DENITRIFICATION OF UREA AND SODIUM NITRITE IN SOME MANITOBA SOILS

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ABSTRACT

The kinds and amounts of nitrogen gas lost during the oxidation of urea were determined. One thousand (1000) ppm-N as urea labelled with 52.4% ^{15}N was uniformly mixed with soil to approximate the concentration of urea - N near the pellet site or fertilizer band. With Wellwood soil, gaseous losses as N_2O and N_2 amounted to approximately 25% of the added N at pH 6.1 in 2 months. Samples of this soil were shown to accumulate NO_2^-. The appearance of NO_2^- coincided with the appearance of gaseous N products in the soil atmosphere. Gaseous production declined as NO_2^- oxidized to NO_3^-. The majority of the N_2 gas (which accounted for 40% of the N evolved) resulted from a van Slyke-type reaction in which one atom of N came from a soil source and one came from the fertilizer. When the pH of the Wellwood soil was increased to 7.6 by the addition of 10% $CaCO_3$, the accumulation of NO_2^- occurred to a greater extent and lasted for the duration of the study. Liming slightly increased losses of nitrogenous gases. The rate of gaseous evolution slowly declined with time even though NO_2^- persisted for the duration of the study. In identical experiments, very little gaseous loss occurred from a Neuenberg soil (pH 7.1) even when the pH was lowered to 6.0 or raised to 8.0.

Nitrate applied as $Ca(NO_3)_2$ to the Wellwood and Neuenberg soils was stable during aerobic incubation.

The rate of gaseous evolution tended to decrease with increased soil

pH and increased with increasing initial NO_2^- concentration when the Wellwood soil was treated with varying concentrations of $NaNO_2$. This trend was especially evident in the case of van Slyke-type N_2 evolution. Further work showed sterilization of soil had little effect on the rate of van Slyke-type N_2 evolution although it greatly decreased the rate of N_2O formation. This indicates the evolution of van Slyke-type N_2 was a chemical process while the production of N_2O was, for the most part, a biological reaction.

Solution studies were performed using the amino acid glycine and NO_2^- between pHs 2 and 8 inclusive. The van Slyke reaction did not occur at any significant rate at pH values in excess of 5.0

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INTRODUCTION

Agricultural scientists are very concerned with improving the plant utilization of applied nitrogen fertilizers. Many studies have been carried out in order to increase the efficiency of fertilizer added to the soil since plant recoveries from applied nitrogen are usually only about 50% of that added. One method of increasing the efficiency of nitrogen fertilizers is to decrease losses by denitrification.

One much-studied fertilizer is urea. This product has several characteristics, which have resulted in its increased use in recent years. However, on occasion, recoveries have been found to low with this product, especially under conditions where nitrite has been found to accumulate.

Gaseous losses of nitrogen as N_2O and N_2 have been recorded by many workers where nitrite accumulates. Factors affecting the rate of loss and the exact mechanism of loss are in doubt. Recent reports have also implicated N_2O formed from these products in the depletion of the ozone layer of the stratosphere.

Many theories have been put forward to explain the mechanism of formation of N_2. One theory suggests N_2 is formed by a van Slyke process where one atom of evolved N_2 comes from the soil N and one comes from nitrite. This theory is generally not accepted as occurring in most agriculturally important soils. Instead, the preponderence of work seems to show N_2 as being a reduction product of N_2O or indicates N_2 is formed via a transitory association of NO_2^- with phenols or carbonyl groups in the soil organic matter. To further elucidate the mechanism

involved in the formation of N_2 in fertilizer-treated soils a study was undertaken with Wellwood soil using urea as the nitrogen source. The work reported in this study involved incubations of soil and urea under aerobic conditions and in this respect was similar to conditions usually found in the field. The concentrations of nitrogen fertilizer used were quite high for, in the field, high concentrations of N occur in the soil adjacent to the fertilizer granule.

Losses of gaseous N were studied using ^{15}N tracer techniques developed in this laboratory. The effect of biological activity, soil pH and nitrite concentration on denitrification were studied.

LITERATURE REVIEW

Organic Nitrogen

At least 90% of the total nitrogen in soils is found in organic combination, (Bremner, 1965b). This nitrogen is quite stable in soils and is only slowly available to plants. Neither the reasons for this stability nor the forms of organic nitrogen are well understood.

Using acid hydrolysis methods, it has been determined that up to 40% of the nitrogen is in the form of amino acids, 10% as hexosamines and 1% as nucleic acids. These compounds themselves are not inherently stable in soils and it is only after microbial decomposition and excretion that resistance to further destruction is conferred upon the product. Several of the theories proposed to account for this stability assume a reaction between partially decomposed lignins, phenols, or other ring structures and amino compounds (Gottlieb and Hendricks 1946, Bennett 1949).

It has also been postulated by Stevenson (1960), and Bremner (1965b) that polymers of glycosamines (termed melanoidins) formed by reactions of soil carbohydrates with amino structures are responsible for some of the nitrogen stability in soils.

Since no single theory can account for all forms of soil nitrogen, it is probable that stabilization is the result of several mechanisms acting on different components at one time.

Inorganic Nitrogen

Inorganic forms make up less than 10% of the soil nitrogen but are the most important agriculturally. It is only as ammonium, nitrite, or nitrate, that plants can assimilate nitrogen. Ammonium and nitrate tend to be the predominant forms of nitrogen in soils. Nitrite is rarely present in large amounts except in cases to be described later.

There exists in soil an interchange between organic and inorganic forms of nitrogen as evidenced by the work of Jansson et al. (1955). They were able to show that through the action of decomposers organic nitrogen enters an inorganic pool - a process termed mineralization. This mineralized nitrogen mixes freely and is available to microbes for protein synthesis. The rate at which this inorganic pool is depleted depends on the C/N ratio of the decomposing substrate. If the ratio is greater than 30:1 nitrogen becomes rate limiting to microbial growth and cells must utilize the inorganic nitrogen for amino acid production. In such a case, the amount of inorganic nitrogen available to plants is diminished and that assimilated by the soil or microflora is

transformed into sparingly available organic forms. This process is termed immobilization. At C/N ratios between 20 and 30, immobilization may or may not occur, depending on the environmental conditions of the soil. If however, the ratio in the decaying tissues is less than 20:1, nitrogen is present in excess of microbial needs and will be released by the decomposers in the form of ammonia. Thus there will occur a net increase in plant-available nitrogen through mineralization.

Urea Hydrolysis

Urea is an organic compound which is deaminated by the enzyme urease to ammonia via the following pathway, (Court et al. 1964).

$$\underset{\text{(urea)}}{CO(NH_2)_2} + H_2O \rightarrow \underset{\text{Ammonium carbamate}}{NH_4CO_2^- \cdot NH_2^+ H_2O} \rightarrow \underset{\text{Ammonium Carbonate}}{(NH_4)_2 CO_3} \rightarrow \underset{\text{Ammonium}}{2NH_4^+} + CO_3^=$$

This process, termed ammonification by Bartholomew (1965), goes to completion without any accumulation of the intermediates. Further oxidation of NH_4^+ to NO_2^- and NO_3^- occurs via the nitrification pathway.

Ammonification of urea requires the presence and activity of the enzyme urease. The enzyme is produced chiefly by the urobacteria (Wallace and Smith 1954), although it is also found in many other microorganisms as well. It is also found in the residues of dead plants. Urease is quite stable in dry soil and its activity will not decrease significantly with prolonged storage (Gould et al. 1973).

Although adsorption may decrease urease activity, adsorbed urease

is much more stable than the free enzyme (Zantua and Bremner, 1976, 1977) Pinck and Allison (1951) suggested adsorption onto soil colloids protected the enzyme. Burns et al. (1972) feel the urease is incorporated into soil organic matter. If this were the case, small molecules such as urea and water could move through pores in the organic matter to the enzyme site and NH_4^+ could move out. Proteolytic enzymes are too large to get in close enough to destroy the urease. This would explain why soil urease is not as vulnerable as free urease to proteolysis. The characteristic urease level of a soil is determined by the degree of protection that soil can provide.

Urease in the soil may be either directly associated with soil microorganisms or adsorbed by soil colloids (Chin and Kroontje, 1963), a situation which raises the Km of the enzyme and lowers its activity (Paulson and Kurtz, 1970). This could be due to a change in enzyme caused by colloidal adsorption which results in a decreased affinity for urea (Gould et al. 1973).

The reaction rate of urea hydrolysis has been shown to follow Michaelis-Menton kinetics and to be first order with respect to concentration of added urea when urea levels are low (Court et al. 1964, Fisher and Parks, 1958, Overrein and Moe, 1967). Simpson and Melsted (1963), as well as Laidler and Hoare (1949), found that saturation of the enzyme complex will occur as concentration of urea becomes high; the upper limit varying with the soil urease level and temperature (Overrein and Moe, 1967). As saturation levels are approached, the hydrolysis reaction becomes zero order and

may even become negative. This shift may be partially explained by end product inhibition for, if excess ammonium is added to the soil, urea hydrolysis slows (Laidler and Hoare 1949). Although urea may be absorbed by soil colloids (Broadbent et al. 1958, Said 1972) and soil urease amounts vary with soil type (Vasilenko, 1962), the levels of soil urease are usually sufficiently high to allow complete hydrolysis of urea in less than a week. Rarely does urea reside in the soil for longer periods.

As microbial tissue is the most important source of soil urease, physical and chemical conditions of the soil (moisture, temperature, pH, etc.) which optimize the microbial growth also optimize urease production and ultimately urea hydrolysis rate (Chin and Kroontje 1962).

The activity of soil urease with respect to temperature may be described by the Arrhenius equation.

$$\log k = \frac{-Ea}{2.303\ RT} + \log A$$

k-rate constant
T-temp ^{o}K
Ea-activation energy
R-gas constant
A-a constant

It is evident that the rate constant increases with a rise in temperature. Using an Arrhenius plot Gould et al.(1973) were able to show that hydrolysis rate increased linearly between 2^{o} and $45^{o}C$. Similar trends have been reported by other workers (Fisher and Parks 1958, Simpson and Melsted 1963, Overrein and Moe 1967). The activation energy determined by Gould et al. (1973) was 9.8 kcal/mole.

Although urease is active over a wide pH range, the optimum pH is around neutrality (Vasilenko 1962).

The effect of moisture content on urea hydrolysis is not pronounced except in cases of very low moisture content (Volk 1966, Gould et al. 1973, Simpson and Melsted 1963, Vaselinko, 1962). Volk found only 7% hydrolysis in six days with air-dry soil; 98% with the same soil at 67% of field capacity. Gould et al. (1973), showed a slight decrease in hydrolysis rate as moisture content rose above field capacity, possibly due to decreased oxygen diffusion and therefore decreased microbial activity.

It has been shown that increased soil organic carbon levels will lead to increased rates of hydrolysis (Simpson and Melsted 1963, Gibson 1930, Gould et al. 1973, Conrad 1940, Zantua and Bremner 1976). Moe (1967) was able to increase urease activity by 40% upon mulching soils. High amounts of organic carbon will promote microbial growth and thereby increase urease content of the soil in question.

Ammonium entering the soil system, be it by desorption of fixed ammonia, mineralization or fertilizer application becomes part of a soil-ammonium equilibrium. Three forms of ammonium participate in this equilibrium; fixed, exchangeable, and soluble.

$$NH_4^+{}_{(sol.)} \longleftrightarrow NH_4^+{}_{(exchangeable)} \longleftrightarrow NH_4^+{}_{(fixed)}$$

Addition or removal of one form of ammonium will therefore result in an equilibrium shift and change the relative amounts of other ammonium forms.

Another equilibrium is that between the ammonium ion and ammonia. Below pH 8, ammonium is the dominant form. As pH rises to 10, the

amount of free ammonia produced increases (Fig. 1).

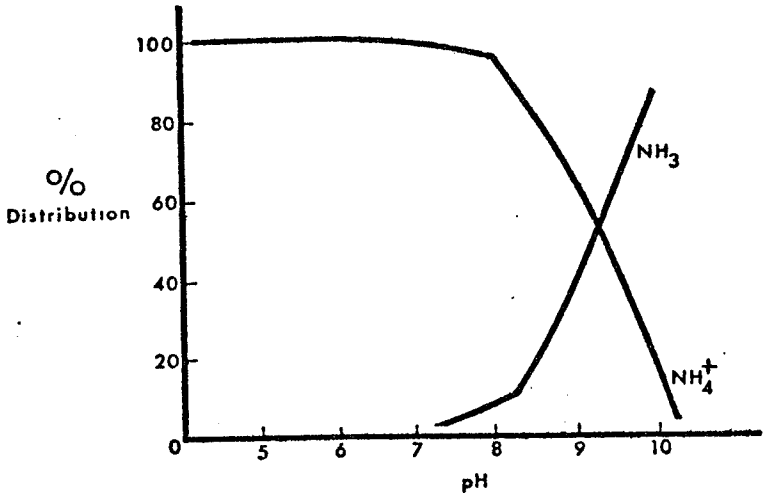

Figure 1. Percent distribution of ammonium and ammonia in aqueous systems as a function of pH (Bates and Barber 1950).

Volatilization

Ammonia volatilization is an important mechanism of nitrogen loss under specific conditions of pH, temperature and concentration. Losses as high as 59% of the added nitrogen have been reported (Volk, 1959).

The following reaction which describes the loss of NH_3 illustrates the importance of pH (Wahhab et al. 1960).

$$NH_4^+ + OH^- \longrightarrow NH_3 + H_2O$$

Although some gaseous loss will occur at pHs as low as 5.0 (Filimonov and Strel'nikova 1974) losses do not become significant until the pH approaches neutrality. Losses continue to increase with increasing pH (Volk 1959, Filmonov and Strel'nikova 1974, Earnst and Massey 1960).

Upon application of urea, volatilization from poorly buffered slightly acid soils may be due to the rise in pH induced by hydrolysis of urea to ammonium carbonate (Earnst and Massey 1960, Moe 1967). Increasing pH by liming also results in an increased volatile loss (Volk 1959). Earnst and Massey (1960) have proffered the following theories in an attempt to explain the increased loss with increased pH;

1) calcium saturation of the soil exchange complex is increased with increasing pH, leaving fewer sites for ammonium adsorption on the colloid,
2) increased OH^- activity pushes the reaction described by Wahhab et al. (1960) to the right, resulting in increased ammonia loss.

Urea in air-dry soil is quite stable due primarily to the inhibition of hydrolysis (Fenn and Escarzaga 1976). However, as soil moisture content increases, volatile ammonia losses increase also, the maximum being reached at a point halfway between air dry status and field capacity (Volk 1959). As moisture increases above this point, volatilization rates decrease. This could be because:

1) more moisture allows more NH_3 to become dissolved throughout,
2) high moisture results in a high nitrification rate, thereby lowering ammonium levels in the soil (Prasad 1976).,
3) As water content increases, the free pore volume decreases, thus limiting diffusion of gaseous ammonia from the soil (Gasser 1964).

Drying the soil from field capacity increases NH_3 losses by increasing the concentration of NH_4^+ in the soil water (Earnst and Massey 1960).

High temperature also promotes volatile losses (Earnst and Massey 1960, Prasad 1976). At low temperature, nitrification can more easily keep up with hydrolysis and so NH_3 accumulation is less likely to occur (Volk 1959). At higher temperatures, hydrolysis is completed quickly and a high concentration of ammonium builds up in the soil with an attendant increase in volatile loss.

Since ammonia is usually in its cationic form in soil, the negatively charged colloids of the soil will hold NH_4^+ and prevent its volatilization. Soils with a cation exchange capacity less than 10 m. eq. per 100 grams are incapable of preventing volatile loss. However, with increasing cation exchange capacity, losses decrease (Filimonov and Strel'nikova 1974). The addition of organic matter will result in an increase in exchange capacity and it is for this reason that incorporation of organic residues will decrease ammonia volatilization (Mortland 1958, Gasser 1964).

It has been shown by many workers that volatilization rates are inversely proportional to the depth at which urea is placed in the soil (Gasser 1964, Earnst and Massey 1960, Overrein and Moe 1967). Earnst and Massey (1960) were able to show a 75% decrease in volatilization when urea was covered to a depth of 3.5 cm.

