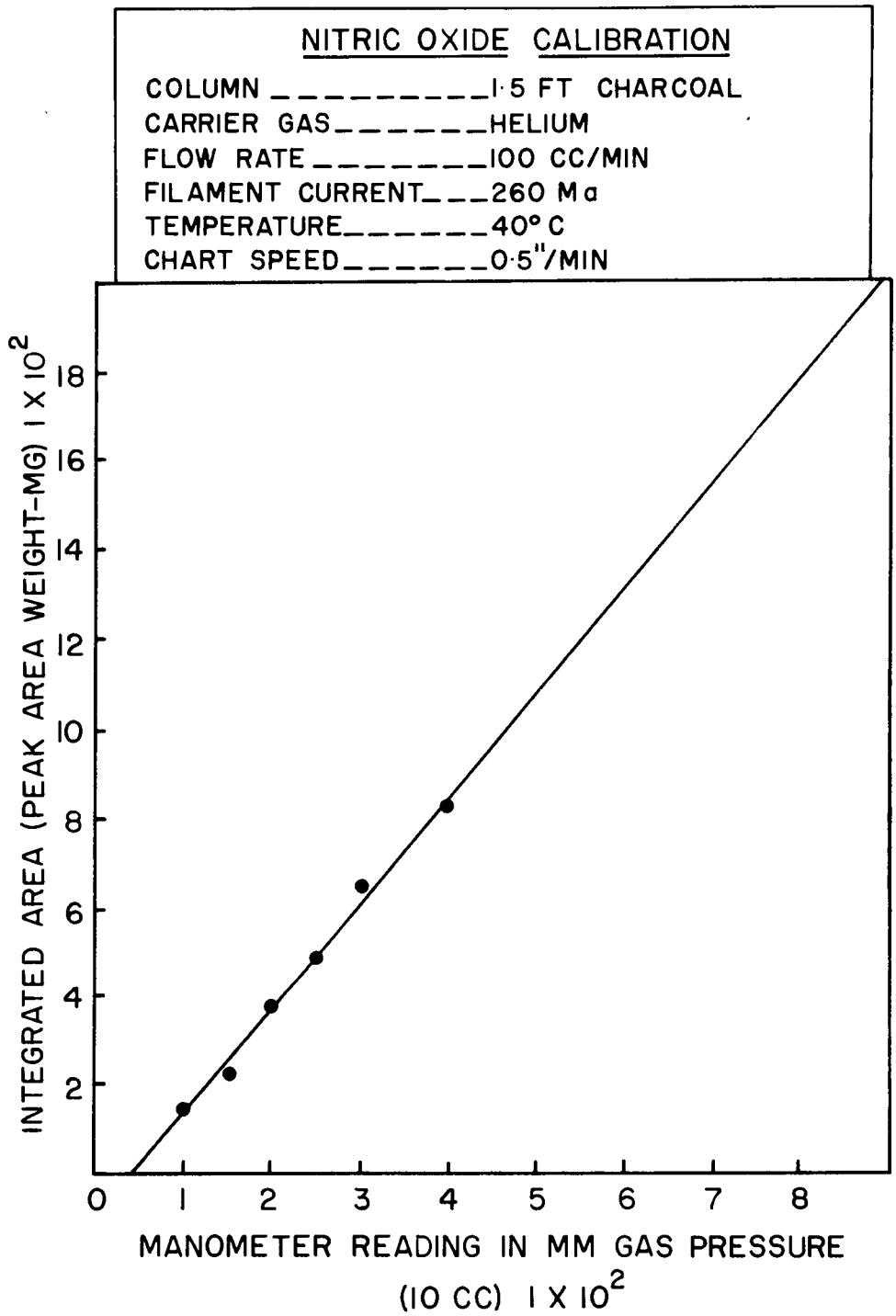


Figure 13. Nitric oxide calibration curve

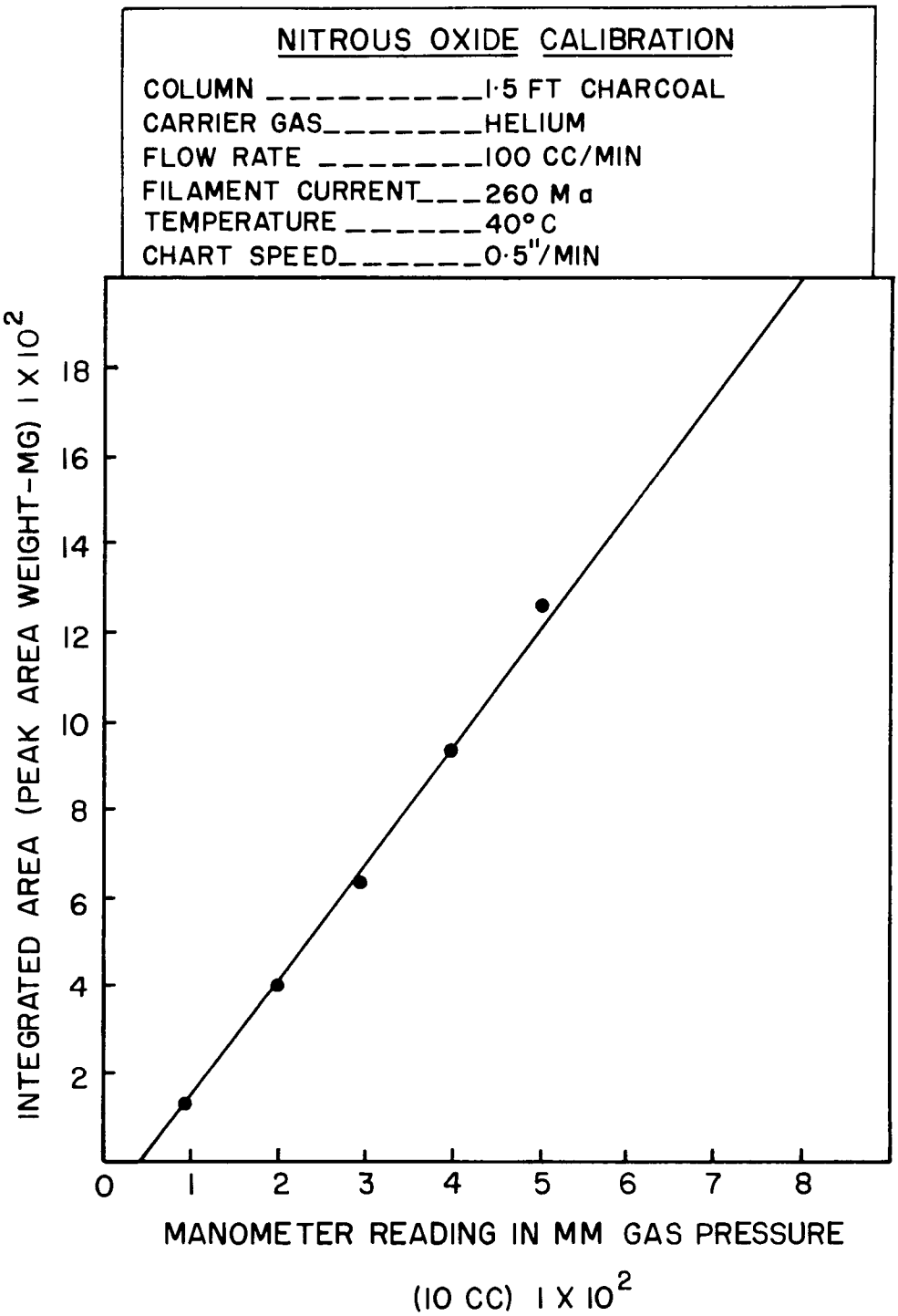


Figure 14. Nitrous oxide calibration curve

Isotope Tracer Studies

To investigate the origin of the gases evolved under sterile conditions, ^{15}N labeled sodium nitrite was utilized. The soil samples were treated and amended in the same fashion as described above. However, 12 and one-half grams of ^{15}N labeled sodium nitrite acquired from the International Chemical and Nuclear Corporation was added along with the same amount of ^{14}N sodium nitrite. The individual flasks were incubated for 14 days at 28 degrees C. and mass spectrometer analyses of the gaseous atmospheres were carried out by Dr. F. E. Broadbent at the University of California, Davis.

Solutions and Media

Several stock cultures of A-62 and P. stutzeri were maintained in the laboratory at 25 degrees C. The P. stutzeri was cultured on a medium consisting of (in grams): potassium nitrite, 1.0; trypticase, 10.0; yeast extract (Difco), 1.0; agar-agar (Difco), 15.0; and one liter of distilled water. A-62 was maintained on nutrient agar medium consisting of (in grams): beef extract (Difco), 3.0; peptone (Difco), 5.0; and agar-agar (Difco), 15.0; and one liter of distilled water.

Cells for nitrate and nitrite respiration studies were grown

in a nitrate reducing test medium (NRT):

Potassium nitrate	1.0 g
Potassium phosphate (dibasic and monobasic, 0.5 g of each)	1.0 g
Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Yeast extract (Difco)	1.0 g
Glucose	10.0 g
Magnesium sulfate	0.5 g
Ammonium chloride	1.0 g

For A-62 cells, nitrite was substituted for nitrate and the concentration changed so as to yield a final nitrite-nitrogen concentration of 200 μgrams per milliliter (200 ppm).

The pH of the media was adjusted to 7.0 and each medium was autoclaved at 121 degrees C. and 15 psig. for 15 minutes. The glucose, nitrate, and nitrite solutions were sterilized separately by membrane filtration and added aseptically to each medium after autoclaving. The median pore size of the membrane filters was 0.22 mμ.