Nitrification

Nitrification is defined by Alexander (1965) as "the biological conversion of nitrogen in organic or inorganic compounds from a reduced to a more oxidized state". This is a very important process in soils for several reasons. Among these is the fact that oxidized nitrogen compounds are subject to losses via leaching and denitrification. Of importance also is the fact that over a period of years nitrification tends to result in acidification of the profile, particularly in poorly buffered soils.

Although some heterotrophs possess the ability to carry out nitrification, they can not use the reaction as a sole source of energy (Hirsch et al. 1961). Nitrosomonas spp. and Nitrobacter spp. which oxidize NH_4^+ to NO_2^- and NO_2^- to NO_3^- respectively, meet all their energy requirements by means of these exothermic reactions. Nitrosomonas spp., characterized by N. europaea, is a rod-shaped organism 0.9-1.0 x 1.1-1.8 μ and is sometimes flagellated with one or two polar flagella. An example of Nitrobacter spp. is N. winogradskyi, a short, gram negative, non-motile rod measuring 0.6 - 0.8 x 1.0 - 1.2 μ (Alexander 1965). Eight other nitrogen autotrophs have been reported, although the taxonomic validity of two is open to question (Buchanan and Gibbons 1974).

Both the oxidation of NH_4^+ to NO_2^- and NO_2^- to NO_3^- are exothermic, the enthalpy being -65.0 to -84.0 kcal/mole and -16.5 to -20.0 kcal/mole respectively (Gibbs and Schiff 1960). It is evident, therefore, that Nitrosomonas spp. get potentially more energy per mole of substrate than Nitrobacter spp. Nitrite concentration is usually rate limiting to growth of Nitrobacter spp. and as a result, rarely accumulates under natural conditions.

Nitrite accumulation does occur however when free NH_3 levels are high. This condition results when ammonia or ammonia-yielding fertilizers are added to soils of high pH. Free ammonia, which is found only at pHs in excess of 7.2 (Morrill and Dawson 1967, Aleem and Alexander 1960) inhibits the Nitrobacter spp. thereby stopping the oxidation of NO_2^-.

Campbell and Lees (1967) have proposed the following pathway for nitrification.

$$NH_3 \xrightarrow{O_2} NH_2OH \xrightarrow{O_2} NO_2\text{-}NHOH \longrightarrow HNO_2 \longrightarrow HNO_3$$

ammonia hydroxylamine nitrohydroxylamine nitrite nitrate

Only NO_2^- and NO_3^- are found in soil, the other intermediates are not detected. Hydroxylamine and nitrohydroxylamine could occur intracellularly (Alexander 1965). During this oxidation, the valency of N changes from -3 in NH_3 to +5 in NO_3^-.

As nitrification is, by definition, strictly biological, environmental factors which affect the growth rates of Nitrosomonas spp. and Nitrobacter spp. will affect nitrification rate as well.

Nitrification patterns are of four basic types, the occurrence of each governed primarily by pH (Morrill and Dawson 1967, Dancer et al. 1973). The patterns observed were:

1) Type I Ammonium rapidly oxidized to NO_2^- which accumulates above pH 7.3.
2) Type II Ammonium and NO_2^- oxidized to NO_3^--pH 6 - 7.3.
3) Type III Ammonium oxidized to NO_3^- without appearance of NO_2^- -pH 5.5 - 5.9.

4) Type IV No ammonium oxidation - pH less than 5.4.

Nitrobacter spp. grow well in neutral or slightly acid soils and under normal conditions can oxidize NO_2^- as soon as it is made available. If, however, the pH rises above neutrality, Nitrobacter spp. activity is limited and a transient accumulation of NO_2^- may occur (Type II) or NO_2^- oxidation may cease completely (Type I).

Aleem and Alexander (1960) demonstrated that with increased pH, the ratio of nonionized to ionized ammonia increases. This results in a suppression of O_2 uptake by Nitrobacter spp. and thereby lowers its activity. Thus NO_2^- oxidation is slowed and an accumulation of this oxide of nitrogen occurs. Work done by Tyler and Broadbent (1960) has shown that the presence of the NO_2^- ion itself will also inhibit its own oxidation by Nitrobacter spp. and thereby promote NO_2^- stability. This inhibition decreased with increasing pH. Nitrosomonas spp. are not as susceptible to inhibition by either ammonia or nitrite.

The optimum pH of Nitrosomonas spp. is in the alkaline range between 7.6 and 8.2 (Morrill and Dawson 1967, Hofman and Lees 1953) and activity is still significant at pH 9.0. This is contrasted with a pH optimum of 6.2 - 7.0 for Nitrobacter spp. Addition of urea to even acid soils will result in local alkaline sites around the pellet (Clark, et al. 1960, Soulides and Clark 1958). Thus addition of urea has two effects on the bacterium's continued growth;

1) it raises the pH as it is hydrolysed which in itself is deleterious to the bacterium's continued growth.

2) the ratio of NH_4^+ to NH_3 decreases as pH rises which is another factor limiting NO_2^- oxidizers.

The effect becomes more pronounced as concentration of added urea increases (Hauck and Stevenson 1965, Aleem and Alexander 1960, Wetselaar et al. 1972). Thus a high concentration of ammonia-N in combination with high pH, both conditions occurring at a pellet site, will result in NO_2^- accumulation. Hauck and Stevenson (1965) and Bezdicek et al. (1971) have shown that with increasing pellet size, higher local concentrations of urea result and NO_2^- accumulation occurs.

As a rule, biological reaction rates rise with increasing temperature. Such is the case with nitrification where rates have been reported to increase over the range of 2° - $40^{\circ}C$ (Justice and Smith 1962, Kowalenko and Cameron 1976). At temperatures above $40^{\circ}C$ denaturation of enzymes occurs and nitrification ceases. Justice and Smith (1962) showed Nitrobacter spp. to be more sensitive to low temperature than Nitrosomonas spp. causing a slight increase in NO_2^- concentration in soils incubated with ammonium sources at $2^{\circ}C$. Work by Broadbent and Tyler (1962) shows similar trends. Anderson (1962) however, found no accumulation of NO_2^- at such low temperatures though the overall nitrification rate was depressed greatly.

Nitrification has been shown to decrease with decreasing soil moisture content between field capacity and the air-dry state (McGill 1971, Justice and Smith 1962 and Reichman et al. 1966). Alexander (1965) suggests the optimum moisture content of a soil for nitrification to proceed is between 50 and 75% of moisture holding capacity. Further

increases in soil moisture result in decreased O_2 diffusion into the soil, thereby diminishing nitrifier activity. The lowered O_2 tension will allow the growth of anaerobic denitrifiers with a concomitant loss of N_2 via denitrification.

Nitrification has been shown to take place on colloidal surfaces (Lees and Quastel 1946). As moisture levels are lowered, the concentration of added NH_4^+ in the water film surrounding the colloid increases. As mentioned previously, NH_4^+ inhibits Nitrobacter spp. with the consequence that at low moisture contents added NH_4^+ may nitrify only as far as NO_2^- and then accumulate (Justice and Smith 1962).

Denitrification

The denitrification process can be defined as "the gaseous loss of nitrogen either by biological or chemical mechanisms but exclusive of ammonia volatilization", (Broadbent and Clark 1965). As mentioned in the above definition, there are two basic types of denitrification - chemical and biological - each of which will be discussed.

A. Chemical

Addition of NH_4^+ or NH_4^+-yielding fertilizers such as urea have, in many cases resulted in losses of the added N. These losses have been found to occur in apparently well-aerated soils, especially when accumulations of NO_2^- have occurred (Jones and Hedlin 1970, Nelson and Bremner 1970, Bollag et al. 1973). Losses have been significant even if the soils in question have been sterilized, confirming the importance of chemo-denitrification (Cady and Bartholomew 1963, Reuss and Smith 1965,

Van Cleemput 1974). It has been postulated by Jansson and Clark (1952) and Greenland (1962) that nitrogen is nitrified in aerobic portions of the soil profile and carried to anaerobic pockets or centres of soil granules where it is subsequently denitrified. However, similar amounts of NO_3^--N added to the aerobic soil are quite stable, a fact which is not consistent with the anaerobic site theory. If anaerobic sites were responsible for denitrification, losses of the added NO_3^--N would occur at similar rates as NH_4^+-N losses.

Broadbent and Clark (1965) suggest four pathways of nitrogen loss via chemo-denitrification.

a) Decomposition of nitrous acid.

The following reaction describing self-decomposition of nitrous acid was proposed by Nelson and Bremner (1970).

$$HNO_2 + H^+ \longrightarrow NO^+ + H_2O$$

$$HNO_2 \longrightarrow H^+ + NO_2^-$$

$$NO^+ + NO_2^- \longrightarrow N_2O_3$$

$$N_2O_3 \longrightarrow NO + NO_2$$

$$2HNO_2 \longrightarrow NO + NO_2 + H_2O$$

Nitric oxide is present only in anaerobic systems (Smith and Clark 1960, Reuss and Smith 1965) for, in the presence of O_2, it is rapidly oxidized to NO_2. Nitric oxide has also been shown to occur when NO_3^--N is added to acidic soils under anaerobic conditions (Wijler and Delwiche 1954, Nommik 1956 Cady and Bartholomew 1960, 1963). Cady and Bartholomew (1963) concluded that this was due to the self-decomposition of nitrite formed during the

reduction of nitrate. Nitrogen dioxide is very soluble in water and in most cases is trapped by soil moisture before it leaves the soil system (Broadbent and Clark 1965, Broadbent and Stevenson 1966). The oxidation of NO and its subsequent hydration are represented as follows (Nelson and Bremner 1970).

$$4\ HNO_2 \longrightarrow 2NO + 2NO_2 + 2H_2O$$

$$2NO + O_2 \longrightarrow 2NO_2$$

$$4NO_2 \longrightarrow 2N_2O_4$$

$$2N_2O_4 + 2H_2O \longrightarrow 2HNO_2 + 2HNO_3$$

$$2HNO_2 + O_2 \longrightarrow 2HNO_3$$

It is evident that the presence of the HNO_2 molecule is obligatory before this disproportionation reaction can commence. Its presence in soil depends greatly on pH for, with a K of 6.0×10^{-4}, only 1.6% NO_2^--N exists in the HNO_2 form at pH 5.

To test the hypothesis that NO and NO_2 are oxidized to NO_3^-, Nelson and Bremner (1970), injected the gases into moist soil samples and then analysed the samples for nitrate content. An increase in nitrate level was recorded. In a further experiment, vials of alkaline $KMnO_4$ were put in the centre of sealed vessels containing soil samples to which nitrite had been added. Alkaline $KMnO_4$ will absorb NO and NO_2 and thereby lower the amount of nitrate produced if NO and NO_2 are intermediates in the conversion of nitrite to nitrate. Nitrate production was lower in samples incubated with the $KMnO_4$ than in controls where $KMnO_4$ was absent.

Further work by Nelson and Bremner (1970) showed that NO_2 production was inversely related to soil pH. They were at a loss, however to explain the continued NO_2 production in neutral soils, even though the amount produced had decreased. Nitrogen dioxide production is not enhanced by addition of organic materials (humic acids or lignin) nor is it affected by soil texture (Reuss and Smith 1965). These workers also showed that the production of this oxide of nitrogen was unaffected by soil sterilization.

Utilization of alkaline $KMnO_4$ to absorb NO and NO_2 also reduced the amounts of N_2 and N_2O produced, leading Nelson and Bremner (1970) to assume that some NO and NO_2 will be reduced to N_2 or N_2O by soil constituents.

Smith and Clark (1960), Tyler and Broadbent (1960) found very little NO_2 evolution from aerobic soil. This could be due to experimental methods for the $KMnO_4$ trap of Nelson and Bremner (1970) absorbs NO_2 quickly whereas the systems of the other authors offered a greater opportunity for absorption by the soil.

Work carried out by Wullstein and Gilmour (1964, 1966) has indicated that the metallic cations Cu, Fe, Mn, and Al will stimulate the evolution of NO from nitrite treated soils. Their studies involved the utilization of very high concentrations of metal (over 5,000 ppm) and nitrogen (10,000 ppm NO_2^--N), conditions which are not normally found in soils. Studies by Nelson and Bremner (1970) failed to show any stimulation of nitrite decomposition by these metals.

b) van Slyke reaction.

The classical van Slyke reaction may be described as follows:

$$RNH_2+HNO_2 \rightarrow ROH + H_2O + N_2$$

It is a reaction in which equal proportions of amino acid-N and nitrous acid-N react to yield dinitrogen and proceeds rapidly to completion in the presence of glacial acetic acid and a NO atmosphere. It is here that the problem lies. The NO atmosphere which is necessary for the reaction to occur at significant speed is not found in the normal soil profile. Nor, as mentioned previously, is HNO_2 of very high concentration over pH 5.

The van Slyke reaction is not considered to be of importance by most researchers (Broadbent and Clark 1965, Allison 1963, Clark et al. 1960). Stevenson et al. (1970) however, proposed that such a reaction may occur to a limited extent. In an experiment in which nitrite and amino acids were added to phthalate buffer (pH 6.0) recovery of amino-N as evolved N_2 varied from 19% with cysteine to 99.6% with glucosamine. Also, hydrolysis of humic acids with 6 N HCl before they were added to the nitrite solution lowered the amount of N_2 produced by 50%. Soils incubated with nitrite produced still more N_2 than unhydrolysed humic acids, possibly due to their higher amino-N content. Such work gives the van Slyke hypothesis some credence.

c) Reaction of HNO_2 with ammonia.

It has been proposed that nitrous acid will react with free ammonia to yield molecular nitrogen (Gerretsen and de Hoop 1957, Allison 1963). The reaction would be:

$$NH_3+HNO_2 \longrightarrow N_2+2H_2O$$

Gerretsen and de Hoop (1957), using sterile buffered solutions of

nitrite and HN_4^+ -N were able to show substantial losses of N as N_2. Smith and Clark (1960) were, however, unable to repeat these experiments. Upon addition of NO_2^- -N and NH_4^+ -N solutions to soils, they were able to show N_2 losses were the same whether NH_4^+ was present or not. They believed the reduction of nitrite to N_2 was promoted by some soil component and not NH_4^+ ions. Allison (1965) later revised his position on this reaction, concluding it to be of little importance.

d) Reaction of nitrite with soil organic constituents.

Many workers have postulated that nitrite can react with some component of the organic matter of acidic soils to yield molecular nitrogen (Clark et al. 1960, Broadbent and Tyler 1962, Nelson and Bremner 1970, Stevenson and Swaby 1964, Stevenson et al. 1970). Stevenson et al. (1970) found that the amount of N_2 recovered from soils treated with nitrite was directly related to soil nitrogen content, indicating that the organic soil fraction is the source of the active N component. Smith and Clark (1960) and Clark et al. (1960) showed that removal of soil organic matter by means of H_2O_2 treatment reduced the nitrogen losses. Nelson and Bremner (1970) noted a similar cessation of N_2 production from previously ignited soils. Incubations of nitrite with quartz and clay samples resulted in no denitrification, showing that inorganic soil constituents do not promote nitrite denitrification. Reuss and Smith (1965) came to a similar conclusion after finding no nitrite denitrification in a calcium-saturated exchange resin buffered to pH 5.2.

Bremner (1957) suggested that lignins and other phenolic substances in the soil undergo nitrosation, a process which commences with the

formation of nitrosophenols (>C-N=O). These nitrosophenols tautomerize to form quinone oximes (>C=NOH) which are further acted upon by HNO_2 to form N_2 and N_2O. Stevenson et al. (1970) studied the reaction of nitrite with lignins, humic substances, and several phenolic products in neutral and slightly acid solutions. They found more gas produced at pH 6.0 than pH 7.0 although significant amounts were still produced at this higher pH. The experiments were carried out anaerobically and so NO was the main gas produced. Nitrogen and N_2O were also found, N_2 being 5 to 10 times as abundant as N_2O. When soils were used instead of pure compounds, the ratio of N_2O to N_2 produced, decreased greatly. This, as mentioned previously, was believed due to the reaction of soil amino groups with nitrite to form N_2.