RESULTS AND DISCUSSION

Soil Denitrification Studies

The primary objectives of the past several years of study have been to establish the fact that nonenzymatic denitrification does occur in certain soil systems, to correlate environmental and inherent soil characteristics with denitrification, and to compare the roles of both enzymatic and nonenzymatic denitrification as they occur in the nitrogen cycle.

It has only been within this decade that nonenzymatic denitrification was seriously considered of some agronomical importance. That nonbiological processes were possible had been realized for some time, but it was not until several years ago that the production of nitrogen gases, end-products of denitrification, was observed and measured (65, p. 20-41) in sterile soil and clay systems. Because soil microbiologists have believed for some time that the biological reduction of inorganic nitrogen such as nitrate and nitrite to nitrogen gas and other nitrogen oxides is the major pathway of soil nitrogen losses, the idea of nonbiological nitrogen reduction was accepted with much reservation.

With these concepts in mind, it was thought that the best approach to this problem was to design some experiments in which several soil systems would be used to prove or disprove the

suggestion of nonenzymatic denitrification. Therefore, three soils obtained from a well-established bench-marked catina from the Willamette valley were chosen. The soils used were Amity silty clay loam, Dayton silty clay loam, and Woodburn silty clay loam. These particular soils were chosen because they had been subjected to different environmental stresses and had not been commercially cropped or fertilized for the past 8 to 10 years. Each catina profile was sampled at the 8, 16, 24, 32, 40, and 48 inch depths. It was believed that the utilization of such samples would provide a broad range of soil types. These samples were then air-dried and pulverized to approximately 100 mesh in an electric shatterbox. After the water-holding capacity of each soil sample had been determined (all ranged between 52 to 56 percent), 50 gram samples, oven-dry basis, were placed in the previously described respirometer flasks and sterilized. Sodium nitrite (10 mg) was added to the flasks, which were then flushed with helium gas and incubated for seven days at 25 degrees C.

Table 1 shows the results obtained from gas chromatographic analyses of the Amity silty clay loam samples. The Amity silty clay loam is an imperfectly drained soil of Podzolic origin. As is shown in Table 7, the pH is on the acid side and there is considerable accumulation of iron and manganese oxide at each profile depth. It was surprising to find nitrogen evolution throughout

Table 1. Observed nitrogen losses with sterile Amity silty clay loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	3.9	0	16.9
16	3.3	0	10.3
24	4.4	0	8.0
32	7.3	0	4.2
40	5.4	0	1.0
48	7.2	0	1.2

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 2. Observed nitrogen losses with sterile Dayton clay loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	3.9	0	16.65
16	3.3	0	26.10
24	4.9	0	10.30
32	7.3	0	0
40	5.4	0	0
48	7.2	0	0

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 3. Observed nitrogen losses with sterile Woodburn silt loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	3.3	0	11.5
16	4.3	0	6.8
24	3.1	0	6.5
32	5.5	0	3.6
40	4.8	0	0
48	2.0	0	0

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 4. Observed nitrogen losses with Amity silty clay loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	3.9	0	17.2
16	3.8	0	11.1
24	4.7	0	7.8
32	8.1	0	3.6
40	6.0	0	0.7
48	8.0	0	1.0

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 5. Observed nitrogen losses with Dayton clay loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	4.2	0	15.8
16	3.9	0	27.1
24	5.8	0	12.8
32	8.6	0	0
40	5.3	0	0
48	7.4	0	0

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 6. Observed nitrogen losses with Woodburn silt loam*

Depth	Nitrogen loss mg (peak height)		
	N_2	N_2O	NO
Inches			
8	3.7	0	12.6
16	5.1	0	8.0
24	3.3	0	6.8
32	5.3	0	3.7
40	5.3	0	0
48	3.1	0	0

* NO_2^--N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity. Soils incubated under anaerobic conditions for seven days at 25 degrees C.

Table 7. Observed nitrogen losses with the nonsterile Amity silty clay loam*

Amity - gray brown podzolic (imperfectly drained)								
		Chemical analysis				Nitrogen loss		
						mg (peak height)		
Depth Inches	Profile description	pH	Base sat.	Fe_2O_3 %	MnO (ppm)	N_2	N_2O	NO
8	Dark brown, fine silt loam	5.6	44	0.90	402	2.3	3.3	0
16	Very dark brown, fine silt loam	5.6	-	-	-	3.0	0	0
24	Dark grayish brown, silty clay loam	5.6	62	0.96	-	2.2	1.5	0
32	Brown, light silty clay loan	5.7	-	-	-	1.5	0	0
40	Brown, light silty clay loam	5.9	83	1.03	462	4.7	0	0
48	Brown, light silty clay loam	6.3	91	1.09	396	1.4	0	0

* Amity taken from a catina soil distribution.
NO_2-N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity.
Soils incubated under anaerobic conditions for seven days at 25 degrees C.

the entire profile. Of immediate significance was the production of nitric oxide, a gas not usually thought to be evolved via denitrification. Significant quantities of both nitric oxide and nitrogen were observed. The origin of the latter gas is not yet known.

Table 2 shows the results of the Dayton soil incubation with sodium nitrite. This silty clay loam is a poorly drained soil and shows characteristics of mottling and rather pronounced formation of manganese granules. The data show that the highest level of nitric oxide was produced by the top soil horizons. In addition, a rather considerable volume if nitrogen was also evolved. The planosol nature of this soil and associated accumulation of reduced metals may account for the more pronounced nonenzymatic reactions. Refer to Table 8.

Table 3 presents data obtained from the Woodburn soil. This soil is characterized as a grey brown of podzolic origin and represents a moderately well-drained profile. The sterile Woodburn silty clay loam evolved both nitric oxide and nitrogen gas. Again, a typical pattern of greater nitric oxide production from the topmost horizons was observed with significant nitrogen gas production throughout the profile. Thus all three of the catina sites - Amity, Dayton, and Woodburn exhibited the same gas pattern under sterile conditions. From these initial experiments it was concluded that nonenzymatic nitrogen gas production was occurring.

Table 8. Observed nitrogen losses with nonsterile Dayton silty clay loam*

Depth Inches	Profile description	Dayton - planosol Chemical analysis pH	Base	Fe_2O_3 %	MnO (ppm)	Nitrogen loss mg (peak height) N_2	N_2O	NO
8	Dark grey silt loam	5.1	46	0.99	459	5.2	0	0
16	Grayish brown silt loam	5.7	61	1.23	396	5.4	0	0
24	Dark grayish brown silt loam	5.8	83	0.87	533	3.3	0	0
32	Olive gray clay	6.0	94	0.74	117	4.2	0	0
40	Olive brown heavy silty clay loam	6.1	95	0.72	478	4.7	0	0
48	Olive brown heavy silty clay loam	6.5	99	0.79	478	3.2	0	0

* Dayton-planosol taken from catina distribution.
NO_2-N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity.
Soils incubated under anaerobic conditions for seven days at 25 degrees C.

The soil samples were checked for sterility after each experiment by plating out one-tenth milliliter portions of the soil extracts, and incubating them under aerobic and anaerobic conditions. No growth occurred on any of the plates.

In order to firmly establish that sterile soils produced a typical pattern of gaseous end-products, control flasks were set up in which nonsterile soil samples were used. Again the Amity, Dayton and Woodburn silty clay loams were used. To these samples the same amount (10 mg) of sodium nitrite was added. The flasks were then flushed with helium gas as before and incubated for seven days under the same conditions prescribed for the sterile set. Tables 4, 5, and 6 show the gas chromatographic analyses of the flask atmospheres. Essentially the same results were obtained although nitrogen gas production was slightly greater in a few instances. It should be pointed out that even though these soil samples were not pre-sterilized, gas production similar to that of the pre-sterilized soils occurred. This indicates that the sodium nitrite exerted a toxic effect on those organisms present. Upon checking the soil samples for sterility by means of streak plates, it was found that no colony formations occurred. Thus this concentration of nitrite produced sterile soils.

Because nitrite is toxic to microorganisms at this concentration, it was decided to apply inorganic nitrogen in the form of

nitrite to nonsterile soils. Ten mg of nitrate-nitrogen as potassium nitrate were added to fifty g of the same three soils - Amity, Dayton, and Woodburn. The flasks were flushed with helium gas in the same manner as described earlier and incubated for seven days at 25 degrees C.

Tables 7, 8, and 9 show the results obtained along with some chemical analyses of the soil profiles. It was not surprising to find nitrogen gas evolution throughout the entire profile for each of the soil samples. With the exception of the Amity silty clay loam, greater nitrogen evolution occurred in the uppermost horizons. This may be attributed to a presumably higher microbial density in the top horizons. Nitrous oxide was evolved only in the Amity soil and may be attributed to an even greater cell population as well as specific bacteria present in the upper horizon of this soil. The highest level of enzymatic denitrification in the nonsterile samples seemed to occur in the Woodburn silty clay loam.

From the data presented in these tables, it can clearly be seen that there is production of nitrogen gases in both sterile and nonsterile soils, that the amounts of gases evolved varies with respect to soil depth, and that the spectra of gases in both sterile and nonsterile systems differ.

Table 9. Observed nitrogen losses with nonsterile Woodburn silt clay loam*

Depth Inches	Profile description	Woodburn - gray brown podzolic Chemical analysis pH	Base sat.	Fe_2O_3 %	MnO (ppm)	Nitrogen loss mg (Peak height) N_2	N_2O	NO
8	Dark brown silt loam	5.4	56	1.02	469	10.7	0	0
16	Dark brown silt loam	5.8	-	-	-	4.3	0	0
24	Dark yellowish brown, silty clay loam	6.0	63	0.97	459	1.5	0	0
32	Brown silty clay loam	6.2	75	1.03	439	0	0	0
40	Brown silt loam	6.2	87	1.14	298	1.6	0	0
48	Brown silt loam	6.3	87	0.87	307	0	0	0

*Woodburn taken from a catina soil distribution.
NO_2-N added to give 200 ppm; moisture adjusted to 60 percent of saturation capacity.
Soils incubated under anaerobic conditions for seven days at 25 degrees C.