Reuss and Smith (1965) found the production of N_2 from nitrite-treated soils tended to be very rapid at first and to subsequently level off even though NO_2^- was still present. It was as if some denitrification-promoting component of the soil organic matter had been exhausted. They postulated that labile NH_2 groups were the source of one of the N atoms of the dinitrogen evolved. Tyler and Broadbent (1960) noted similar trends.

B. Biological

The accepted pathway of biological denitrification is:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow N_2O \longrightarrow N_2$$

During dissimilatory nitrate reduction, accumulation of the intermediates (NO_2^- and N_2O) occurs before the final product (N_2) is

formed (Cady and Bartholomew 1960, Renner and Becker 1970, Payne 1976, Payne and Riley 1969, Rolston et al. 1976). This could be due to either

i) a delay in induction of enzymes necessary for further reduction of a product until the precursor of that product is depleted i.e. NO_3^- would inhibit induction of NO_2^- reducing enzymes or

ii) repression by the precursors of the functioning of reductive enzymes further along in the reaction sequence.

The second theory seems more plausible for Payne and Riley (1969) were able to show that all the enzymes necessary for the reduction of nitrate to molecular nitrogen were present in bacteria grown in liquid cultures five hours after nitrate was added. The presence of the enzymes did not prevent the accumulation of NO_2^- though it was not stoichiometric, causing them to conclude that nitrate was inhibiting the reducing complexes to some extent.

Some bacteria, notably Corynebacterium nephridii have been shown to reduce nitrate stoichiometrically to nitrous oxide but no further (Renner and Becker 1970). This bacterium apparently lacks the enzymes necessary to carry reduction on to N_2.

Many workers working with anaerobic systems have found a transitory accumulation of nitric oxide to occur. This has led Payne (1976) and Payne and Riley (1969) to conclude that NO is a precursor to N_2O formation for NO decreases as N_2O levels increase. If it were a precursor of N_2O, however, facultative anaerobes should be able to grow using NO

as a terminal electron acceptor. It does not support growth.

Earlier work by Cady and Bartholomew (1960) as well as the preponderance of work since indicates that NO is solely a product of nitrite decomposition. Nitrite accumulated during reduction of nitrate forms a pH-dependent equilibrium with NO. The equilibrium requires a HNO_2 molecule and so is almost non-existent at higher pHs. Cady and Bartholomew (1960) were able to show that NO formation from anaerobic nitrite-treated soils was not affected by sterilization. The rate of NO formation was, however, directly tied to the level of nitrite added and decreased as nitrite was denitrified further. As mentioned in the previous section, nitrogen loss as NO is not considered to be significant.

The bacteria responsible for denitrification are facultative anaerobes which have the ability to use both oxides of nitrogen and oxygen as hydrogen acceptors provided sufficient energy sources are available. Although both oxides of nitrogen and O_2 are suitable terminal electron acceptors, bacteria utilize O_2 preferentially to nitrate and therefore only in situations of high O_2 demand is nitrate denitrified (Bremner and Shaw 1958).

Complete anaerobiosis is not necessary for biological denitrification to occur but O_2 levels must be quite low. Even an increase in O_2 pressure from 0 to 5 mm resulted in a decrease in denitrification rate of 90% (Wijler and Delwiche 1954). Reports of aerobic biological denitrification are probably situations in which anaerobic sites in the soil have developed due to high biological oxidation demand (Allison et al. 1960, Broadbent and Clark 1965). Such conditions can occur when

biological activity is very great or when O_2 diffusion through the soil profile is hindered.

It has been shown that high moisture levels in soils promote denitrification for, when water fills the pore spaces of soil, decreased oxygen diffusion occurs resulting in establishment of anaerobic sites (Bailey and Beauchamp 1973, Greenland 1962, Bremner and Shaw 1958, Cady and Bartholomew 1960). As expected, soils which are fine textured show the effect of increased moisture levels more dramatically (Greenland 1962, Focht 1974).

Biological oxygen demand is an important factor in the establishment of anaerobic sites in soils. Physical factors which increase the numbers and respiration rate of bacteria result in the hydrogen acceptor supply becoming the rate limiting step in growth. In such a case, rapid utilization of nitrate will occur. Much work has been done on the effect of organic matter on denitrification.

Greater losses have been reported from cropped as opposed to fallow soils (Ketcheson and Jackovlijaic 1970, Volz et al. 1976, Flühler et al. 1976, Bailey 1976). Plant roots can release up to 14% of the total C fixed by photosynthesis (Barber and Martin 1975) thereby greatly enhancing microbial activity. Addition of nitrate not only provides an alternate hydrogen acceptor, it stimulates plant growth and thereby increases organic C levels in the soil. McGarity and Meyers (1968) were able to show a direct correlation between available organic C and

rates of denitrification. Burford and Bremner (1975) found an even higher correlation between water-soluble carbon than total organic carbon ($r=0.99$ vs $r=0.77$) leading them to suggest that water-soluble organic carbon is a good index of a soil's denitrifying capacity. Similar trends were found when the energy source added was simply glucose (MacGregor 1972). Allison et al. (1960) found a 5-fold increase in denitrification rate when soils were amended with 0.5% glucose. The increased C promotes bacterial respiration to such an extent that O_2 supply becomes rate limiting. In such cases, oxides of nitrogen become alternate hydrogen acceptors. McGarity (1962) showed that freezing and thawing as well as air drying increased the subsequent denitrifying capacity of soils, presumably by releasing available organic matter.

Biological reactions tend to occur faster as temperature is increased and the denitrification process is no exception (Bailey and Beauchamp 1973, Bailey 1976). Bremner and Shaw (1958) reported that the upper temperature limit of denitrification was $70^{o}C$, the lower limit being $2^{o}C$. Bollag et al. (1973) suggested the optimum temperature is around $30^{o}C$. The pH of soil also affects microbial activity, the optimum being slightly over neutrality (Renner and Becker 1970, Bremner and Shaw 1958).

How closely the reduction of nitrate approaches completion depends on the rate of the reaction and time of sampling since different gases predominate during different stages of the reduction. If conditions are optimum for denitrification i.e. pH, temperature, carbon source, restricted O_2 supply, bacterial activity will be so great that all oxides

of nitrogen will be reduced rapidly to N_2. If however, some physical or chemical parameter is insufficient to allow complete denitrification, some intermediate such as NO_2^- or N_2O will accumulate. This would account for some of the reports of N_2O being the final gaseous product under adverse conditions.

Occasionally, especially in cases where nitrite accumulation results from the use of NH_4^+ or NH_4^+-yielding fertilizers, both biological and chemical denitrification occur simultaneously. In such cases, nitrogen gas is formed by reduction of N_2O as well as via a soil-nitrite interaction. MacGregor (1972) found that although the ^{15}N content in N_2O was the same as the denitrified nitrate, the ^{15}N content of the N_2 gas was approximately one thirtieth of the source NO_3^-. This led him to conclude that the nitrogen gas came from both non-biological sources and biological sources.

^{15}N Methodology

When using ^{15}N, two basic assumptions must be made. First, it must be assumed that the ^{15}N content of all compounds in their natural state is constant. Secondly, it must also be assumed that living organisms cannot distinguish between the heavy and light isotopes (Bremner 1965a, Hauck 1973).

The validity of these assumptions has been questioned by several authors. Failure to meet these requirements is important when working with ^{15}N levels near that of natural abundance (here taken to be 0.366% [Nier 1950]). Microbial reactions have been shown to favour ^{14}N over ^{15}N resulting in an increase in substrate ^{15}N concentration (Wellman et

al. 1968, Delwiche and Steyn 1970, Blackmer and Bremner 1977, Chien et al. 1977). This discrimination is only at a level of 2%, however. The natural abundance of ^{15}N has also been shown to vary within a soil profile (Delwich, and Styne 1970). Again the variations were low and the abundance of ^{15}N does not exceed 0.38%.

The extent to which the two assumptions are not met does not invalidate results where the ^{15}N to ^{14}N ratio is high (Blackmer and Bremner 1977, Hauck 1973, Edwards 1973). Small errors in natural abundance or discrimination during microbial reactions are significant only when % ^{15}N drops to very low levels.

If labelled and unlabelled nitrogen atoms are allowed to combine randomly, be it nitrogen atoms released during ammonia oxidation or due to the combination of nitrogen from two nitrite ions during denitrification, the distribution of ^{15}N and ^{14}N in the resultant molecules can be described by the following equation:

$$(p + q)^2 = p^2 + 2pq + q^2$$

where p is the atom fraction of ^{14}N and q is the atom fraction of ^{15}N. The terms on the right hand side of the equation correspond to the mass species 28, 29, and 30, respectively. A similar distribution occurs with N_2O (Hauck et al. 1958).

If however, one atom of labelled nitrite combines with soil nitrogen to yield nitrogen gas, essentially the only nitrogen molecules formed will correspond to masses 28 ($^{14}N^{14}N$) and 29($^{14}N^{15}N$).

The isotopic equilibrium developed between existing individual samples of N_2 gas is shown in the reaction following:

$$^{14}N^{14}N + {}^{15}N^{15}N \longleftrightarrow 2\,{}^{14}N^{15}N \qquad Keq = 4.000$$

This reaction does not proceed at moderate temperatures and so a mixture of different nitrogen samples with different mass distributions will remain as distinct molecules and not yield an isotopic equilibrium. Thus if enrichment is great, it is possible to differentiate between nitrogen formed via a random combination of labelled ions and that formed via an association of one soil nitrogen atom and one nitrite atom.[1]

The atom percent N can be calculated using the following formula.

$$\%^{15}N = 100\ \frac{\text{number of } {}^{15}N \text{ atoms}}{\text{number of } ({}^{14}N + {}^{15}N) \text{ atoms}}$$

$$= \frac{100\ \frac{R^1}{2}}{1 + R^1/2}$$

$$= \frac{100\ R^1}{R^1 + 2} \qquad \text{where } R^1 = \frac{(29)}{(28)}$$

Using only 29 and 30, the formula becomes

$$\%\ {}^{15}N = \frac{200}{R + 2} \qquad \text{where } R = \frac{(29)}{(30)}$$

Mass intensities at 44, 45 and 46 may also be used resulting in a R^1 value derived from (45)/(44) and an R value from (45)/(46).

Sample Atmosphere

Most denitrification studies are carried out in nitrogen-free atmospheres so that any N_2 evolved can be readily measured (Nelson and Bremner 1970, Meek and MacKenzie 1965, Reuss and Smith 1965). This use of non-nitrogen atmospheres may cause problems for different incubation

[1] Cho, C.M. Personal communication

atmospheres have been shown to result in different growth rates for Neurospora crassa. Schreiner et al. (1962). Working with this organism, it was found that the rate of filament growth was linearly related to the square root of the molecular weight of the incubation gas. Parr et al. (1970) and Blackmer and Bremner (1977) were not able to confirm these results in soils, noting no variation in respiration or denitrification rates under different atmospheres, provided sufficient inorganic nitrogen was present. If samples were incubated without the inorganic nitrogen amendments, soils incubated with N_2 in the air had greater microbial activity. This was probably due to nitrogen fixation by some anaerobes thereby supplying nitrate for non-fixers and allowing growth of a more diverse soil population.

METHODS AND MATERIALS

Soils

Samples of soil were collected from the Ap horizon (0-15 cm) of Keld and Wellwood associations, and Neuenberg series, air dried and ground to pass through a 2 mm sieve. Legal locations of the soils as well as some chemical and physical properties are listed in Table 1.

Apparatus

Soil samples were incubated in a container which consisted of a 10.5 cm long sample tube with an interior diameter of 2.4 cm (Fig. 2). The upper portion of the unit consisted of a 2 mm stopcock to which was attached a ₹ 10/18 cone ground glass joint. The two sections were connected to each other at a B24 ground glass joint and sealed with Corning high vacuum grease. The total volume of the sealed container with the stopcock closed was adjusted to 42.1 ml by addition of glass beads.

To sample the gases produced during the experiment, the incubation container was fitted to a sampling apparatus (Fig. 3) at a ₹ 10/18 ground glass joint. To this sampling apparatus were also fitted two gas sample containers (Fig. 4), one containing 3 ml of conc. H_2SO_4, the other containing solid KOH. The containers were topped with a right angle 2 mm stopcock. To this was attached a ₹ 10/18 socket glass joint. The gas container stopcocks were opened and the

TABLE 1

Subgroup designation, legal location and some physical and chemical properties of the soils.

Soil Name	Keld	Wellwood	Neuenberg
Subgroup	gleyed black	chernozemic black	gleyed rego black
Legal location	SE 15-25-20	NE 24-12-14	NW 6-12-16
Texture	FSL	FSL	L
pH	5.5	6.1	7.3
C.E.C. (meq/100g)	33.2	24.1	26.5
Conductivity (mmho/cm)	0.15	0.6	0.3
% organic matter	8.1%	4.6%	5.28%
% $CaCO_3$	0.5%	0.2%	0.2%

whole sampling apparatus was evacuated to a pressure of 5 Torr. The stopcock between the vacuum pump and sampling apparatus was then closed and the incubation container opened. The gas containers were closed, thereby trapping some of the gas originally in the incubation vessel.

The gas containers were left to stand for approximately 30 minutes. This was done to allow removal of moisture and CO_2 by the solid KOH and moisture alone by the concentrated H_2SO_4.

Gas was then introduced into a V.G. Micromass 602 isotopic ratio mass spectrometer through a ₹ 10/18 joint on the machine.

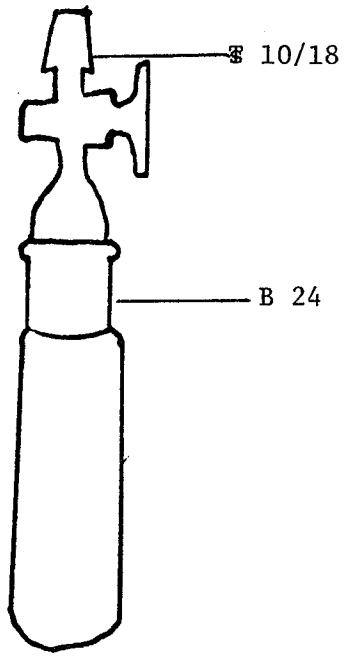

Fig. 2

Incubation vessel

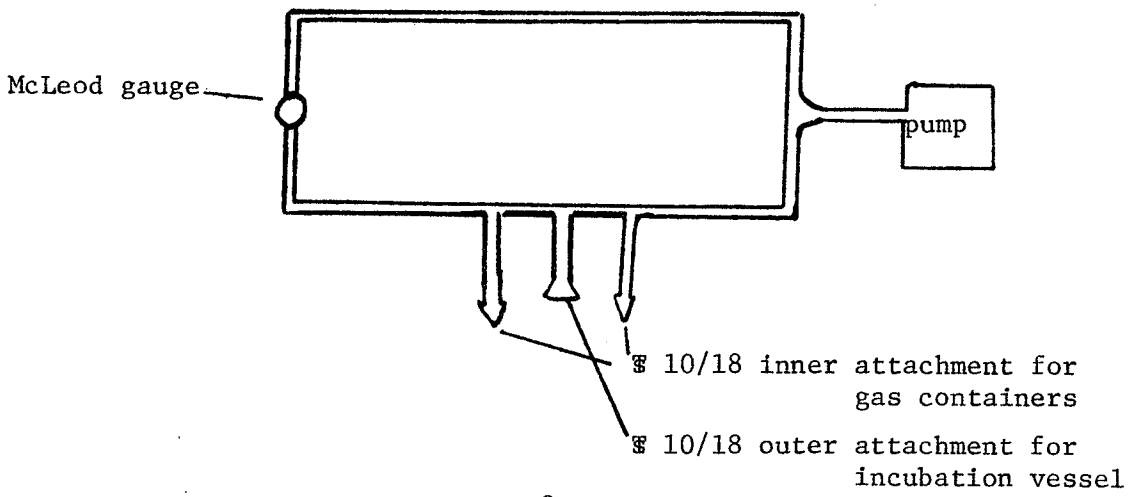

Fig. 3

Sampling apparatus

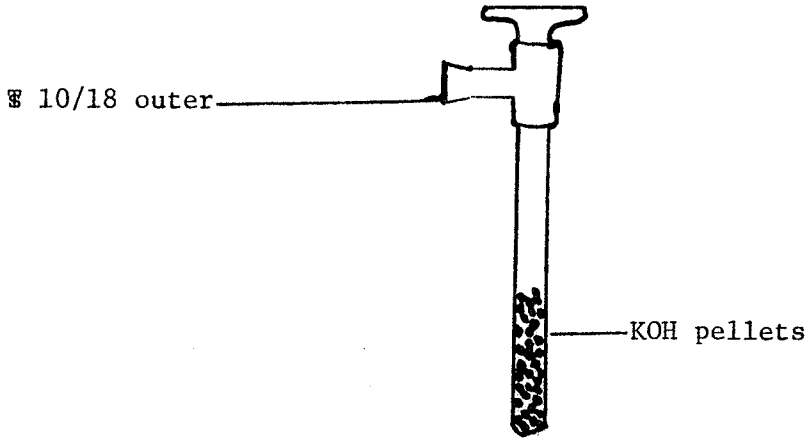

Fig. 4

Gas sample container

Experiment I Oxidative Denitrification During Incubation with Added Urea.

Samples of Wellwood soil, pH 6.1 and limed to pH 7.6 and Neuenberg soil pH 7.1, limed to pH 8.0 and acidified to pH 6.0 were pre-incubated at 15% moisture content for 3 days at room temperature to allow the establishment of a soil microbial population. Sufficient water and urea were mixed with pre-incubated soil samples in order to adjust the moisture content to 25% and result in 1000 ppm-N as urea. The urea was enriched with 52.3% ^{15}N.

Twenty-five grams of this moist soil was added to incubation vessels which were subsequently closed and sealed with high vacuum grease. The vessels were incubated at 20°C for 3 days and the gas was sampled, after which the tops were removed for 15 minutes. This was to allow the oxygen levels in the vessel to return to near normal levels. The tops were then replaced, ungreased, with the stopcock open and left to incubate for 3 days under aerobic conditions. After three days of aerobic incubation the tops were again removed for 15 minutes then replaced, sealed with grease, and re-incubated as before. This cycle was completed 10 times over the next 60 days. Water was added as needed to maintain the moisture content at 25%.

Identical incubations were carried out with another set of samples in an alternate series. These tubes had been opened for the first 3 days, closed the next 3 days and sampled. This was carried out over 10 sampling periods thereby resulting in samples being made available for analysis every 3 days. Each series was incubated in duplicate.

Analysis of the gas was carried out mass spectrometrically, scanning from 28 to 46 a.m.u. inclusive. The gases N_2, NO, O_2, CO_2 and N_2O have masses within this range.

Other incubations were carried out in the same manner as outlined above using unlabelled urea, tubes being sampled at intervals to determine the levels of ammonium, nitrite and nitrate in the soils as well as pH.

The gas of one of these unlabelled urea tubes was sampled and scanned to determine the contribution to a.m.u. 30 by sources other than $^{15}N^{16}O$.

Experiment 2 Denitrification of $Ca(NO_3)_2$ in Wellwood Soil.

a) In order to determine if denitrification of nitrates occurs under conditions which allowed urea denitrification in the Wellwood soil incubations were carried out in a like manner to those of Experiment I save that 1000 ppm-N as $Ca(NO_3)_2$ containing, 52.4% ^{15}N was substituted for urea as a N source. Incubations were in alternate series and lasted for 15 days with Wellwood soil at pH 6.1 and the same soil limed to pH 7.6.

b) As very little gas production was found in part a), it was thought that possibly NO_3^- inhibition of microbial activity had occurred. The experiment was therefore repeated as before except that the concentration of $Ca(NO_3)_2$ employed was only 100 ppm-N.

In both parts of this study, gas analysis was carried out mass spectrometrically.

Experiment 3 Denitrification of $NaNO_2$ in Wellwood and Neuenberg soils.

Untreated samples of Wellwood and Neuenberg soils and samples of these soils to which 10% $CaCO_3$ had been added were incubated as in Experiment I. This was a preliminary study carried out to determine the effect of accumulations of NO_2^- on denitrification rates.

Nitrogen was added at 100 ppm-N as $NaNO_2$ containing 52% excess ^{15}N. The duration of the experiment was 21 days with Wellwood soils and 15 days with the Neuenberg sample.

Gas production was again studied mass spectrometrically.

Experiment 4 Effect of Nitrite Concentration on Denitrification

Samples of untreated Wellwood soil and Wellwood to which 10% $CaCO_3$ had been added were pre-incubated as in Experiment I. To these samples was added 52% ^{15}N enriched $NaNO_2$ at the following rates; 50, 100, 150, 200, 250, 400 and 500 ppm-N. Samples were sealed and incubated for 3 days after which time gas analysis and inorganic-N analysis were carried out.

Experiment 5 Effect of soil pH and concentration of N upon $NaNO_2$ denitrification.

The pH of samples of Wellwood soil was adjusted from an initial value of 6.1 to 6.8 and 7.2 by means of addition of 0.3 N $Ca(OH)_2$ previous to pre-incubation. Other samples were treated with 10% $CaCO_3$, thus raising the pH to 7.6. Nitrite-N in the form of 52% ^{15}N enriched $NaNO_2$ was added at the following rates to samples at each pH: 50, 100, 200, 400 ppm. Incubations lasted 3 days.

Analyses of incubation gases and inorganic nitrogen were performed.

Experiment 6 Effect of steam sterilization and glucose addition on the denitrification of NO_2^- with time.

a) This series of experiments was carried out to determine the effect of the activity of the microbial population on denitrification rates. Three sets of incubations were prepared.

1) To pre-incubated Wellwood soil (pH 6.1) was added 0.5% glucose (by weight).

2) Wellwood soil was steam-sterilized as were the incubation vessels and the grease. The $NaNO_2$ used in this portion of the study was also sterilized by means of membrane filtration. All work with sterilized soils was performed in a laminar flow unit.

3) A control experiment was also carried out using Wellwood which had been pre-incubated but was otherwise left untreated prior to addition of nitrite.

To all soil treatments was added 54% ^{15}N-labelled $NaNO_2$ at 100 and 200 ppm-N. These samples were sealed and incubated for the following periods of time; 1, 2, 3, 4, 5, 7, 9, 11 and 13 days. The air pressure in the incubation tubes was lowered from about 75 cm Hg (atmospheric pressure) to 65 cm Hg with the use of a vacuum pump. This lowering of the total pressure in the containers was needed in order to accommodate the gases produced during prolonged incubation.

A further incubation using 100 ppm-N as NO_2^- and Keld soil was performed in the same manner as the controls. This portion of the

study was carried out to determine if van Slyke-like nitrogen formation was peculiar to the Wellwood soil.

All incubations were done in duplicate. Samples were scanned between a.m.u. 28 and 46 and analysis of inorganic-N levels was carried out.

b) It was noted in the case of glucose studies that as nitrite became very low in the soil, the $\%^{15}N$ appeared to decline. This often coincided with an absence of O_2 and therefore could have been due to reduction of soil NO_3^- to NO_2^- which then diluted the nitrite pool. There was some question as to whether or not this also occurred when NO_2^- levels were high in the soil.

Therefore, parallel experiments were carried out in which Wellwood soil, to which had been added 100 ppmN levels of both NO_2^- and NO_3^-, was incubated for 3 days. In the first case, the NO_2^- was labelled with 54% ^{15}N excess and the NO_3^- was unlabelled. In the second case the converse was true with NO_3^- being labelled with 52.4% ^{15}N and NO_2^- unlabelled. After incubation, gas samples were scanned mass spectrometrically and the NH_4^+, NO_2^- and NO_3^- content of the soil determined.

c) There was some concern that the nitrite was being converted to NH_4^+ and then reoxidized to NO_2^- or that an exchange of ^{15}N was occurring between NO_2^- and NH_4^+. Some of the 2N KCl soil extract which was generally used for NH_4^+ analysis was used to determine ^{15}N content in NH_4^+.

Since one requires a sample of at least 1.0 mgm of N to accurately determine ^{15}N content and levels of NH_4^+-N were not high in

the sample aliquot, direct oxidation of NH_4^+ with NaOBr and analysis of the N_2 produced was not possible. Therefore, the isotopic dilution technique was used to determine % ^{15}N in the ammonium sample.

The method used was as follows: 1.0 ml of the sample was added to test tubes containing 1.0, 2.0, 3.0, or 4.0 ml of a solution containing 1000 ppm-N NH_4Cl. These were mixed and oxidized to N_2 gas by treatment with NaOBr via the method outlined by Bremner (1965a). The % ^{15}N of the N_2 gas was determined by mass spectrometry.

Experiment 7 Effect of pH on the van Slyke Reaction in Buffered Solutions.

The classical van Slyke reaction involves the treatment of an amino acid solution with HNO_2 in a NO atmosphere and results in the complete recovery of amino-N as N_2 - a reaction in which the amino acid and HNO_2 each contribute one N atom to the N_2 formed. The reaction is generally believed to be almost non-existent in solutions above pH5 yet experimental results obtained so far indicated it to be of importance at pH values in soil up to neutrality.

This experiment was carried out to determine the effect, in solution, of pH on the rate of formation of van Slyke nitrogen from an amino acid-HNO_2 mixture.

Potassium hydrogen phthalate buffers were made up to the following pH values: 2.3, 3.0 , 4.0, 5.0, 6.0, 7.0 and 8.0. To eight ml of each buffer were added 1.0 ml of 1000 ppm-N glycine (a common amino acid in soil organic matter) (Bremner, 1965b) and 1.0 ml of a solution containing 1000 ppm-N as $NaNO_2$ in an incubation vessel as described in Experiment I.

The experiment was carried out, in duplicate, originally with air as the incubation atmosphere at 65 cm Hg. However, results of this test indicated that at low pH (2.3 and 3.0), the NO_2^- in the system oxidized to NO_3^- and failed to react completely with the amino acid-N.

The experiment was repeated in a like manner save that argon was substituted for air as the incubation atmosphere in the second

case. The pressure was again lowered to 65 cm Hg.

Samples were incubated for 3 days, sampled and, as in the first case, analysed for NO_2^- and NO_3^-.

Chemical Analysis

pH

pH was determined using a Fisher model 520 digital pH/ion meter equipped with a combination electrode. The soil-water ratio was 1:10. Samples were shaken 30 minutes prior to pH determination.

$CaCO_3$

The $CaCO_3$ content was determined by the method of Skinner et al. (1959).

Organic Matter

The potassium dichromate-conc. H_2SO_4 method described by Peech et al. (1947) was used to determine soil organic matter.

Conductivity

Conductivity was measured with a Radiometer conductivity meter with a soil-water ratio of 1:4.

Cation Exchange Capacity

The cation exchange capacity was determined by the ammonium acetate method of Chapman (1965).

Ammonium Nitrogen

Ammonium -N levels were determined with an Orion ammonia electrode coupled to a Fisher model 520 digital pH/ion meter in a 1:10 soil-2N KCl extract which had been shaken for 30 minutes.

Nitrite and Nitrate Nitrogen

Nitrite-N and nitrate-N were determined using the 1:10 soil-

water solution previously used for pH determination. The method used was a variation of that of Kamphake et al. (1967) employing a Technicon autoanalyser.

Nitrite standards were run with and without reducing agent. Percent transmittance was converted to optical density and plotted against concentration.

In order to determine the amount of endogenous nitrite present during nitrate reduction, the previously measured nitrite concentration curve found without reducing agent was applied to the graph of nitrite with reducing agent. From this was determined the optical density corresponding to the endogenous nitrite. This was subtracted from the optical density of the sample with reducing agent in order to determine the optical density due to nitrate alone.

Gas Analysis

The gas sample was scanned mass spectrometrically between a.m.u. 28 and 46. Argon (mass 40) was chosen as an internal standard and before scanning the sample, the argon peak was set to a pre-arranged height by varying the inlet pressure. The peak at 40 was set to 100 and all other peaks were normalized to this figure (see Table 2). In cases where the incubation atmosphere was argon, the argon peak was set to 5000 and all other gases were normalized to this figure. An air sample treated with KOH in order to remove CO_2 was scanned in an identical manner to determine the contribution of air to each a.m.u. scanned.

Measurements of a.m.u. 28 to 46 correspond to the molecules listed

TABLE 2

Normalized peak heights of a scan between a.m.u. 28 and 46 of atmosphere of Keld soil, 100 ppm-N as NO_2^-, 6 days.

a.m.u.	Measured Peak Height	Normalized Peak Height
28	18.9	3513.0
29	1.88	34.944
30	0.25	4.647
31	0.12	2.230
32	22.4	416.36
40	5.38	100
44	0.74	13.755
45	0.812	15.093
46	0.273	5.074

Normalized Peak Heights where Ar becomes 100

in Table 3.

Samples taken from gas containers containing H_2SO_4 possessed CO_2 in similar proportions to that originally in the incubation vessel. Scans of these samples were used only to determine the amount of CO_2 evolved.

The probability of fragmentation of N_2O to NO and N_2 in air was determined at a fixed mass spectrometric setting. The percentages of fragmentation of N_2O to NO and N_2O to N_2 were found to be 17.7% and 5.3% respectively.

Use of KOH in gas tubes eliminated most CO_2 present and therefore peaks at 45 and 46 were considered to be of N_2O only. Using these peaks, the $\%^{15}N$ was calculated using the formula

$$\%^{15}N = \frac{200}{R+2} \qquad (1)$$

where R equals the ratio of (45) to (46) where brackets denote the measured peak height at each a.m.u.

Since O_2 was present in most scans, some oxidation of carbon which was adsorbed to the mass spectrometer's gas inlet capillaries and near the ion source resulted in the formation of CO_2. The peak at 44 was not considered clean and was calculated using the equilibrium equation

$$4 = \frac{(^{14}N\ ^{15}N)^2}{(^{14}N\ ^{14}N)\ (^{15}N\ ^{15}N)} \qquad (2)$$

$$= \frac{(45)^2}{(44)\ (46)} \qquad (3)$$

TABLE 3

Molecular species found between a.m.u. 28 and 46.

Mass	Molecule
28	$^{14}N\ ^{14}N$, $^{12}C\ ^{16}O$
29	$^{14}N\ ^{15}N$, $^{13}C\ ^{16}O$
30	$^{15}N\ ^{15}N$, $^{14}N\ ^{16}O$
31	$^{15}N^{16}O$
32	$^{16}O\ ^{16}O$
40	^{40}Ar
44	$^{12}C\ ^{16}O\ ^{16}O$, $^{14}N\ ^{14}N\ ^{16}O$
45	$^{14}N\ ^{15}N\ ^{16}O$, $^{13}C\ ^{16}O\ ^{16}O$
46	$^{15}N\ ^{15}N\ ^{16}O$

$$(44) = \frac{(45)^2}{4\ (46)} \qquad (4)$$

The total peak height due to N_2O was the sum of $(44)_{(N_2O)}$, (45) and (46).

Using the % ^{15}N determined in equation 1 and mass peak 31, the mass peak at 30 due to NO was determined as follows:

$$\left(\frac{(31)}{(30)+(31)}\right)\ 100 = \%\ ^{15}N \qquad (5)$$

$$\frac{(31)100}{\%^{15}N} - (31) = (30) \qquad (6)$$

The sum of NO was divided by the sum of N_2O to determine if any NO was produced other than as a fragmentation product of N_2O. The ratio obtained should approach 0.177 if all NO was due to fragmentation of N_2O.

Since the mass intensity at 30 is the sum of $^{14}N\ ^{16}O$ and $^{15}N^{15}N$, subtraction of the calculated $^{14}N\ ^{16}O$ from a.m.u. 30 yields the peak height at 30 due to $^{15}N\ ^{15}N$.

The mass intensities at 28, 29 and 30 are now the result of N_2 formed via both a van Slyke-like process and a reduction of N_2O, hereafter designated $N_2{}^2$ and $N_2{}^1$ respectively.