In all horizons of the three soil samples, nitrogen gas production occurred. The only exception to this was the 48-inch, non-sterile Woodburn sample. The production of the various gases seemed to be affected by soil depth. As can be seen, the sterile Amity, Dayton, and Woodburn samples showed a trend in the quantity of nitrogen and nitric oxide evolved at different levels. In all of the sterile systems nitric oxide evolution was much greater in the top three depths than in the lower ones. In fact, no nitric oxide was produced in the lower three depths of the Dayton sample and the lower two depths of the Woodburn sample. On the other hand, nitrogen gas production was greater in the lower horizons of the soil profiles than in the upper ones. These results suggest that certain soil characteristics and environmental conditions affect the nonenzymatic reaction mechanisms of denitrification in such a manner so as to liberate different gaseous end-products.

Horizon Differentiation

Because the data obtained in the above experiments showed a trend in gas production with different soils, it was decided that a series of soil systems should be set up to follow the time course evolution of these gases. Therefore, distinctly different horizon samples were chosen to afford their differentiation with respect to the types and sequence of gas evolution.

The soil samples (50 g) were placed in the respirometer flasks, sterilized, and amended with 80 mg of nitrite-nitrogen as sodium nitrite and flushed with helium. They were then incubated as described earlier. At various time intervals the flask atmospheres were sampled and analyzed on the gas chromatograph. Table 10 shows the results of these analyses.

As is apparent by the data shown here, both the specific horizon and soil type determines the magnitude and rate of gas production. In the case of the Amity soil, an immediate high level of nitrogen was produced with correspondingly less evolution of nitric oxide. With the exception of the Dayton sample, the other soil types eventually (in 96 hours) produced significant amounts of both nitric oxide and nitrogen. Again, these data contribute to the idea that certain soil characteristics, probably physical and chemical, play an important role in the nonenzymatic denitrification reactions.

Nitrogen Balance Studies

As can be seen from the previous experiments nitrogen gases are formed via biological and nonbiological routes. However, one feature of both the sterile and nonsterile systems is that complete conversion to the gaseous state of the nitrate and nitrite added to the soils did not occur. The question which arose at this juncture

Table 10. Gas spectrum for selective profile horizons*

Soil		mg (peak height) Hours 24	48	72	96
Dayton-C_1-51"	N_2	1.25	1.95	1.95	7.55
	NO	0	0	0	0.14
Woodburn-A_p-5"	N_2	.70	1.55	2.10	10.15
	NO	5.55	7.30	4.20	5.60
Woodburn-B_{22}-30"	N_2	1.80	2.55	2.15	8.65
	NO	.70	1.65	1.15	9.55
Amity-A_{11}-6"	N_2	25.20	24.65	25.00	97.80
	NO	1.20	3.90	3.30	27.35

* NO_2-N--80,000μg.

Soil was sterilized for six hours at 121 degrees C. and 15 psig. Atmosphere--anaerobic.

was one concerning the fate of the inorganic nitrogen. Therefore, more refined experiments were set up in a manner to make possible a recovery account of the added nitrogen.

The soil chosen for such investigations was the Dayton silty clay loam. Fifty g of the 8, 16, 32, and 40-inch samples were added to two series of flasks. One series was sterilized, the other left untouched. To the sterile samples, 25 mg of nitrite-nitrogen and the same amount of nitrate-nitrogen were added to the non-sterile samples. All flasks were then flushed with helium gas and

incubated for 14 days at 25 degrees C. in an air-tight cupboard. In addition, 10 percent potassium hydroxide traps were placed within center wells of all the respirometer flasks before flushing. No additional hydrogen donor was added to the soil samples.

Table 11 shows the results of all analyses performed on each system. From the spectra of gases produced under sterile and nonsterile conditions, it is quite evident that nitric oxide and nitrogen comprise the major gases evolved from nitrite-nitrogen in the absence of biological activity. On the other hand, classical denitrification is depicted with nitrogen as the primary end-product. It is interesting to note that at the eight-inch level approximately three percent of the added nitrite-nitrogen was converted to nitrogen under sterile conditions, whereas four percent was evolved via the biological route. A similar comparison can be made for the 16, 32, and 40-inch depths. However, in this connection it should be remembered that no oxidizable carbon had been added to the Dayton soil, thereby minimizing the role of the active microbial denitrifiers. Yet, some such carbon probably occurs under normal field conditions.

Of some additional significance is the gradual decrease of 6.61 to 0.98 milligrams in nitric oxide production with depth along with a gradual increase in nitrogen production in the sterile Dayton soil. In these cases, there is a slight reduction in

Table 11. Relative fate of nitrate and nitrite nitrogen in Dayton silty clay loam*

		Sterile					Nonsterile				
		Soil extract		Gas analysis			Soil extract		Gas analysis		
Depth	pH	NO_3^-	NO_2^-	KOH**	NO	N_2	NO_3	NO_2	KOH	NO	N_2
Inches		mg			mg		mg			mg	
8	6.17	0	2.08	0.62	6.61	0.86	11.10	0.45	0.11	0	1.00
16	6.05	0	1.30	0.62	3.99	0.84	11.85	0	0	0	0.92
32	6.91	0	11.60	1.12	2.72	1.28	10.38	5.10	0.12	0	0.45
40	6.67	0	11.40	1.20	0.98	2.36	3.12	3.50	0.23	0	0.79

* Total input of NO_3 and NO_2-N = 25 mgs.

** NO absorbed in KOH and detected as nitrite-nitrogen.

hydrogen ion concentration with depth attesting to the supporting role of pH.

Other observations made were that there was some conversion of nitrate to nitrite in the nonsterile system but no conversion of nitrite to nitrate in the sterile system, thus producing some doubt as to the probability of nonenzymatic nitrification. Another point which should be made is that complete recovery of the nitrogen added as inorganic ion in both systems was not accomplished. No concrete conclusions can be drawn from this deficit. That large deficits existed in each soil sample is evident from Table 11. One explanation which may be of some validity is that there was formation of a complex ion with the nitrite. If so, this may have tied up the nitrite in both systems and rendered its detection impossible.

The data depicted in Table 11, along with similar data using other soil types, have brought into focus two points: the importance of nitrogen as an end-product of nitrite decomposition, and the relative magnitude of nitrogen loss in the sterile system.

Complex Ion Formation

Incomplete recovery of added nitrogen to the soil systems studied prompted inquiry as to the mechanism rendering such deficits. Therefore an experiment was designed so that mineral nitrogen analyses could be performed on influent and effluent

nitrite solutions.

Fifty ml of a 5000 ppm nitrite-nitrogen solution were passed at the rate of one drop per second through a glass column containing a mixture of washed and ignited sand, plus five parts molybdate, and one part ferrous iron. Mineralogical analysis of the effluent showed no nitrite to be present.

Conclusions which might be drawn are that the nitrite complexed with the molybdate and iron to render it no longer available or that the nitrite in such a complex was immediately converted to nitrogen gases. It is reasonable to assume that the same type of complex could be formed within the soil. If this occurred in the previously described experiments, the latter conclusion is probably not valid because only relatively small amounts of nitrogen gases were detected.

^{15}N Studies

To firmly establish the fact that nonenzymatic denitrification can occur, it was decided to determine the origin of the nitrogen gases evolved in sterile soil systems by applying appropriate tracer techniques.

In the first of such experiments, two soils, Woodburn-Ap-5 and Dayton-A_2-11, were selected. These soils were pulverized

in an electric shatterbox to approximately 100 mesh and 50 g of each were placed in respirometer flasks, sterilized, and brought to 60 percent of their water-holding capacity by the addition of a filter-sterilized nitrite solution containing 15 ml of sodium nitrite (85 mg of nitrite-nitrogen) and five ml of a solution containing 0.3 mg of ^{15}N tagged nitrate-nitrogen. The flasks were then flushed with helium gas and incubated for 14 days at 25 degrees C. as previously described.

The ^{15}N stock solution was in the form of 13 molar nitric acid which was obtained from the International Chemical and Nuclear Corporation. The ^{15}N sodium nitrite was prepared by reduction of the nitrate with an appropriate reductive agent. A six molar solution of ^{15}N labeled nitric acid was first made from the stock solution and this was neutralized to a pH of 7.1 by very careful pipetting with dilute sodium hydroxide solutions and using a Beckman pH meter. After neutralizing the ^{15}N nitric acid, the formed ^{15}N sodium nitrate was partially reduced to ^{15}N sodium nitrite by constant swirling of the solution in a tightly sealed flask containing zinc granules. The solution was constantly mixed in this fashion for about 12 hours after which five ml were added to each of the soil systems as mentioned above.

At the end of the incubation period, samples of each of the flask atmospheres were carefully transferred to 10 ml glass

sampling vials provided with a stop-cock at one end. Mass spectrometer analyses were then performed on the gas samples by Dr. F. E. Broadbent at Davis, California.

Reference to Table 12 calls attention to the fact that nitric oxide showed a significant ^{15}N enrichment whereas the nitrogen did not. However, there was a very small quantity of nitrogen present and this may explain the lack of enrichment for the latter gas.

Presumably any gas formed directly from nitrite would exhibit ^{15}N enrichment. Because the nitric oxide formed in the flask holding the Woodburn soil contained 1.4 percent ^{15}N and the nitric oxide samples from the Dayton soil system contained 0.86 percent ^{15}N, compared to the normal 0.36, it was concluded that this gas was formed directly from the nitrite under sterile conditions.