Therefore:

$$(29)_{N_2} = (29)_{N_2}1 + (29)_{N_2}2 \qquad (7)$$

and

$$(30)_{N_2} = (30)_{N_2}1 + (30)_{N_2}2 \qquad (8)$$

In the above equations, $(29)_{N_2}$ and $(30)_{N_2}$ refer to the total contribution of N_2 to a.m.u. 29 and 30. If it is assumed that isotopic separation is negligible during the formation of $N_2{}^1$, the distribution of ^{15}N in this form of N_2 should be the same as that of N_20. Therefore $(29)_{N_2}1 = (30)_{N_2}1 \; R$ (9)

The distribution of a.m.u.s 28, 29 and 30 in $N_2{}^1$ and $N_2{}^2$ could be described by the following expressions:

$$(28)_{N_2}1: \; (29)_{N_2}1: \; (30)_{N_2}1 = P_1{}^2: \; 2p_1q_1: \; q_1{}^2 \qquad (10)$$

$$(28)_{N_2}2: \; (29)_{N_2}2: \; (30)_{N_2}2 = P_1P_2: \; (p_1q_2 + p_2q_1): \; q_1q_2 \qquad (11)$$

in which $p_1 + q_1 = 1$, and $p_2 + q_2 = 1$. In the above expression p_1 and p_2 are the fraction of ^{14}N in the labelled and natural N respectively, and q signifies ^{15}N fraction. The value of q_2, natural abundance of ^{15}N, as determined by the machine, Micromass 602, is 0.0037. The relationship between q_1 and R is

$$q_1 = \frac{2}{2 + R} \qquad (12)$$

The ratio of $(29)_{N_2}2$ to $(30)_{N_2}2$ becomes

$$\frac{(29)_{N_2}2}{(30)_{N_2}2} = \frac{\frac{R}{2 + R} \; (0.0037) + (0.9967) \; \frac{2}{2 + R}}{\frac{2}{2 + R} \; (0.0037)}$$

$$= 269.27 + \frac{R}{2} \qquad (13)$$

Substitution of eq. (9) and (13) into eq. (7) yields

$$(29)_{N_2} = R(30)_{N_2}1 + (269.27 + \frac{R}{2}) \; (30)_{N_2}2 \qquad (14)$$

The solution of simultaneous equations (8) and (14) yields

$$(30)_{N_2}1 = \frac{(269.27 + \frac{R}{2})\ (30)_{N_2} - (29)_{N_2}}{269.27 - \frac{R}{2}} \qquad (15)$$

$$(30)_{N_2}2 = \frac{(29)_{N_2} - R\ (30)_{N_2}}{269.27 - R/2} \qquad (16)$$

The values of $(29)_{N_2}1$, $(29)_{N_2}2$, $(30)_{N_2}1$ and $(30)_{N_2}2$ can be calculated using eq (9), for $(29)_{N_2}1$, eq (13), for $(29)_{N_2}2$ and the relationships

$$(28)_{N_2}1 = \frac{R^2}{4}(30)_{N_2}1 \qquad (17)$$

and

$$(28)_{N_2}2 = \frac{R}{2 + 0.0037R}(29)_{N_2}2 \qquad (18)$$

The sums

$$(N_2{}^1) = (28)_{N_2}1 + (29)_{N_2}1 + (30)_{N_2}1$$

$$(N_2{}^2) = (28)_{N_2}2 + (29)_{N_2}2 + (30)_{N_2}2$$

were used to calculate the total nitrogen production after making corrections for fragmentation of N_2O.

Standard curves were developed to obtain a relationship between partial pressure and peak height by scanning known amounts of pure N_2O mixed in varying proportions with air. For N_2, the peak heights

in air scans were used to calculate the partial pressure of produced gases.

In cases where argon was the incubation atmosphere new standard curves for each gas were made by scanning known amounts of pure N_2O or N_2 in varying proportions with argon.

Calculations for Experiment 6

The equation necessary to solve for the $\%^{15}N$ of the ammonia in the soil sample involves 4 variables, 3 of which are easily measured.

The variables are:

q^1 = quantity of N added as 1000 ppm NH_4Cl solution

q = quantity of NH_4-N in original extract - measured by ammonia electrode

x^1 = $\%^{15}N$ excess of extract and NH_4Cl mixture - determined by mass spectrometer

x = $\%^{15}N$ excess of NH_4^+ in original extract.

From this it follows that

$$x^1 = \frac{qx}{q+q^1} .$$

Inverting

$$1/x^1 = 1/x + q^1/qx.$$

Plotting $1/x^1$ versus q^1 results in a graph with $1/qx$ becoming the slope and $1/x$ the intercept. A slope of 0 indicates no enrichment of x over natural abundance.

RESULTS AND DISCUSSION

Experiment 1 Oxidative Denitrification During Incubation with 1000 ppm-N Urea.

a) Wellwood pH 6.1

The results of the incubation study using 1000 ppm-N urea and uncarbonated Wellwood (pH 6.1) indicated that the oxidation of NH_4^+ (the hydrolysis product of urea) occurred quite rapidly for the first 12 days after which the rate of oxidation slowed to a fairly constant level (Fig. 5a). Nitrite accumulation began at day 3 and quickly reached a peak of 8 mg-N by day 12. Oxygen consumption and CO_2 production were both high during the initial 12 days and then dipped after this point. It may be that as NO_2^- accumulated inhibition of the microbial population occurred. From day 12, NO_2^- levels slowly decreased to 0.2 mg by day 60. Nitrate was evident from day 3 although its production rate was not very rapid until NO_2^- concentrations began to decline.

Throughout the course of urea hydrolysis and subsequent nitrification the pH was found to shift from an initial value of 6.1 to a peak of 8.3 on day 3 and down to 5.7 at the end of the experiment (Fig. 5a).

Gaseous losses (Fig. 5b), appear to follow the formation and oxidation of NO_2^-. The production rate of N_2, the nitrogen formed by van Slyke-type reaction, was very low during the first nine days and then increased rapidly to a maximum of 0.11 mg/day for the

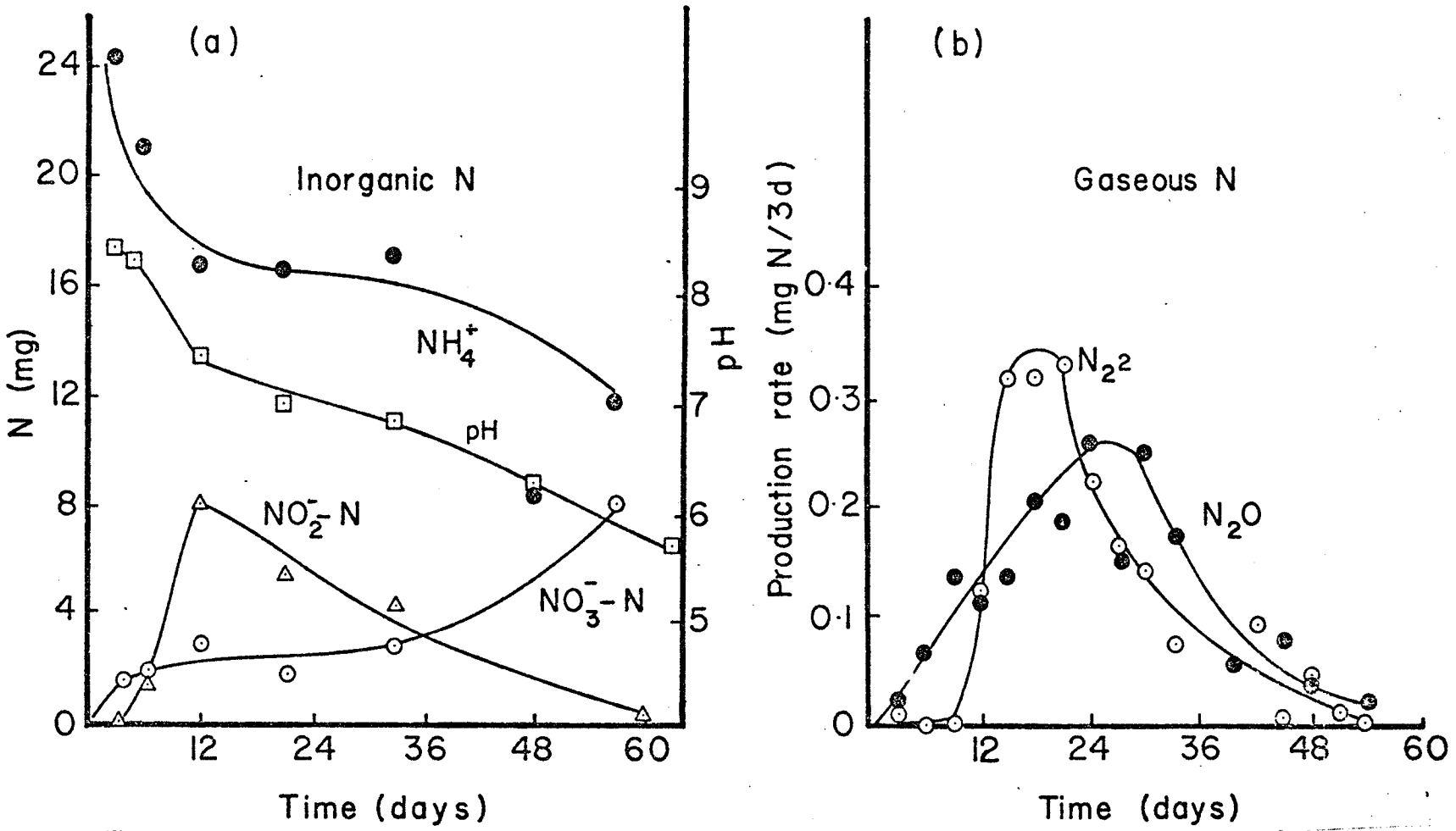

Fig 5. Transformation of Urea to various N compounds, Wellwood, pH 6·1

3-day periods ending at days 15, 18 and 21. Thereafter N_2^2 production dropped steadily to a zero point by day 50. At the point of maximum van Slyke-like N_2 formation, the pH was approximately 7.1.

Throughout the duration of the experiment, N_2O was found in the incubation atmosphere. The rate of formation increased steadily to a maximum of 0.087 mg/day on day 27. A considerable scatter of points was found in the graph of N_2O formation rates. Although trends were evident, it was difficult to draw firm conclusions as to the relationship between concentration of soil NO_2^- and evolution of the gas.

Nitrogen (N_2^1) derived solely from added labelled urea, was found from day 3 but formation was low throughout the experiment and tended to follow similar trends to N_2O production. Total losses of N as N_2^1 were 0.847 mg over the course of the experiment (Table 4). Thus losses as N_2O or N_2 derived from N_2O were 60% of all N lost during this study. These losses were spread out over the course of the experiment. Such was not the case where N_2^2 was concerned. Formation started at day 12 and was essentially finished by day 48. The majority of the 1.970 mg N lost from the system via the van Slyke-like reaction was evolved between days 15 and 21.

Total gaseous loss of N was 4.058 mg or 20.3% of the added N. Total recovery was 97% of added N (Table 4).

TABLE 4

Gaseous recovery of N from 1000 ppm-N as urea (20 mg per 20 gm soil).

Soil	pH	Gas production (mg) N_2O	N_2 [1]	N_2 [2]	Total loss of added N mg*	Inorganic N Remaining mg	Recovery mg	% Recovery
Wellwood	6.1	2.226	0.847	1.97	4.058	15.39	19.45	97%
Wellwood	7.6	2.162	0.61	2.585	4.065	15.64	19.7	99%
Neuenberg	6.0	0.015	-	-	0.015			
Neuenberg	7.1	0.071	-	-	0.071			
Neuenberg	8.0	.269	.009	.013	0.291			

Note: Inorganic analysis of Neuenberg samples not carried out

* In computing total losses - since N_2 [2] had only half its N coming from added N, half of the production value of N_2 [2] was used.

b) Carbonated - Wellwood, pH 7.6

Ammonium oxidation approached completion more closely in this study than with the soil at pH 6.1, possibly due to increased Nitrosomonas spp. activity at higher pH. Ammonium levels were only 3.0 mg by the termination of the experiment in contrast to 11.6 mg in the non-carbonated samples (Fig. 6a). Nitrite was found from day 3 and increased rapidly to a maximum value of approximately 11 mg by day 24. For the duration of the study, NO_2^--N remained near this level. Nitrate was also present from day 3 although its continued formation via the further oxidation of NO_2^- seemed to be very slow until day 36. Even after this point in time NO_3^- levels increased slowly and reached only 5.4 mg by the end of the experiment. It seemed that after the peak in NO_2^- formation had been reached, further oxidation of NO_2^- to NO_3^- was balanced by oxidation of NH_4^+ to NO_2^-. The soil pH in this experiment rose from 7.6 to 8.3 on day 3 but had levelled off at 7.7 by day 21 (Fig. 6a). It did not decrease with continued incubation.

It is quite evident from the results shown in Figs. 5b and 6b that N_2 and N_2O were formed while the oxidation of urea was taking place. The nitrogen produced was mainly N_2, van Slyke-like nitrogen. Thus, half of the N atoms of the N_2 gas evolved during the oxidation of urea originated from the soil. Such formation of N_2 was not prevented by lime application.

No van Slyke-like N_2 was found during the first 9 days when NO_2^- levels were low. As NO_2^- concentration rose, so did the rate of

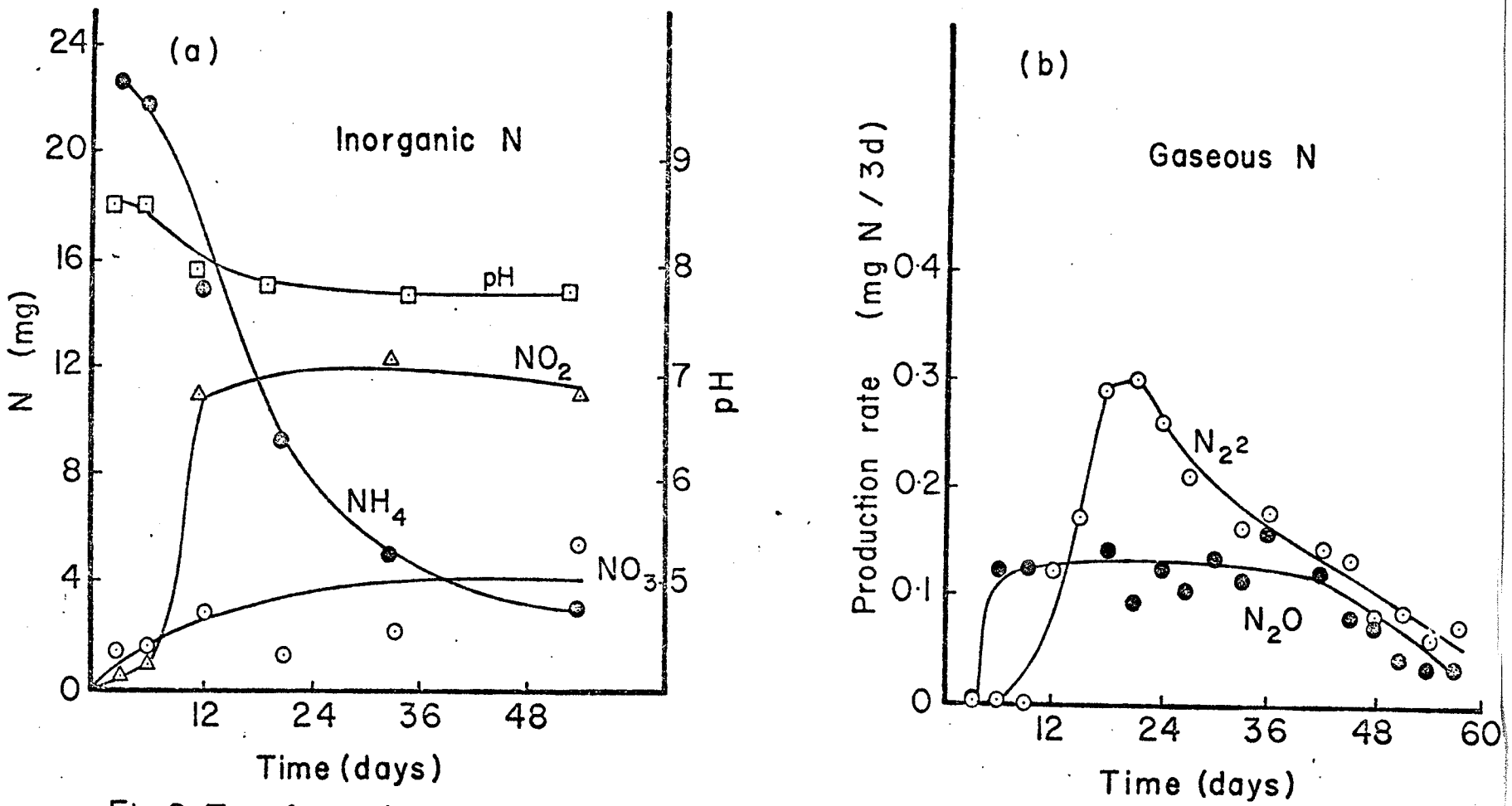

Fig 6. Transformation of Urea to various N compounds, Wellwood with 10 % $CaCO_3$, pH 7·6

N_2^2 formation. After attaining a peak production rate of 0.1 mg/day on day 21, the rate of formation steadily decreased until the end of the experiment when it was being evolved at approximately 0.02 mg/day. The total nitrogen loss via this pathway was 2.585 mg which is approximately 31% more than was lost as N_2^2 in the non-carbonated sample (Table 4). This increased loss may be attributed to the sustained presence of NO_2^- after day 12.

It is difficult to determine why the decrease in van Slyke-like N_2 production occurred from day 18 despite the continued presence of high levels of NO_2^- and stable pH. The decrease could possibly be due to a depletion of the type of soil N necessary to combine with nitrite in this reaction.

Production of N_2O rose from zero on day 3 to 0.045 mg/day by day 6 and continued at about the same level for the next 39 days. After this, bacterial activity declined as is evidenced by a decline in O_2 consumption and CO_2 evolution rates. This decreased activity resulted in a lowered O_2 demand thereby causing less NO_2^- to be denitrified to N_2O. The decreased biological activity may have been due to a decreased level of readily available soil carbon by day 45.

The formation of N_2^1 parallelled the curve of N_2O formation although maximum rates never exceeded 0.023 mg, respectively, over the course of this study (Table 4). Total inorganic nitrogen recovered was 15.64 mg and total gaseous losses reached 4.065 mg. Recovery of added N was 99%.

In comparing Figs. 5b and 6b, two trends are immediately evident: 1) the N_2 production did not decline as rapidly after reaching a peak in the carbonated samples as it did in the unamended soil; 2) the N_2O formation curve was a broad, flat-topped curve in Fig. 6a with no well-defined maximum point such as was found with Fig. 5a.

These differences were attributed to the continued presence of high levels of soil NO_2^- throughout the experiment in carbonated samples.

c) Unlabelled urea-treated Wellwood

Mass spectrometric scanning of a mixture of gas separates the various components depending on their m/e (mass to charge ratio). Thus, for example, a.m.u. 28 could be N_2^+ or CO^+. Some doubts could be cast upon the identity of a.m.u. 29, 31, 45 and 46 as they are not normally abundant in the atmosphere. They could be produced by the addition of ^{15}N or could be produced by other components. Thus scans of gas produced by incubating unlabelled urea were carried out to confirm that the a.m.u.s listed above were solely the result of ^{15}N presence in the gases.

Scans of unlabelled urea at day 12 with both carbonated (pH 7.6) and non-carbonated (pH 6.1) samples showed no significant peaks at masses 29, 31, 45 or 46 above the levels found in an air scan (see Table 5). This indicates that peaks with a m/e ratio of 29, 31, 45 or 46 found during scans of labelled urea gaseous products were the result of ^{15}N atoms in the gas mixtures.

TABLE 5

Relative peak heights of a.m.u. 28 to 46 from an air scan as compared to scan of the gas above soil samples incubated with 1000 ppm of added urea-N.

a.m.u.	Normalized peak heights		
	Wellwood pH 6.1	Wellwood pH 7.6	Air
28	3477.4	3427.1	3532.8
29	25.865	25.574	26.454
30	7.876	6.519	-
31	-	-	
32	34.023	6.296	883.68
40	100	100	100
44	44.559	37.000	2.251
45	0.357	0.018	-
46	-	-	-

time 12 days

$\%^{15}N = 0.37\%$

d) Neuenberg Soils

Incubation of 1000 ppm-N as urea with Neuenberg soil showed (Table 6) that the production pattern of various gases from this soil was quite different from that of Wellwood. Losses of N from the Neuenberg soil were very much lower than those from the Wellwood samples.

Nitrogen dioxide and N_2 were found only when there was almost complete consumption of O_2 in the system. The consumption rate of O_2 by the Wellwood soil during the three-day interval was not as great as observed with Neuenberg soil, indicating that the biological activity of the latter soil must have been greater than the Wellwood soil.

The major form of gas derived from urea was N_2O in the Neuenberg soil. There was virtually no N_2, whether N_2^1 or N_2^2, formation from the Neuenberg soil. The nitrogen lost during the incubation period was 0.269 mg and it amounted to 1.3% of the N added as urea. The percentage of ^{15}N in N_2O was nearly constant but was quite low in the early stages of incubation. It is not known whether it was due to isotopic separation or experimental error because of the low N_2O content.

Losses started from day 14 and continued to day 27 in this sample. Dinitrogen was found in small amounts only during the final sampling period. Losses of nitrogen from unamended Neuenberg and acidified Neuenberg soils occurred at approximately the same times but were not as large.

The production rate of N_2O from unamended Neuenberg was lower than that of carbonated Neuenberg. Total loss of nitrogen was only 0.071

mg from unamended Neuenberg as compared to 0.269 mg from carbonated samples. It is not known why the addition of lime to an alkaline soil increased the production of N_2O while urea was incubated in an initially aerobic system. It could be due to the inhibitory effect of free lime on the activity of Nitrobacter spp., thereby causing the accumulation of NO_2^-. It could also be due to increased microbial activity in the limed soil thereby inducing anaerobic conditions quickly during the three days of closed incubation. No investigation regarding the activity of Nitrobacter spp. or the rate of O_2 consumption due to the addition of lime was carried out. The development of anaerobic conditions during incubation may have reduced the oxidized forms, such as NO_2^- and NO_3^- which had been formed during the oxidation of urea.

Acidification of the Neuenberg soil to pH 6.0 did not promote the evolution of nitrogen gases to any significant extent. This was probably due to decreased microbial activity at lower pH thereby preventing the development of anaerobic conditions. It was therefore not necessary for the soil population to reduce NO_2^- or NO_3^- to respire.

Table 6 Gaseous production during incubation of Neuenberg soil samples with 54% ^{15}N-enriched urea.

a) Neuenberg pH 6.0, (acidified)

time (days)	N_2O (mg)	N_2^1 (mg)	N_2^2 (mg)	$\%O_2$ left	$\%^{15}N$
15-18	0.001	-	-	1.0%	34.8%
14-21	0.014	-	-	1.3%	19.9%

b) Neuenberg pH 7.3, (uncarbonated)

time (days)	N_2O (mg)	N_2^1 (mg)	N_2^2 (mg)	$\%O_2$ left	$\%^{15}N$
10-14	0.017	-	-	2%	36.4%
15-18	0.017	-	-	1.7%	18.1%
19-21	0.027	-	-	0.2%	35.3%
22-24	0.010	-	-	0.2%	33.27%
	$\Sigma = 0.071$				

c) Neuenberg pH 8.0, (carbonated)

time (days)	N_2O (mg)	N_2^1 (mg)	N_2^2 (mg)	$\%O_2$ left	$\%^{15}N$
10-14	0.016	-	-	3%	29.9%
15-18	0.055	-	0.004	0%	41.7%
19-21	0.057	-	-	0.3%	43.1%
22-24	0.07	-	-	2.0%	44.6%
25-27	0.071	0.009	0.009	0%	44.7%
Σ	0.269	0.009	.013		
	Σ= 0.291 mg				

Experiment 2 Denitrification of Ca $(NO_3)_2$ in Wellwood Soil

Table 7 illustrates the per cent recovery of O_2 at the end of each sampling period where 100% would indicate no O_2 consumption over 3 days.

It is evident from the table that the higher concentration of added NO_3^- had an inhibitory effect on the rate of O_2 consumption by the soil microorganisms in Wellwood soil. In the case of 100 ppm-N as NO_3^- the biological activity in the carbonated sample seemed to be higher than the non-carbonated sample for the first nine days of the experiment. The pH of the carbonated soil was higher than the non-carbonated sample and this factor probably promoted soil respiration. After day 9 however, the rates of O_2 consumption in both samples appeared to be similar.

With 1000 ppm-N as NO_3^-, no losses were recorded as van Slyke-like nitrogen. Although very slight losses as N_2O were noted, the amounts produced were too low to be quantified using the mass spectrometric methods employed in these studies. This lack of N_2O formation is due to the fact that the biological demand for O_2 was quite low under the conditions of this study. Therefore, NO_3^- was not used as an alternate electron acceptor and N_2O was not found.

When 100 ppm-N as NO_3^- was the N source, slight losses of N were recorded in the uncarbonated samples only. Total losses, which occur between day 4 and day 15 amounted to only 0.324 mg and were in the form of N_2O only(Table 7). It is surprising that any losses would have occurred via this pathway in the light of the apparently sufficient

TABLE 7

Percent of original atmospheric O_2 remaining after 3 days of incubation with nitrate and loss of N_2O from 100 ppm-N-nitrate sample.

time period days	1000 ppm-N- NO_3^-		100 ppm-N- NO_3		loss from unlimed sample (mg N_2O) (100 ppm-N-NO_3^-)
	unlimed	10% $CaCO_3$	unlimed	10% $CaCO_3$	
0-3	68	60	47.8	15.8	-
4-6	77	66	55.1	16.5	0.073
7-9	77.5	66	64.5	29.1	0.080
10-12	85	75	60.0	56.6	0.076
13-15	87	77	62.6	54.8	0.095
16-18	62	64	62.4	63.6	-
				Σ	0.324 mg.

supply of O_2 in the system. Possibly some denitrification may have occurred in the centres of soil aggregates where O_2 diffusion rates were slow.

Even though the carbonated samples used O_2 faster, no measurable denitrification products were recovered. Addition of $CaCO_3$ made the soil more friable than non-carbonated samples so that even though the O_2 consumption rate was greater in these soils, few aggregates were present and so O_2 diffusion to any soil site was not a limiting factor to microbial growth. Therefore no reduction of NO_3^- to N_2O occurred.

Thus it seems that under aerobic field conditions, NO_3^- will not react with soil organic matter to yield van Slyke-like nitrogen, nor will it be biologically reduced to N_2O or N_2[1] unless microbial oxygen demand is high and/or O_2 diffusion through the soil is inhibited. That is, NO_3^- is usually quite a stable form of inorganic nitrogen in soil.

Experiment 3 Denitrification of $NaNO_2$ in Wellwood and Neuenberg soils.

a) Wellwood Soil

The rate of formation of all the denitrification gases is greatest in the Wellwood soil at pH 6.1 (Table 8). In this case 63% of the added nitrogen was evolved via the denitrification process, the majority being as N_2O. The rate of formation of N_2O was rapid over the first two sampling periods and thereafter remained relatively steady. By day 15, most of the nitrite was gone and soil nitrite was entering the NO_2^- pool, diluting the % ^{15}N. This made accurate measurement of evolved gases very difficult. Thus, measurement error may have been the cause of the apparent rise in rate of N_2O formation by day 15.

The rate of formation of N_2[1] seemed quite low and stable until the last sampling period which, as previously mentioned, could have been a measurement error. As O_2 levels in the samples were relatively high throughout the study, the microbes had little need to reduce N_2O further.

The rate of formation of van Slyke-like nitrogen decreased rapidly from the initial period and by day 12, none was being produced. The total loss via this reaction was only 0.206 mg or slightly less than 5% of the nitrogen added for only half the N atoms came from the fertilizer source. As will be shown in later experiments, a threshold value of approximately 50 ppm -N as NO_2^- is necessary in Wellwood soil at pH 6.1 to allow the rate of formation of N_2[2] to be great enough to be measured in a 3 day period. Probably by this point in time, less nitrite was

TABLE 8

Gaseous nitrogen losses from Wellwood and Neuenberg soils treated with 100 ppm -N as Na NO_2 (mg).

	Wellwood pH 6.1				Wellwood pH 7.6				Neuenberg pH 7.1			
time period (days)	N_2O	N_2^1	N_2^2	% O_2 left	N_2O	N_2^1	N_2^2	% O_2 left	N_2O	N_2^1	N_2^2	% O_2 left
0-3	0.619	0.012	0.173	55%	0.153	0.010	0.011	30%	0.0226	0.006	0.014	48.9%
4-6	0.114	0.014	0.025	74%	0.031	-	-	34%	0.014	0.008	0.011	46.9%
7-9	0.077	0.022	0.008	64%	0.076	0.006	0.035	20%	-	-	-	-
10-12	0.078	0.016	0	65%	0.079	-	-	54%	0.014	0.012		47.9%
13-15	0.119	0.084	0	60%	0.043	0.004	-	63%	-	-		91. %
	1.007	0.148	0.206		0.382	0.020	0.046		0.051	0.026	0.025	

sum = 1.258 % of fertilizer added=63% sum = 0.425 % of fertilizer added=34% sum = 0.076 % of fertilizer added=4%

Note: In determining % recovery only one half the loss as N_2^2 was calculated as coming from the fertilizer.

present in the soil than was necessary to allow the van Slyke-like reaction to proceed at a measurable rate. Total losses in all gaseous forms amounted to 1.258 mg or 63% of the fertilizer nitrogen added.

When the Wellwood soil was carbonated similar trends in N gas formation were recorded although the overall rates of formation were greatly reduced from those of the Wellwood soil at pH 6.1. Total loss amounted to 0.425 mg or 34% of that lost from non-carbonated samples (Table 8). The breakdown of losses was 0.382 mg N_2O, 0.020 mg N_2[1] and 0.046 mg N_2[2]. Although O_2 consumption was higher in the carbonated samples, the formation of N_2O was lower. This could be due to the increased friability of the Wellwood soil when free lime was present. Another factor could be increased nitrite stability with increased pH.

It appears that formation of N_2O and N_2[2] are pH-dependent reactions and that the rate of formation of these gases decreases with time for a given initial concentration of nitrite; i.e. the rate decreases as nitrite is used up over time.

b) Neuenberg Soil

When Neuenberg soil was incubated under similar conditions to Wellwood, losses of gaseous nitrogen were very much reduced (Table 8).

Losses of N_2O were maximum during the first 3 day period but the maximum in this soil was much lower than the minimum production rate in any 3 day period for the Wellwood soils. Total loss of N_2O was 0.051 mg over the 15 day period. (The nine day sample was lost and so an average value for N_2O loss between the period previous and the period after was used as the loss for that period.) Production rates of both types of nitrogen gas were also very much decreased, totals equalling only 0.051 mg. Overall gaseous loss of N from Neuenberg soil was 0.101 mg being approximately 4% of the fertilizer added.

It is evident that nitrite is quite stable in the Neuenberg soil which has a higher native pH than Wellwood.

Experiment 4 Effect of Nitrite Concentration on Denitrification.

The levels of NO_2^- chosen for this experiment corresponded approximately to the range of levels found in Experiment 1 where 1000 ppm-N was added as urea. High concentrations of NO_2^- can be found in the area immediately around the fertilizer pellet during urea hydrolysis. The object of this experiment was to determine the effect of a high concentration of NO_2^- over a short period of time on the rate of denitrification.

a) pH 6.1

Incubation of NO_2^- with the Wellwood soil resulted in the formation of N_2 and N_2O (Fig. 7). It appears that the rate of van Slyke-like N_2 formation varies linearly with concentration of added NO_2^-. At 500 ppm -N NO_2^-, 8.2% of the added N combined with an equal amount of soil N resulting in the loss corresponding to 16.4% of added NO_2^--N from the soil system in 3 days. Extrapolation of the curve back to the abscissa indicates that the threshold value of NO_2^- necessary to allow this reaction to proceed is approximately 42 ppm -N as NO_2^-. Below this concentration, the evolution rate of this form of nitrogen is too slow to be measured over three days.