To further investigate the origin of nitrogen and nitric oxide, several soils which had been subjected to varying environmental stresses were chosen. To these samples were added 12.5 mgm of ^{15}N labeled sodium nitrite and the same amount of carrier sodium nitrite to yield a final concentration of 500 ppm nitrite-nitrogen. The ^{15}N labeled sodium nitrite was acquired from the International Chemical and Nuclear Corporation.

Table 12. Origin of nitric oxide.

Gas	Gas analyses	
	Dayton-A_2-11	Woodburn-A_p-5
Helium	98.30	92.70
Nitrogen	0.76	0.53
Nitric Oxide	1.10 (0.86% ^{15}N)	5.82 (1.40% ^{15}N)
Oxygen	0.02	0.03
Argon	0.08	0.08
Carbon dixoide	0.20	0.41

The soil samples were pre-sterilized in the usual manner after which they were aseptically amended, sparged, and incubated for seven days at 25 degrees C. Percent excess ^{15}N in the flask atmospheres was determined by Dr. F. E. Broadbent at Davis, California. The data given in Table 13 represents the first direct proof that nitrogen gas accumulation, as observed in these nitrite-nitrogen studies, evolves from nitrite and therefore must be regarded as a major nitrogen loss product, mediated via non-biological factors. The heavy enrichment shown by nitric oxide was as expected since this gas is a direct decomposition product of nitrite. The appearance of nitrous oxide as an ^{15}N enrichment

Table 13. Origin of gaseous end-products of nitrite decomposition*

Soil	% ^{15}N Excess			Relative proportion
	N_2	NO	N_2O	
Amity silty clay loam	4.10	29.4	5.22	NO > N_2 > N_2O
Cove clay	3.13	14.7	11.7	NO > N_2 > N_2O
Chehalis silty clay loam	2.94	19.4	14.2	N_2 > NO > N_2O
Cloquato silt loam	2.48	14.6	9.7	NO > N_2 > N_2O
Willamette silty clay loam	2.09	14.8	11.8	N_2 > NO > N_2O

* 500 ppm NO_2-N added to sterile soils and incubated for seven days at 25 degrees C. Analysis by Dr. F. E. Broadbent, Davis, California.

product is difficult to explain, because this gas had not been previously detected as a product of the nonenzymatic system. However, the possibility exists that it could be derived via nitric oxide. Also, nitric oxide was the predominant gas formed in three of the five soil samples.

Magnitude and Gas Spectra of Enzymatic and Nonenzymatic Denitrification

It has already been established that certain transformations of nitrogen lead to a net loss of the element from the soil through volatilization. In enzymatic denitrification, nitrogen is lost to the atmosphere, and is no longer available to the cell. The same is true for nonenzymatic denitrification. However, the nonenzymatic process differs from the enzymatic in several ways, particularly in the end-products. The spectra of gases evolved via both mechanisms appears to be different in all cases.

The present thinking relative to the balance that exists between enzymatic and nonenzymatic routes of gas loss is shown in Figure 15. It seems reasonable to assume that both are operative in normal field soils. However, because nitric oxide was not detected with the nonsterile soils, it appears that microbial nitrification drains the major part of nitrite to nitrate and under reduced oxygen tension the nitrate reductase probably drains

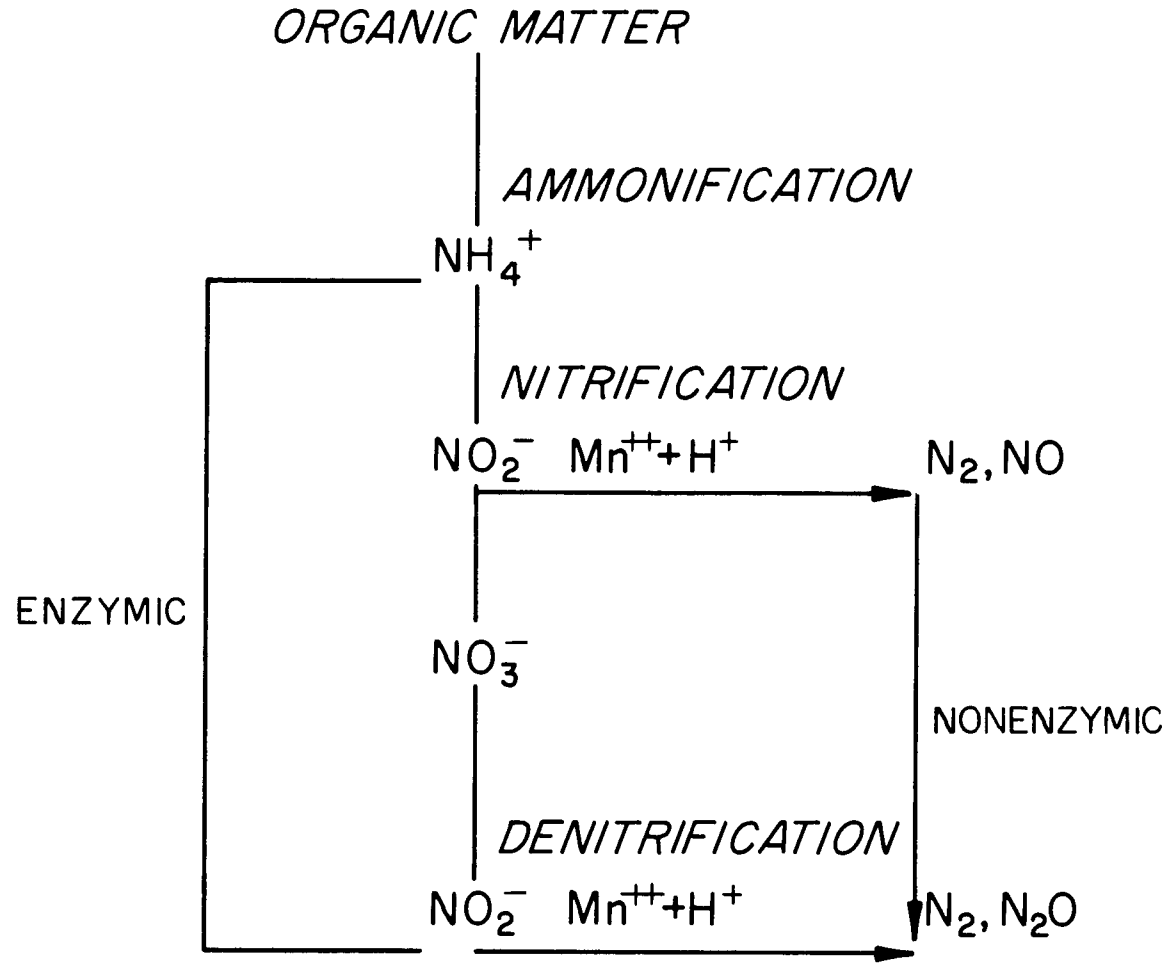


Figure 15. Enzymic and nonenzymic routes of gas loss

nitrate to nitrogen and nitrous oxide. One other alternative would be the possible role of nitric oxide as a precursor of nitrogen. Under such conditions any formed nitric oxide would be converted to nitrogen gas as a result of microbial activity and thus would not be detected in a nonsterile system.

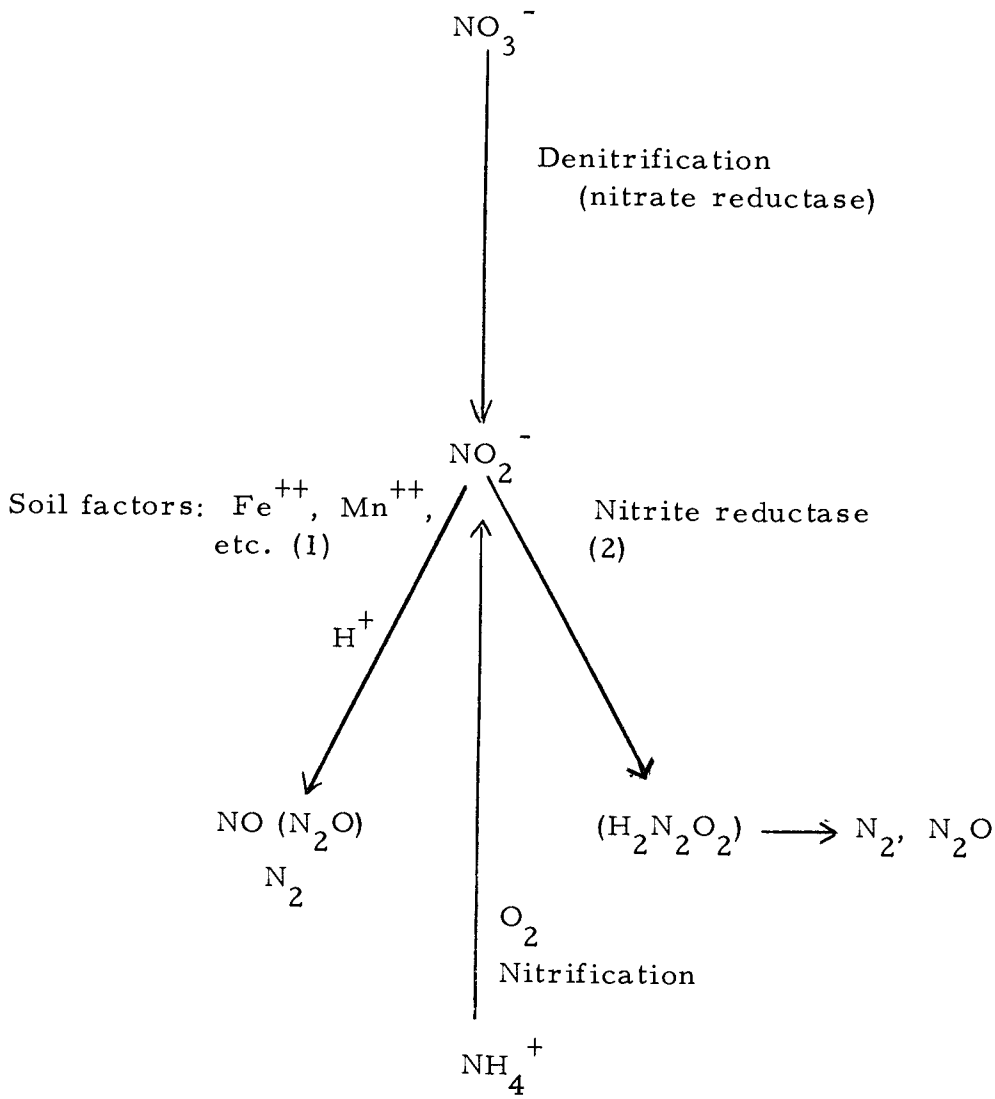
With these points in mind, experiments were designed to demonstrate the magnitude and the spectra of gases evolved via biological and nonbiological pathways. A variety of soil types were selected and treated in the previously described manner. Glucose was added as an additional hydrogen acceptor.