The rate of nitrous oxide formation increased with increasing NO_2^- up to 100 ppm N_1 (Fig. 7). In three days 0.430 mg N as N_2O was produced. The rate of formation decreased to 0.095 mg/3 days with

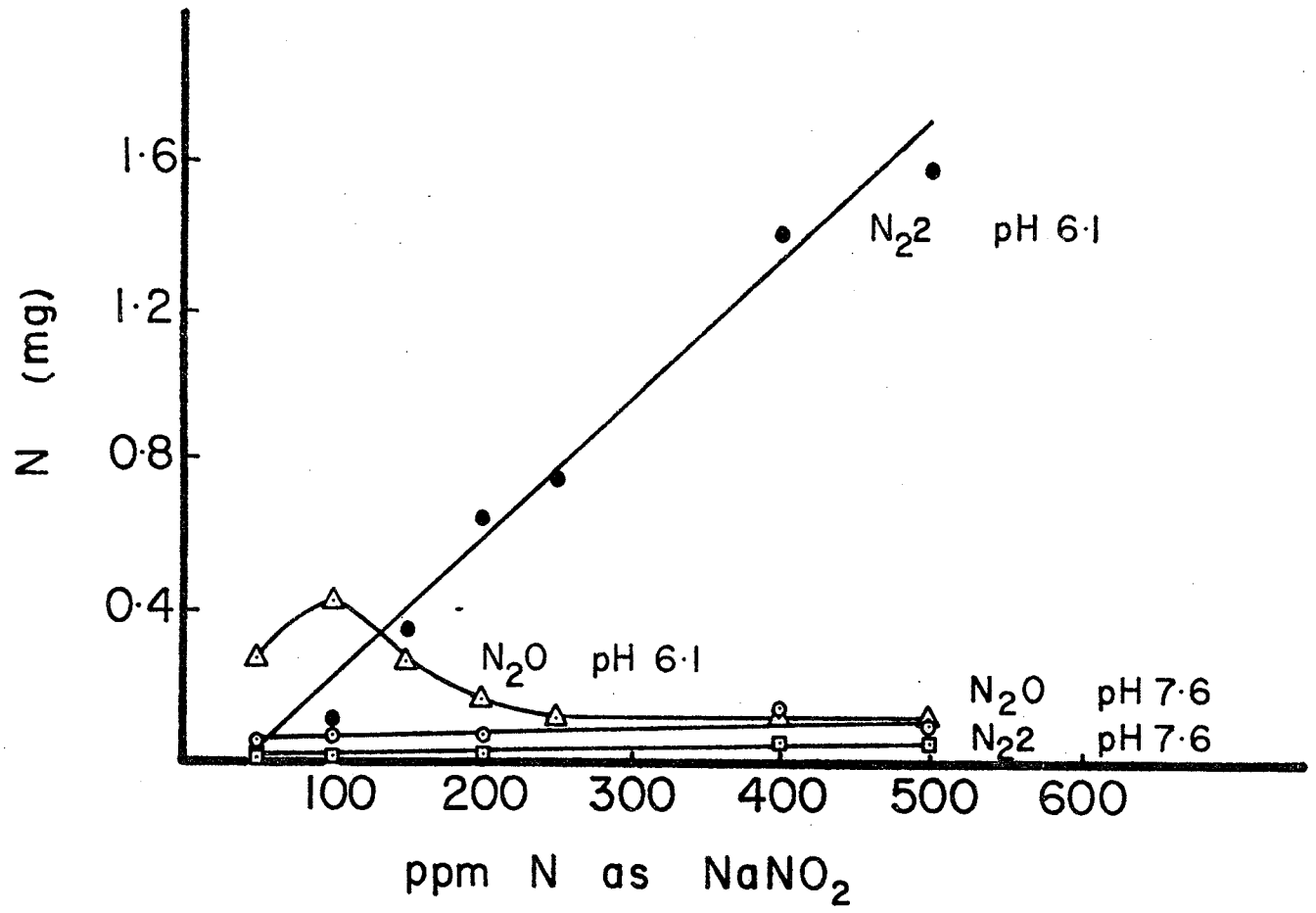

Fig 7. Effect of Soil pH and Concentration of N upon $NaNO_2$ Denitrification

500 ppm -N as NO_2^-. This could be due to NO_2^- induced inhibition of the soil microbial population. The toxicity of this ion can be quite pronounced at low pH (Tyler and Broadbent, 1960). Losses of nitrogen as N_2O were not very large at higher levels of soil nitrite over a 3 day period.

Production of N_2 via the reduction of N_2O was very low decreasing from 1.8% (0.023 mg/3 days) at 50 ppm to 0.3% (0.028 mg/3 days) at 500 ppm-N.

b) pH 7.6

In the carbonated samples of Wellwood soil, gaseous loss of nitrogen due to interaction of NO_2^- and the soil was greatly decreased. Even at initial levels of nitrite of 500 ppm -N, losses as N_2O and N_2 amounted to only 0.108 and 0.046 mg -N respectively. Losses of van Slyke-like nitrogen in carbonated Wellwood are only 3% of those of unamended samples (Fig. 7).

The results were contrary to those of Experiment 1. The results of Experiment 1 showed significant formation of N_2, van Slyke-type N_2, when carbonated Wellwood soil was treated with urea. In that study, losses of N as N_2 were recorded when nitrite began to accumulate despite the fact that the pH measured throughout the experiment was in excess of 7.6. A possible explanation for this apparent anomaly may be found by considering the pH of the microsites in the soil.

In a soil suspension, hydrogen ions are not distributed uniformly but are clustered around the negatively charged clay micelles.

Therefore when an electrode is placed in a soil suspension, the pH measured is only an average measure of the pH of the system and does not accurately reflect the pH of the area near the soil colloid. Ammonium ions would behave in a like manner to the hydrogen ion and therefore would tend to be either adsorbed to soil colloids or exist in the vicinity of the colloid. Lees and Quastel (1946) concluded that nitrifiers preferentially oxidize NH_4^+ ions adsorbed on soil particles. This would tend to result in a lower pH in the area of the colloid surface as NO_2^- is formed via the following reaction:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

Thus, even though the gross pH of soil-water system was high in the carbonated urea - Wellwood experiment, the pH near the microsite where the NO_2^- was being produced may have been lower.

In cases where nitrite alone is added to soils, little alteration of the pH near the colloid is expected as NH_4^+ oxidation is not taking place. Thus the pH near the colloid is relatively constant and it seems reasonable to suppose that when NO_2^- is added, the pH which is measured more accurately reflects the pH of the entire soil system.

Thus it is possible to explain the high production rate of N_2 when urea was added to the carbonated soils even though similar additions of NO_2^- to similar soils showed little van Slyke-like N_2 formation.

Experiment 5 Effect of Soil pH and Concentration of N on Na NO_2 Denitrification.

In the previous study, only two soil pH values, namely 6.1 and 7.6 were used. This study involved Wellwood soil at pH values of 6.1, 6.8, 7.2 and 7.6.

It is evident (Fig. 8) that at lower levels of added NO_2^-, N_2O production varied inversely with pH. With 50 ppm-N as NO_2^-, the N_2O formation rate went steadily down from a peak of 0.218 mg/3 days at pH 6.1 to 0.053 mg/3 days at pH 7.6. Very similar trends were noted at 100 ppm -N where N_2O production again went from a high at pH 6.1 (0.430 mg/3 days) to a low at pH 7.6 (0.067 mg/3 days).

At concentrations of 200 ppm -N, less N_2O formation was found at pH 6.1 than at pH 6.8. This was probably due to inhibition of soil microorganisms by the combination of low pH and high NO_2^- concentration. This observation has been reported previously by Broadbent and Tyler (1962). From pH 6.8 to 7.6, N_2O production decreased with increasing pH in a manner similar to that which occurred at the 50 and 100 ppm rates of NO_2^- -N. Results with 400 ppm NO_2^- -N were the same as those with 200 ppm NO_2^- -N except that inhibition was observed at pH values of 6.1 and 6.8.

The formation of N_2 via the reduction of N_2O was very low over the 3 day study period (Fig. 9). A general trend of decreasing N_2[1] formation with increasing pH was apparent. The rate of loss seemed to decrease, in a given pH range, with decreasing concentration of added NO_2^-. The greatest loss recorded via this pathway occurred

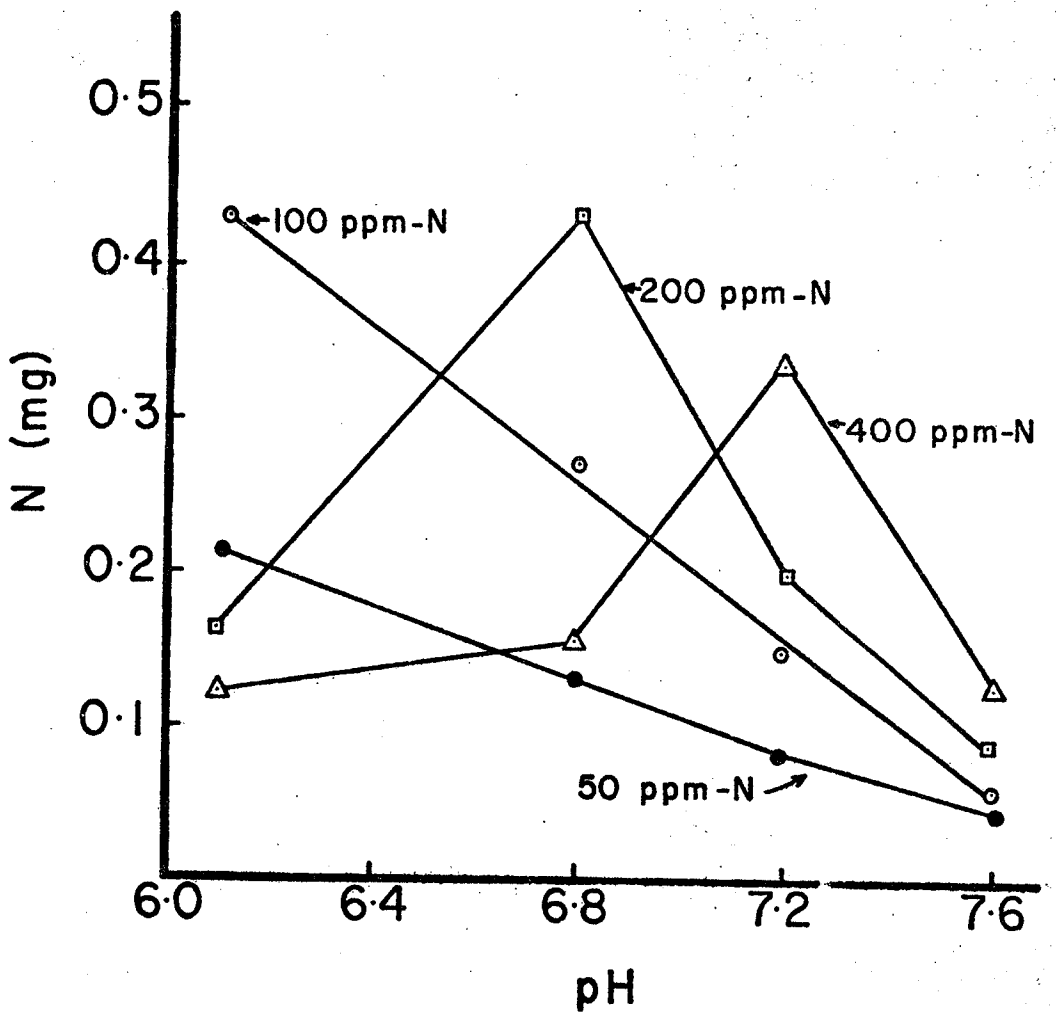

Fig 8. Effect of pH and $NaNO_2$ concentration on N_2O production

time = 3 days

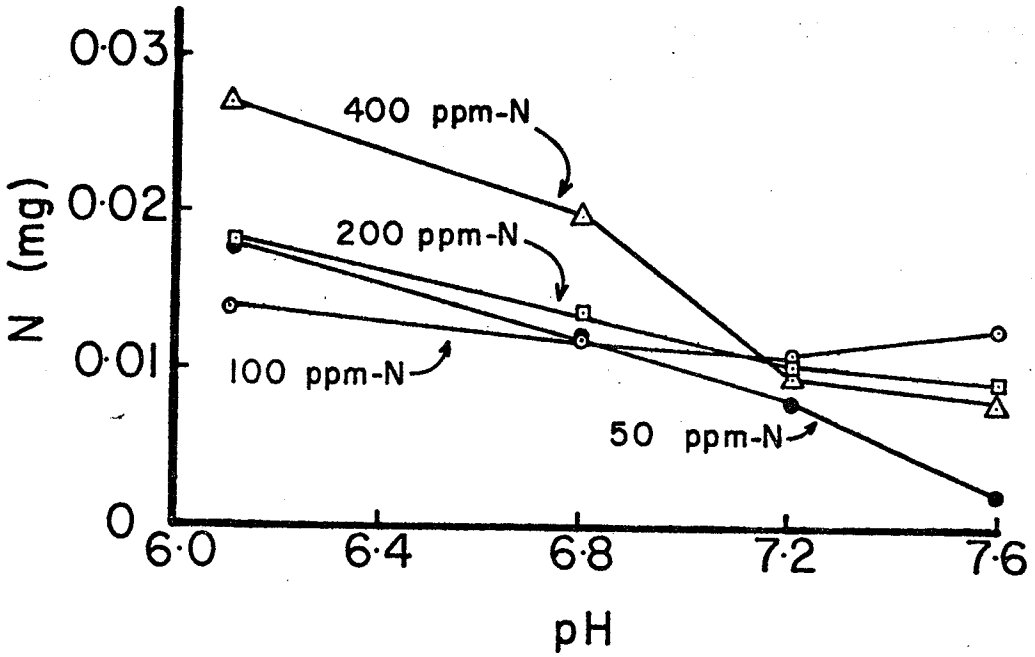

Fig 9 Effect of pH and $NaNO_2$ concentration on N_2 derived from N_2O

time = 3 days

at pH 6.1 with 400 ppm-N as NO_2^- and accounted for only 0.027 mg over 3 days. The losses of N through this pathway were not very great as the oxygen demand was not sufficient to result in reduction of N_2O. With a higher O_2 demand, this could be a more important source of N loss from the system.

As is evident from Fig. 10, losses of NO_2^- and soil N via the van Slyke-like reaction were quite extensive. Again, the same trends of decreasing loss with increasing pH were evident. Total loss increased with an increasing concentration of added N. The greatest loss over 3 days occurred at pH 6.1 and 400 ppm-N as NO_2^- where 1.409 mg were lost. Losses were quite low at pH 7.2, the highest loss, again recorded with 400 ppm-N, being only 0.055 mg/3 days. Losses of N when only 50 ppm-N NO_2^- were present were quite low, 0.035 mg/3 days, and decreased to zero at pH 7.2. Losses from 100 and 200 ppm-N NO_2^- also decreased linearly with pH and reached zero at pH 7.2

No inhibition of this reaction occurred even at high N levels suggesting that the van Slyke-like reaction is chemical in nature.

By plotting the amount of nitrogen formed in this manner versus the amount of NO_2^--N added it was possible to determine the K value (rate constant) for the first order reaction of N_2^2 formation, at each pH used. The K values, listed below, were then plotted against pH to give the following equation relating pH to rate of van Slyke-like N_2 formation.