The data shown in Table 14 attest to the importance of the following transformations of nitrate and nitrite-nitrogen in soil. Nitrite becomes the critical intermediate formed via the denitrification and nitrification reaction sequences. In this regard, it is probable that the nitrate reductase enzyme is more active in the production of nitrite.

Table 14. Comparative percentage of nitrogen and oxides of nitrogen evolved with various soils under sterile and nonsterile conditions

Soil	pH	% NO_2 or NO_3 converted to gaseous state					
		Nonsterile*			Sterile**		
		N_2	N_2O	NO	N_2	NO	N_2O
Gooch sandy clay loam	6.1	64	0	0	14	8	0
Olympic sandy clay loam	6.7	83	0	0	2	20	0
Walla Walla sandy clay loam	7.3	90	0	0	14	7	0
Willamette sandy clay loam	6.2	105	0	0	26	63	0
Cloquato sandy loam	6.1	98	0	0	9	66	0
Amity sandy clay loam	6.1	62	0	0	18	57	0
Cove clay	5.8	112	0	0	14	37	0
Concord sandy clay loam	5.3	9	0	0	14	37	0
Marine sediment	8.9	30	4	0	2	0	0

* Nonsterile: input 28 mg NO_3-N; 2 percent glucose.
** Sterile: 28 mg NO_2-N; 2 per cent glucose.



With the above concept in mind it becomes quite apparent that the total volume or weight of nitrogen and nitric oxide evolved via route 1, nonbiological, approaches that of route 2, biological. It can be seen in Table 14 that enzymatic conversion exceeds the nonenzymatic. However, a consistent ratio of these values is not shown by the different spectra of gases evolved. It should also be

stressed that the extensive denitrification rates in nonsterile soils are probably much higher than one would observe in the field, because a rather high level of glucose was added to each test soil. On the other hand, the addition of glucose did not contribute to the nitrogen loss in the sterile soil systems. It appears, therefore, that under normal conditions of low levels of oxidizable carbon that biological reduction of nitrate to nitrite, and/or oxidation of ammonium to nitrite, plus the nonenzymatic conversion of nitrite to nitric oxide and nitrogen constitutes the primary loss pathway via nitrate to nitrite.

Thus it can be conceived that both enzymatic and nonenzymatic conversions are important in the over-all nitrogen cycle. One aspect which should be considered is that biological conversions of nitrite do also occur but to what extent, is not known. However, due to toxic effects, no biological nitrite conversion can occur under high concentrations of nitrite (>100 ppm). It can be postulated that the nonenzymatic process in situations of high nitrite concentration reduces this concentration in soil to a level tolerated by microorganisms, and that at these lower levels both enzymatic and nonenzymatic nitrite conversions can occur. To what extent nonenzymatic nitrite conversion occurs in field conditions it not known.

It should be reiterated that characteristically those gases evolved from nitrite under sterile conditions are nitrogen and

nitric oxide (Table 14). The gases evolved from nitrite under non-sterile conditions in some instances are nitrogen and nitrous oxide. No gas evolution has been observed when nitrate was added to sterile soil systems. Therefore it must be concluded that nitrite which is a critical intermediate in the denitrification and nitrification sequences is degraded nonbiologically and that nitrate, unless it is converted first to nitrite, cannot be degraded nonbiologically.

pH Effect

Nitrogen gas evolution via nitrite nitrogen may be explained in several ways:

Van Slyke (pH mediated) -

A) $RNH_2 + NHO_2 \longrightarrow N_2 + ROH + H_2O$

$NH_3 + HNO_2 \longrightarrow N_2 + 2H_2O$

(via $H_2N_2O_2$)

B) $5HNO_2 \longrightarrow N_2 + 3HNO_2 + H_2O$

C) Unknown mechanism.

In A, one would expect pH of 3.5 to 4.0 and in like manner with the B reaction sequence one would expect to have a pH of at least 4.0 to form nitric oxide. In addition, with the B reaction sequence, nitrate should be detected along with the nitrogen gas.

All reactions relating to the breakdown of sodium nitrite to gaseous end-products in soil involve hydrogen ions:

$$NaNO_2 + HX \longrightarrow NaX + HNO_2$$

$$HNO_2 \xrightarrow{H^+} H_2O + NO$$

$$\text{or } 4HNO_2 \longrightarrow 2NO + 2NO_2 + 2H_2O.$$

The more important question relates to other mediating soil factors, such as transition metals, organic matter, and cation exchange capacity. Figure 16 depicts the pH at which maximum conversion of sodium nitrite to nitric acid occurs and subsequent liberation of nitric oxide. At pH 3.0, approximately 50 percent of the added sodium nitrite was converted to nitric oxide; at pH 4.0, 20 percent; at pH 5.0, 10 percent; at pH 6.0, zero percent; and at pH 7.0, zero percent. It is apparent that a pH of 5.0 or less is required to bring about any significant breakdown of the added nitrite-nitrogen. It is significant that no apparent decomposition of nitrite occurred at pH 6.0 or above. One might theorize at this point that at a pH of 6.0 or above, the limiting reaction becomes the conversion of sodium nitrite to hyponitrous acid. At the same time, it becomes evident that because most of the soils studied showed a pH of 6.0 or more that other soil-associated factors are operative in the breakdown of nitrite. It is proposed that transition metals are implicated:

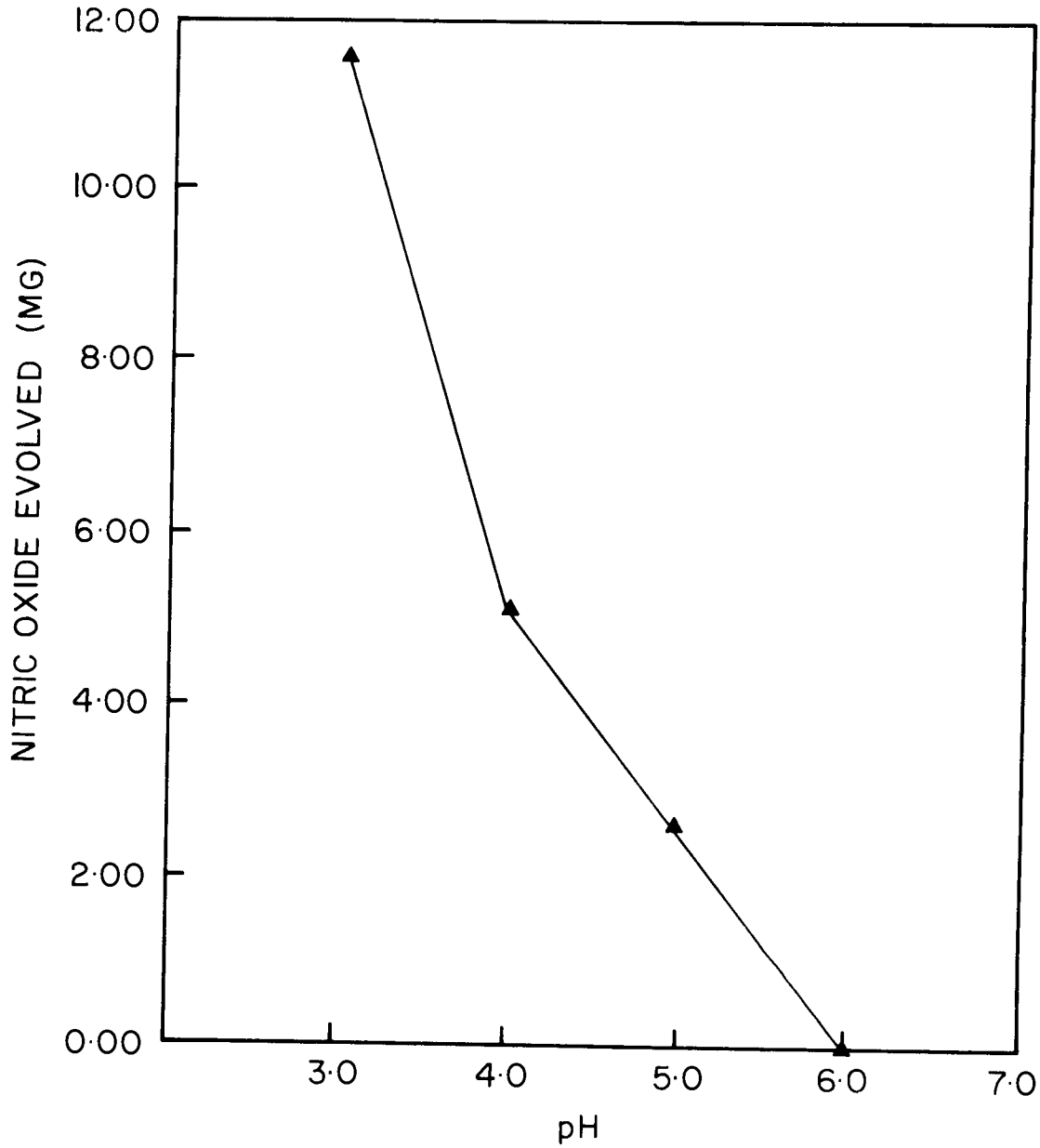


Figure 16. Effect of pH on conversion of sodium nitrite to nitric oxide

$$NaNO_2 + H^+ \longrightarrow HNO_2 + N_2$$

$$HNO_2 + Mn^{++} + 2H^+ \longrightarrow Mn^{+++} + NO + H_2O$$

$$N_2$$

A critical point is that nitrogen evolution was not observed in the same nitrite-nitrogen experiments. Nitric oxide was the only product. Thus nitrogen production appears to require other soil-contained factors not associated with the immediate acid decomposition of nitrite-nitrogen. This observation is also exemplified by the data on the Astoria silt loam and Cloquato silt loam soils given in Figures 17 and 18. Higher nitrogen values were obtained with the Cloquato soil (pH 6.1) and lower nitric oxide values than were observed for the Astoria soil (pH 4.8).

A-62 Studies

The use of a bacterium, designated as A-62, which was isolated from marine sediment (24, p. 71) to study the enzymatic denitrification process in soils of varying pH values was quite helpful. The organism involved in this study is unique in that it degrades only nitrite and not nitrate with the production of nitrogen gas. Because nitrite is an intermediate in the denitrification scheme, and because nitrite becomes toxic to microorganisms at high concentrations, the use of this particular bacterium became invaluable

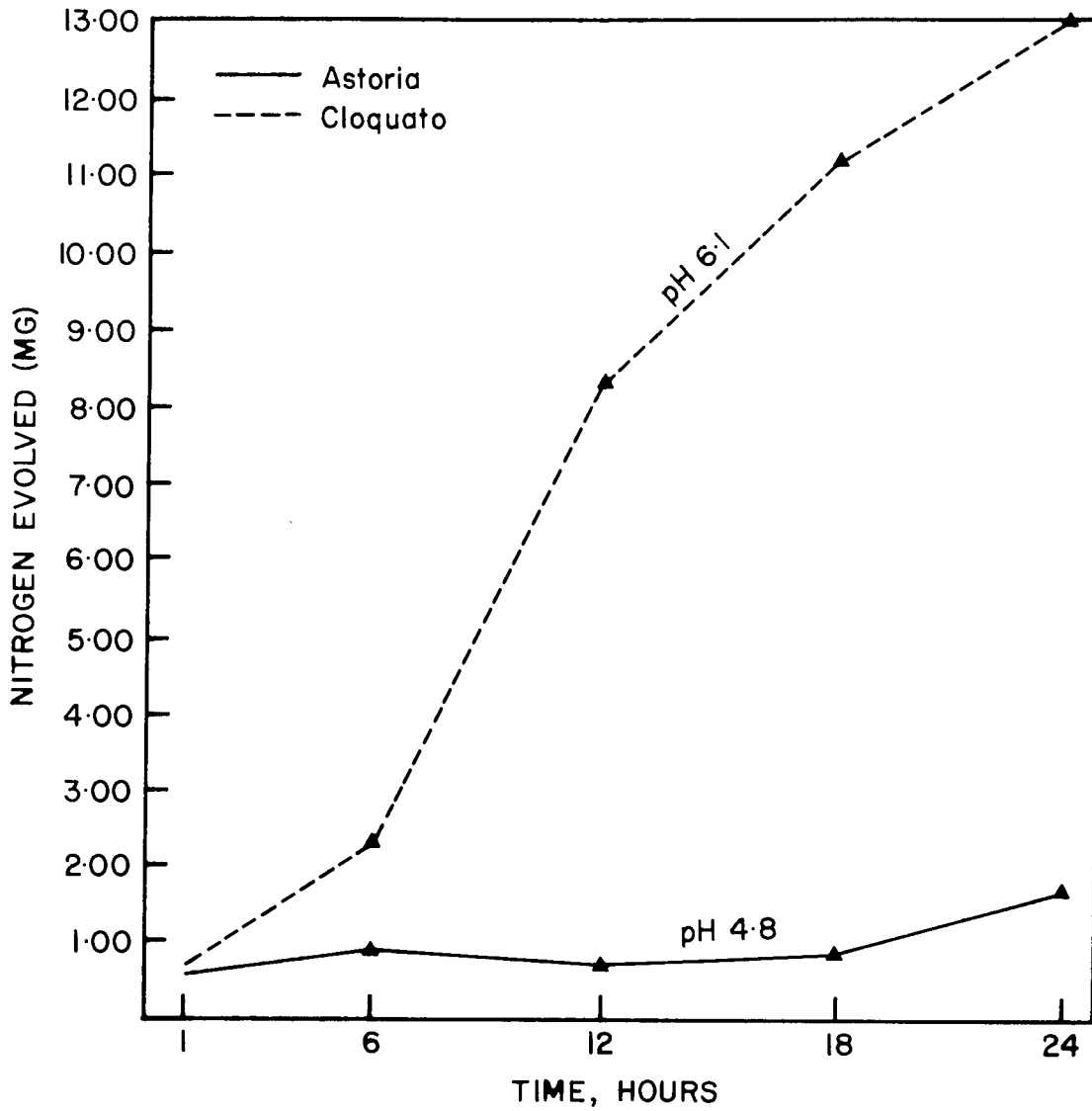


Figure 17. Effect of soil pH on nonbiological nitrogen production

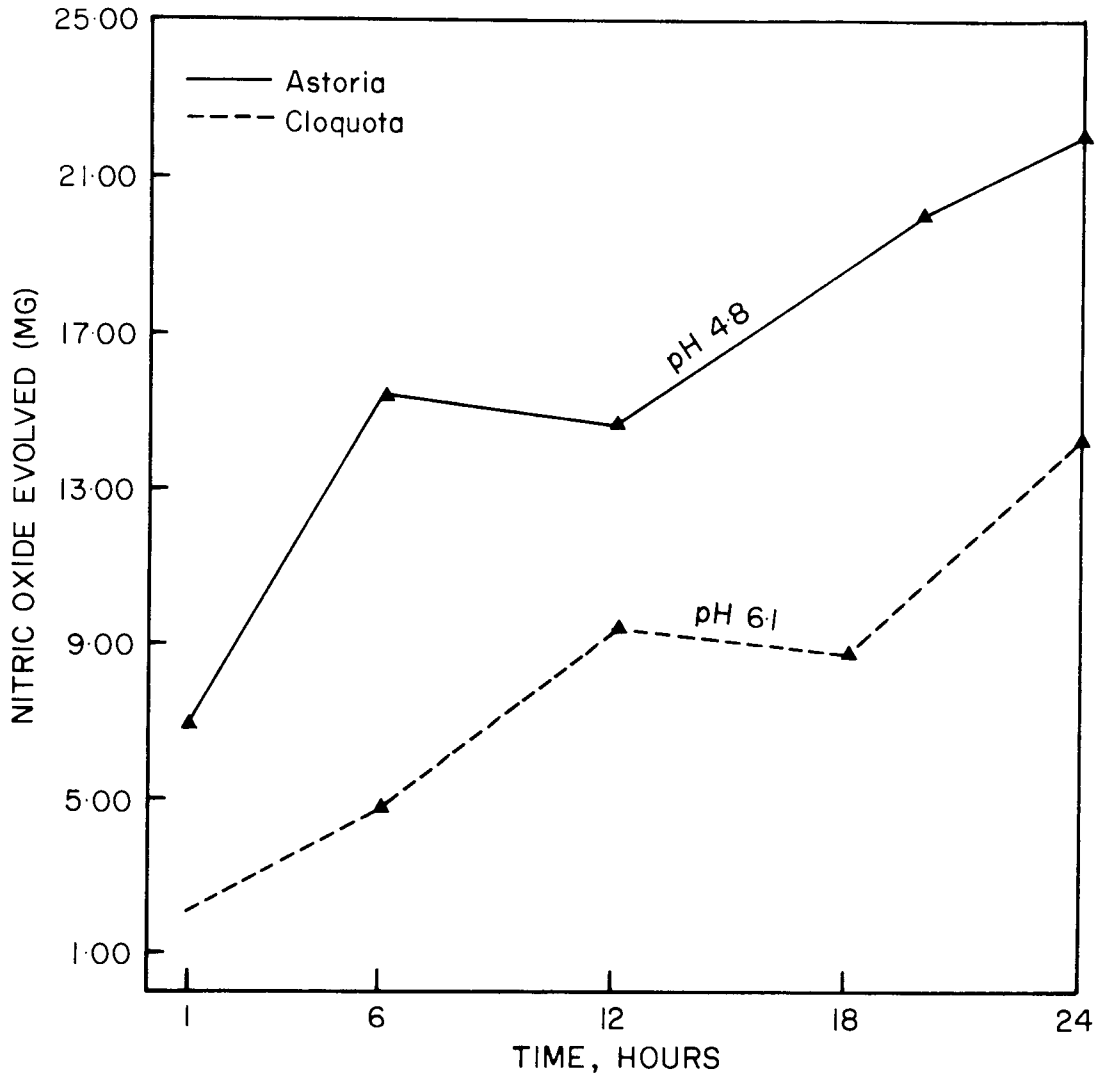


Figure 18. Effect of soil pH on nonbiological nitric oxide production

in studying both the enzymatic and nonenzymatic mechanisms of denitrification.

This particular series of experiments included the incorporation of A-62. The soil samples to which this organism was added were pre-sterilized as described previously. Five ml of a heavy slurry of cells, approximately 20 mgm./ml, washed in a buffer adjusted to pH 7.0, were asceptically added to the soil samples. These were incubated for 14 days at 25 degrees C. Control flasks which were used included sterile soils with no organism added, sterile soils with nitrite and Ps. stutzeri added, and sterile soils with nitrate and A-62 added. With the latter two controls no gas evolution was detected.

That the relationship of both denitrification processes is somewhat complex was further indicated by the experiments involving A-62. As can be seen from Table 15, nitrogen was the only gas evolved. The conversion of nitrite seems to have occurred enzymatically. Because no nitric oxide occurred, one might conclude that the nonenzymatic process was not functioning. Again, see Table 15 for a comparison of the gas spectra under both sterile and nonsterile conditions.

With the above mentioned facts in mind, it appears that the nonenzymatic and enzymatic denitrification processes are not related, in that their reaction sequences are probably different. But

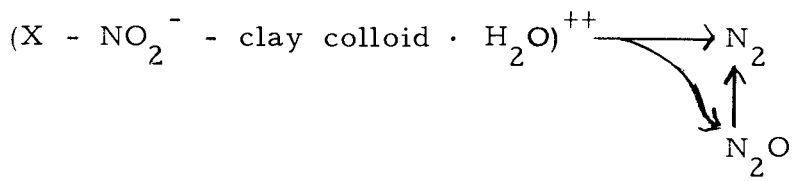
Table 15. Comparative percentage of nitrogen and nitrogen oxides evolved with various sterile soils amended with A-62

Soil sample	pH	Percent NO_2^- converted to gaseous state by A-62 in sterile soils*			Percent NO_2^- converted to gaseous state in sterile soils*		
		N_2	N_2O	NO	N_2	N_2O	NO
Astoria silt loam	4.8	98	0	0	7.0	0	71
Chehalis silty clay loam	6.1	93	0	0	23.0	0	33
Walla Walla silty clay loam	7.3	92	0	0	15.0	0	11
Corvallis sandy loam	8.9	74	0	0	0.9	0	11

* Total input NO_2^--N, 10 mg.; 2 percent glucose.

it should be pointed out that the nonenzymatic process may be extremely important in causing soil nitrogen deficits under field conditions, especially in situations in which a high C:N ratio and/or low oxygen tension exist.

One particular aspect of the data in Table 15 which has already been pointed out should be re-emphasized. The only gas evolved was nitrogen. This suggests that the conversion of nitrite to nitrogen does not involve nitric oxide with this particular organism. This idea is, of course, contrary to some other investigators' beliefs (15). Nevertheless, because no accumulation of nitric oxide occurred, it may be difficult to accept the idea of nitric oxide as an intermediate in this particular reaction. As for nonenzymatic denitrification, this question is unanswered. Whether nitric oxide is an intermediate is not known. The production of nitrogen does occur. The important question at hand is how does this reaction take place. The following scheme shows a postulated mechanism for production of nitrogen, in which transition metals (X) are included:



Role of Soil Characteristics and Environmental Factors

Up to this point, the major concern in the denitrification process has been the role of hydrogen ions in the conversion of nitrite to nitrogen gases. However, as has already been shown, pH alone cannot fully explain the instability of nitrite in a sterile soil system. Therefore, more thorough analyses of the different soil types were carried out in order to try to establish relationships between soil characteristics and the denitrification process, both enzymatic and nonenzymatic.

As has already been mentioned, the soils used in all of the experiments were chosen so as to afford a fairly broad spectrum of texture and pH. Along with this, certain other analyses were performed which characterized the individual soil systems used. Analyses were made for cation exchange capacity, exchangeable potassium, calcium, magnesium, and sodium; and organic matter, carbon-nitrogen ratio, particle size distribution, and moisture tension. The results of these analyses are found in Tables 16 and 17.

As can be seen from the data in Tables 15, 16, and 17, no clear-cut relationships can be definitely drawn between certain of these soil characteristics and nonenzymatic denitrification. No parallelism exists such as can be observed with enzymatic

Table 16. Chemical analyses of all soils studied

Sample	Soil pH	H_2O %	Ash %	Total C %	Kjeldahl N%	C/N
Amity 8"	5.6	1.93	95.21	0.9391	0.09	10
16"	5.6	1.91	95.16	0.8681	0.08	11
24"	5.6	2.45	95.16	0.3985	0.05	8
32"	5.7	2.92	95.78	0.2825	0.03	9
40"	5.9	2.92	95.14	0.2430	0.03	8
48"	6.3	3.01	95.10	0.2580	0.03	9
Dayton 8"	5.6	1.59	95.29	0.8490	0.09	9
16"	5.7	2.30	95.10	0.4095	0.05	8
24"	5.8	3.75	93.38	0.2989	0.04	7
32"	6.0	3.48	94.28	0.1624	0.02	8
40"	6.1	2.99	94.92	0.1542	0.02	7
48"	6.5	2.18	96.17	0.1228	0.02	6
Woodburn						
8"	5.4	2.02	94.53	1.4701	0.12	12
16"	5.8	1.77	94.42	0.9855	0.10	10
24"	6.0	1.91	94.76	0.5023	0.06	8
32"	6.2	3.08	95.25	0.1911	0.03	6
40"	6.2	2.71	94.55	0.1460	0.03	5
48"	6.3	2.72	94.52	0.1979	0.02	10
Chehalis	6.1	2.62	92.05	1.7881	0.16	11
Cloquato	6.1	2.10	93.40	1.5042	0.13	12
Concord	5.3	2.09	95.09	1.5288	0.13	12
Corvallis	8.9	0.98	96.74	1.1343	0.09	13
Cove Clay	5.8	5.46	87.53	4.7666	0.30	16
Gooch	6.1	2.08	92.21	2.8705	0.18	16
Olympic	6.7	3.90	86.23	1.5297	0.13	12

Continued

Table 16. Chemical analyses of all soils studied (Cont'd)

Sample	Soil pH	H_2O %	Ash %	Total C %	Kjeldahl N%	C/N
Walla Walla	7.3	1.77	95.47	1.7840	0.15	12
Willamette	6.2	2.01	94.07	2.3819	0.17	14
Astoria	4.8	2.01	92.03	1.5301	0.13	12

Table 17. Mechanical and physical analyses of all soils studied

Sample		Total sand 2.0-.05	Total silt .05-.002	Total clay .002	Moisture Tension 1/3 ATM	Moisture Tension 15.0 ATM	CEC me/100g	K me/100g	Ca me/100g	Mg me/100g	OM %	Na me/100g
Amity	8"	5.35	73.05	21.62	30.63	10.60	16.77	0.32	7.8	2.5	2.74	0.14
	16"	5.18	73.24	21.59	29.95	10.47	19.42	0.20	8.0	2.8	0.11	0.16
	24"	3.95	70.32	25.76	33.32	13.13	23.45	0.36	11.8	4.8	0.05	0.21
	32"	3.80	68.96	27.22	40.46	15.89	33.42	0.48	15.5	6.9	0.21	0.32
	40"	3.87	68.98	27.16	42.03	16.10	33.80	0.53	15.8	6.9	0.26	0.34
	48"	3.80	69.88	26.29	41.67	14.85	31.42	0.50	15.3	7.1	0.37	0.32
Dayton	8"	8.21	74.22	17.55	31.66	8.23	19.35	0.18	3.8	2.3	1.63	0.16
	16"	6.06	67.22	26.15	30.88	12.42	23.33	0.32	6.2	6.1	0.79	0.39
	24"	1.98	51.42	46.62	44.17	25.00	34.78	0.57	16.0	14.2	0.63	1.10
	32"	2.10	64.49	33.36	41.52	18.59	36.74	0.55	14.0	13.6	0.37	1.15
	40"	1.08	70.66	28.32	43.21	16.88	35.69	0.45	12.9	12.4	0.32	1.15
	48"	12.69	69.01	18.26	34.22	11.98	21.03	0.43	10.5	8.8	0.32	0.80
Woodburn	8"	8.18	71.09	20.75	30.25	9.58	15.16	0.65	8.5	1.6	2.42	0.10
	16"	3.49	70.00	26.49	32.33	14.04	16.40	0.53	8.2	2.8	2.00	0.13
	24"	4.22	66.31	29.47	32.32	15.06	18.06	0.55	10.1	3.7	1.00	0.14
	32"	3.41	63.90	32.70	38.09	17.86	24.21	0.72	14.0	5.6	0.53	0.18
	40"	4.90	64.81	30.25	38.81	17.18	23.23	0.67	14.0	6.1	0.37	0.21
	48"	4.44	65.85	29.69	39.73	17.16	22.95	0.67	11.8	5.8	0.42	0.24
Corvallis		68.87	23.12	7.96	11.70	5.34	8.47	0.54	11.8	5.8	2.16	1.38
Chehalis		11.07	61.88	27.11	29.26	16.67	28.21	0.80	17.2	6.5	3.16	0.27
Cloquato		37.86	46.46	15.73	27.36	12.06	23.24	0.93	12.9	5.1	2.52	0.22
Gooch		8.30	63.43	23.20	30.62	11.71	21.36	1.08	9.8	2.3	5.63	0.12
Olympic		10.77	53.01	36.22	34.58	21.44	32.39	1.91	10.8	9.0	5.58	0.11
Walla Walla		16.74	67.84	15.41	23.48	8.87	19.39	1.6	11.6	3.7	3.26	0.06
Willamette		2.97	74.27	22.76	32.73	9.70	16.15	0.82	7.3	1.9	4.05	0.05
Cove clay		11.93	41.03	47.04	33.10	21.06	47.64	0.77	18.3	11.2	8.47	0.25
Concord		6.48	70.91	22.60	33.07	9.81	15.43	0.29	3.5	1.6	2.68	0.14
Astoria		12.40	49.10	38.50	48.00	29.00	55.20	0.94	0.4	1.0	2.11	0.08

denitrification. Tables 15, 16, and 17 show that with decreases in the cation exchange capacity, moisture tension, and clay fraction, there is a corresponding decrease in nitrogen production. However, nitrogen production decreases with increasing pH values. The same general scheme exists with nitric oxide production in the nonenzymatic process. Nevertheless, a low pH does affect nonenzymatic nitrogen production. On the other hand, lower cation exchange capacity, clay fraction, and moisture tension along with an increased pH appear to render the nonenzymatic denitrification process practically ineffective in nitrogen gas production.

SUMMARY AND CONCLUSIONS

Determinations of the magnitude of nitrogen loss mediated via biological (enzymatic) and nonbiological (nonenzymatic) have been made. Verification of the origin of the nitrogen gases evolved via the enzymatic versus nonenzymatic routes by using appropriate tracer techniques has also been accomplished. Further, the effect of various soil characteristics and environmental factors on nitrite and nitrate decomposition were evaluated.

From the initial experiments involving various soil horizons, both sterile and nonsterile, it was shown that nonenzymatic and enzymatic of nitrogen gases occurred. The amounts of gases evolved vary with respect to soil depth, and the spectra of gases in both sterile and nonsterile systems differ. In virtually all horizons of the soil samples used, nitrogen gas production occurred. In all of the sterile systems, nitric oxide evolution was much greater in the upper horizons than in the lower ones. On the other hand, nitrogen gas production was greater in the lower horizons than in the upper ones. The data suggest that certain soil characteristics and environmental conditions affect nonenzymatic denitrification in such a way so as to cause liberation of different gaseous end-products.

The initial studies also showed that both the soil type and the specific horizon determine to a certain extent the magnitude and rate of gas production.

The nitrogen balance studies coupled with the investigations of pH and other soil characteristics in regard to the magnitude and spectra of gases produced established the importance of nitrogen gas as an end-product of nitrite decomposition and the relative magnitude of nitrogen loss in sterile soil systems. The spectra of gases produced under sterile and nonsterile conditions differ. It is quite evident that nitric oxide and nitrogen comprise the major gases evolved from nitrite-nitrogen in the absence of biological activity. On the other hand, classical denitrification is depicted with nitrogen gas as the primary end-product.

In experiments designed to show the magnitude and spectra of gases formed via biological and nonbiological routes, it was observed that enzymatic conversion exceeds the nonenzymatic. However, a consistent ratio of the value obtained was not found for the different spectra of gases evolved.

The data obtained from ^{15}N tracer experiments represents the first direct proof that nitrogen gas evolves from nitrite and therefore must be regarded as a major nitrogen loss product, mediated via nonbiological factors. The heavy enrichment shown by nitric oxide was an expected result because this gas is a direct

decomposition product of nitrite.

Both enzymatic and nonenzymatic conversions are important in the over-all nitrogen cycle. To what extent biological conversion of nitrite occurs is not known. However, under high concentrations of nitrite (>100 ppm) no biological nitrite conversion can occur due to toxic effects. Therefore, it is probable that the nonenzymatic process does not occur in soil at nitrite levels tolerated by microorganisms, and that at these lower levels both enzymatic and nonenzymatic nitrite conversions can occur. The extent of nonenzymatic nitrite conversion occurring in field conditions is not actually known.

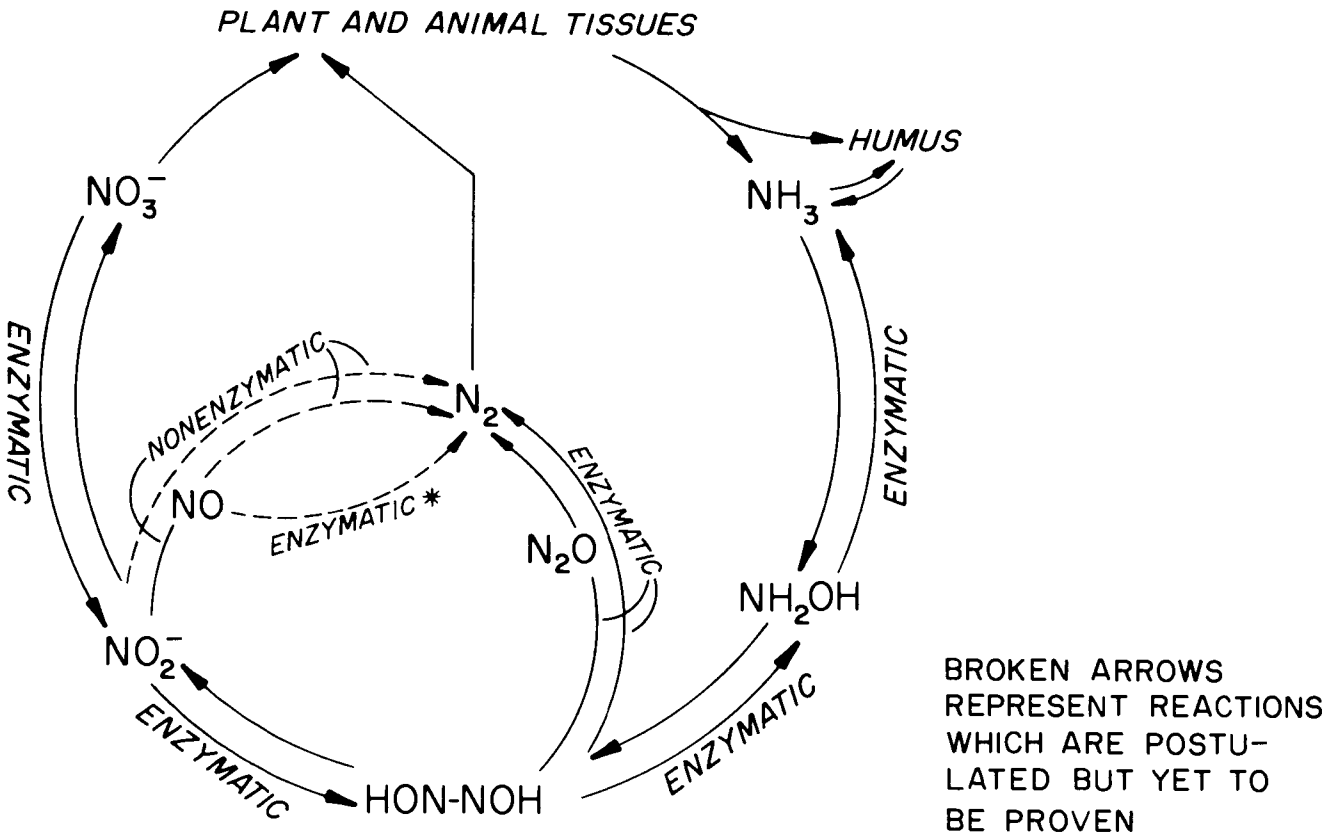
The relationship of both denitrification processes is somewhat complex. This was exemplified in experiments involving a bacterium isolated from marine sediment. In these studies it was noted that no measurable nitric oxide production occurred. It was concluded from this that the nonenzymatic process was not functioning. Apparently the nonenzymatic and enzymatic denitrification processes are not related because their reaction sequences appear to be different. Because nitrogen was the only gas evolved, it is believed that the biological conversion of nitrite to nitrogen does not involve nitric oxide, which was not found to be an intermediate in this reaction. As for nonenzymatic denitrification, the mechanism of the reaction is unknown. The production of

nitrogen gas does occur, but by what reaction sequences remains undetermined. A reasonable explanation is that transition metals are involved. Complex formation with transition metals, nitrite ion, and clay colloidial particles is possible and may be the basis for nonbiological denitrification.

The factors affecting the instability of nitrite in sterile soil systems are pH, cation exchange capacity, water tension, organic matter, and clay fraction. The enzymatic process is also affected by these factors. No clear-cut relationships can be definitely drawn between these soil characteristics and nonenzymatic denitrification. No parallelism exists such as was observed with enzymatic denitrification. Decreases in the cation exchange capacity, moisture tension, and clay fraction, along with increasing pH appeared to decrease enzymatic nitrogen production and nonenzymatic nitric oxide production.

The results obtained from the experiments outlined in this dissertation provide evidence that nonbiological route(s) of soil nitrogen loss do exist and must be given equal emphasis in the classifical denitrification pathway. Because nitrogen represents a major end-product of the nonenzymatic mediated breakdown of nitrite-nitrogen, it is difficult to differentiate between the relative contribution of these routes to soil nitrogen losses. However, it becomes clear that with nitrate- and nitrite-nitrogen, greater

field losses of nitrogen occur than have been previously considered possible, particularly because pH represents only one soil factor influencing the conversion of nitrate or nitrite salts to the gaseous state. With this in mind, a few changes can be made in the classical drawing of the nitrogen cycle. The production of nitrogen gas should now be depicted as in Figure 19.



*Chung, C.W. and V.A. Najjar. J. Biol. Chem. 218, 1956. Postulated this reaction in which they stated that NO is the key intermediate.

Figure 19. Role of denitrification in the nitrogen cycle

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