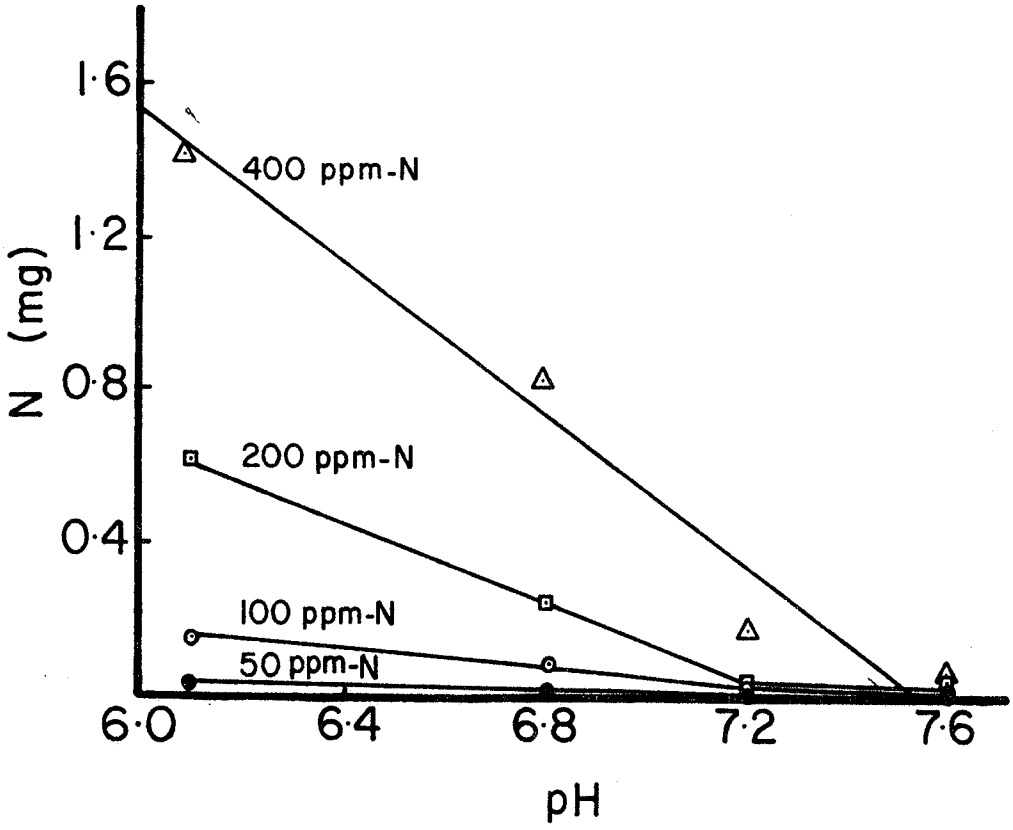

Fig 10. Effect of pH and $NaNO_2$ concentration on van Slyke-like N_2 production time = 3 days

$$\frac{d\ (N_2)vs}{dt} = K\ (NO_2^-)$$

$$\frac{d\ (N_2)vs}{dT'} = K\ (NO_2^-)_o$$

pH	K	$(\frac{mg}{20g - 3\ days - ppm})$
6.1	0.0040	
6.8	0.0028	
7.2	0.0005	
7.6	0.0002	

$$K = 0.0206 - 0.00272\ pH$$

Theoretically the maximum pH for this reaction to occur in this soil at this moisture content is 7.57. The slight deviation of the data from this expected termination point is probably due to the error in measurement of N gases when such low amounts are evolved.

Experiment 6 Effect of Steam Sterilization and Glucose Addition on the Denitrification of NO_2^- with Time.

This study was carried out to determine the effect of microbial activity on the rate of formation of the various denitrification products of nitrite. Microbial activity was, in one case stimulated by the addition of 0.5% (by weight) glucose to a soil - NO_2^- mixture and in the other case essentially stopped by steam sterilization. Controls were run with unaltered Wellwood soil. A sample of Keld soil was also included to determine if the van Slyke-like reaction was peculiar to the Wellwood soil.

a) 200 ppm - N NO_2^-

When the soil was amended with glucose (Fig. 11a) the rate of N_2O formation was very rapid after a delay of 3 days. The production of this gas had a maximum of 2.224 mg over 7 days. After this period, so much CO_2, N_2O and N_2[1] had been produced that the pressure in the vessel became too high and eventually the gas leaked out. The N_2O curve was sigmoidal in shape.

In the unamended soils (Fig. 11b) initiation of N_2O formation took longer to start. Inhibition of the denitrifying bacteria seemed to occur over the first 3 days but was slowly overcome. Nitrogen dioxide production was very rapid between days 3 and 9. It was only when nitrite levels became very low by day 9 that the rate of N_2O formation started to decrease. The total amount of nitrogen lost as N_2O produced in this soil was 3.065 mg.

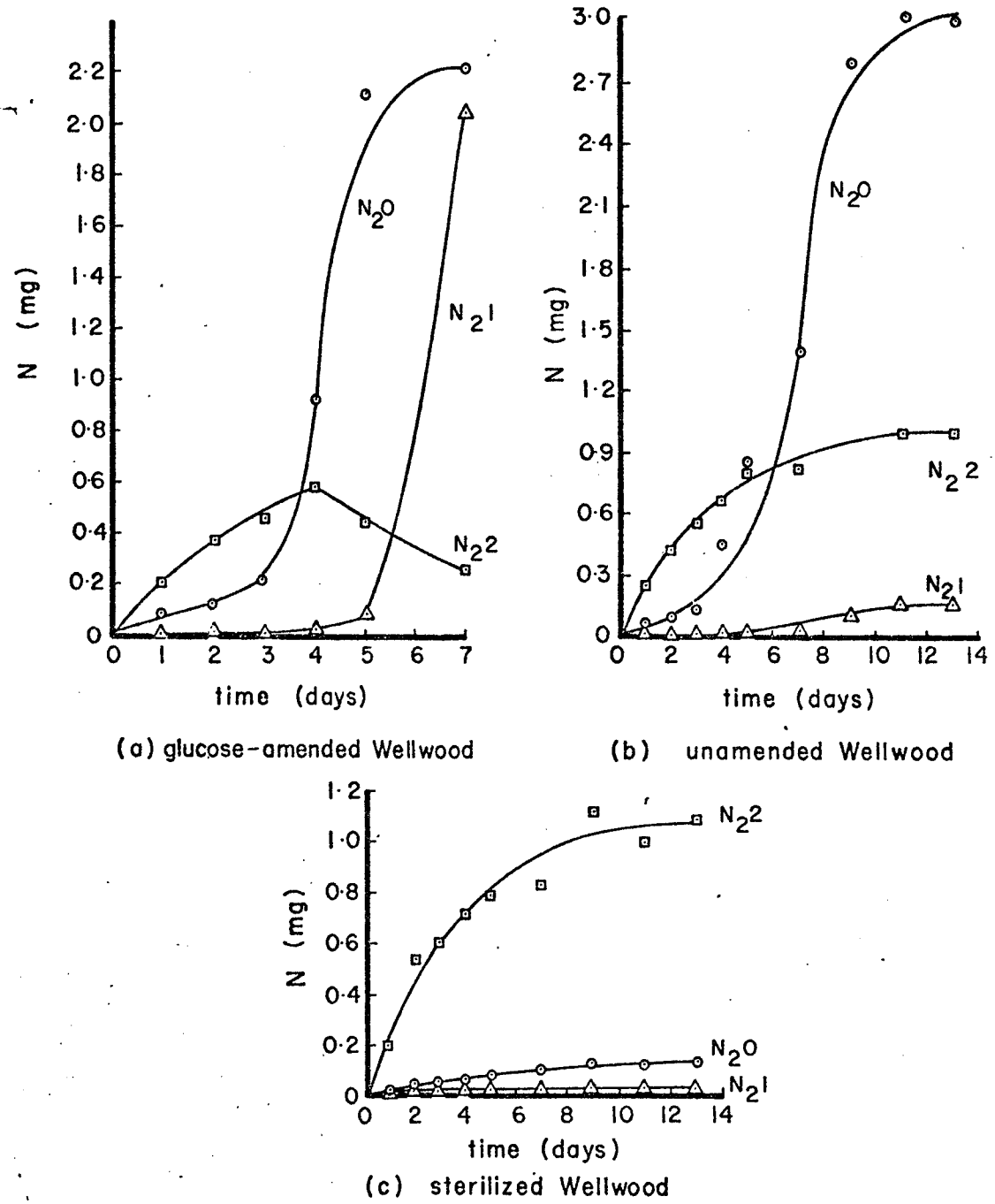

Fig 11. Effect of microbial activity on rate of production of various gaseous denitrification products.

200 ppm-N as NO_2^-

When Wellwood soil was sterilized, N_2O formation decreased (Fig. 11c). Total loss of this gas amounted to only 0.139 mg over 13 days. Formation of N_2O started on day 1 but amounts lost were not high. This loss could be due to the contamination of the soil sample, which could allow some microbial production of N_2O or the gas may have been formed by a chemical soil - NO_2^- interaction. Reactions in which nitrite combines at phenolic sites in organic matter (Nelson and Bremner 1970) or carbonyl sites of ketones (Stevenson et al. 1970) have been postulated as eventually releasing nitrogen as N_2O or N_2[1].

Nitrogen formed via the reduction of N_2O was not a significant loss pathway except in the case of the glucose-amended samples (Fig. 11a). Even here, N_2[1] was very low until day 5 when 0.091 mg had been produced. By day 7 however, 2.064 mg of this gas had accumulated. High biological activity was reflected in the absence of oxygen in the containers. This led to the utilization of N_2O by the soil microflora as a terminal electron acceptor.

In the unamended and sterilized soils the demand rate for O_2 by the microbial population was not great enough to result in the further reduction of N_2O to N_2[1] during the experimental period so only 0.092 mg and 0.038 mg were found at day 13 in each soil respectively (Fig. 11b, 11c).

The rate of formation of van Slyke-like N_2 seemed to be independent of soil treatment so long as nitrite was present. When glucose-amended soil was used, N_2[2] production was very similar in rate to that of the other soil treatments for the first 4 days. However, with the glucose treatment by day 5, 66% of the added NO_2^- had been

lost as a gas and O_2 was depleted greatly. This probably led to the reduction of NO_3^- originally in the soil to NO_2^-, thereby diluting the labelled NO_2^- pool. The change in $\%^{15}N$ of labelled NO_2^- with time due to the addition of an external source of unlabelled NO_2^- has serious consequences with respect to the calculation of $\%^{15}N$ in the resultant gaseous compounds. The $\%^{15}N$ in N_2^1 and N_2^2 was calculated using equation (1). There is a tacit assumption in the use of that equation that there exists an isotopic equilibrium within each form of dinitrogen studied (i.e. N_2^1 and N_2^2). Such an assumption was necessary to calculate $\%^{15}N$ in N_2^1, for example, without measuring a.m.u. 28 peak height.

If a nitrogen sample was non-equilibrium with respect to ^{15}N, then one has to measure all 3 peaks, a.m.u. 28, 29 and 30, in order to calculate $\%^{15}$. Such a method could be used to calculate $\%^{15}N$ in N_2^1 in the absence of N_2^2. However, if the sample was a mixture of N_2^1 and N_2^2 and the $\%^{15}N$ in each component changed with time, i.e., each fraction is non-equilibrium with respect to ^{15}N, then there is no method presently available that enables us to calculate the contribution of N_2^1 and N_2^2 in each a.m.u. 28, 29 and 30. Thus, when the experimental results showed that labelled NO_2^- was being diluted by natural NO_3^-, no further calculation regarding the quantities of N_2^1 and N_2^2 was undertaken.

When unamended Wellwood soil was incubated with 200 ppm-N as NO_2^-, van Slyke-like N_2 production increased steadily until day 9, after which its rate of formation levelled off. This coincided with an almost complete depletion of soil nitrite. In the sterilized soil,

the production rate of N_2 was constant between days 2 and 11. The rate of formation of the gas was somewhat higher than the other two treatments of Wellwood, possibly due to an increase in availability of organic matter due to the steam sterilization.

Losses of N_2 accounted for 1.084 mg, 1.011 mg and 0.585 mg in the sterile, unamended and glucose-treated samples respectively. There was no apparent lag phase before the onset of N_2 formation. Production of van Slyke-like nitrogen appears to be chemical in origin in light of the fact that altering the biological activity had little effect on the rate of N_2 formation. High microbial activity resulted in rapid depletion of soil nitrite and, as nitrite disappeared, N_2 formation slowed.

b) 100 ppm - N as NO_2^-

When 100 ppm-N was the level of nitrite source, similar trends to those of 200 ppm-N were recorded (Fig. 12, a-d). Losses as N_2O were very high in the glucose-amended soil, accounting for 97% of the added NO_2^- by day 4. The delay in initiation of gas production was shorter than found with 200 ppm -N, possibly due to the fact that NO_2^- inhibition upon microbial activity decreased with the decreased concentration of added nitrite (Fig. 12a). Nitrous oxide formation in the unamended Wellwood yielded a sigmoidal curve, reaching a maximum by day 11 of 1.271 mg (Fig. 12b) Again, the inhibitory phase of the reaction was less apparent with the lower concentration. After day 11, the level of N_2O measured in the soil atmosphere started to decrease. This was probably due to further reduction of

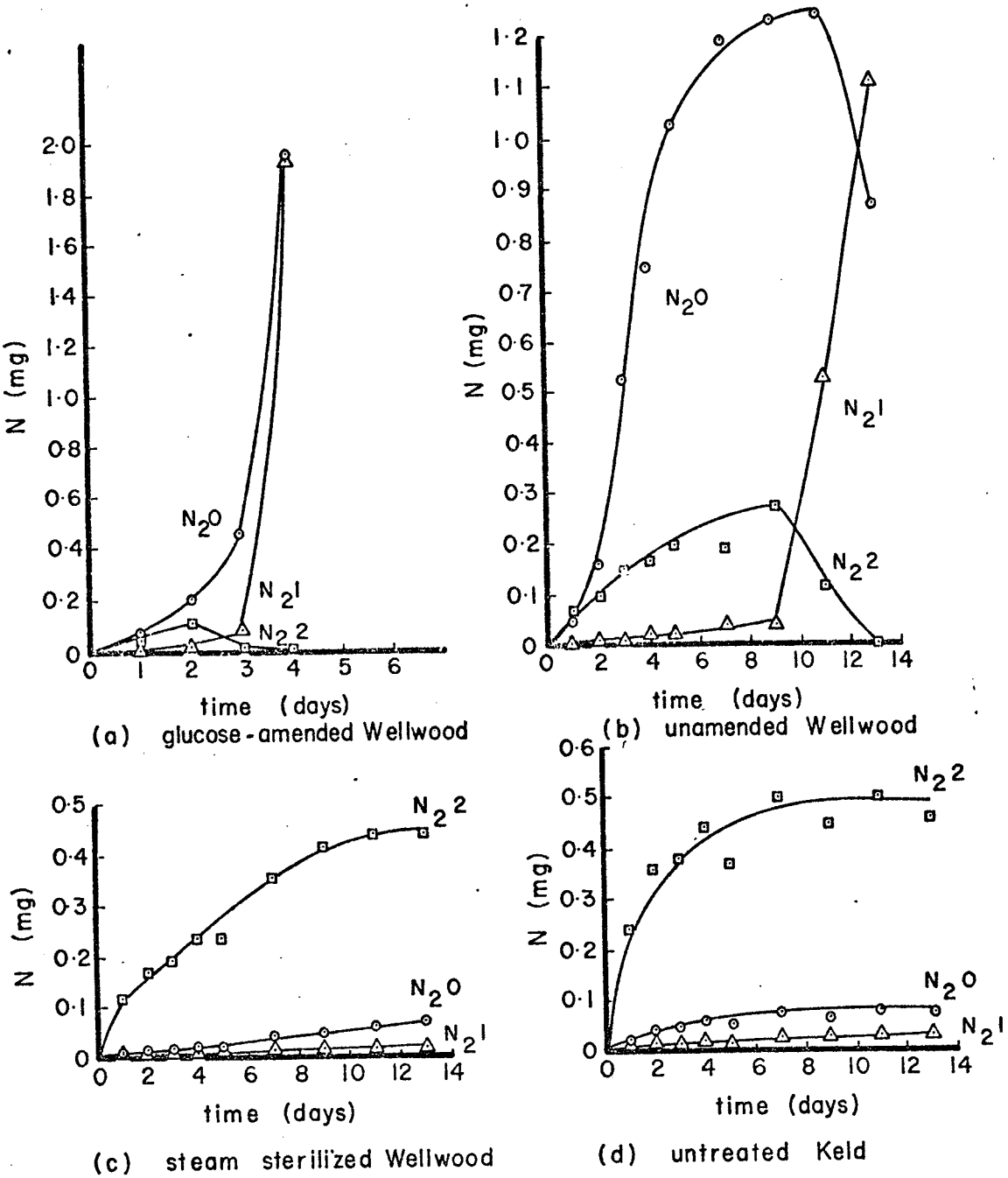

Fig 12 Effect of microbial activity on rate of production of various gaseous denitrification products.

100 ppm-N as NO_2^-

N_2O to N_2[1]. The final N_2O level at the end of day 13 was 0.868 mg; a decrease of almost 33% in 2 days. Sterilization resulted in much lower production of N_2O. The amount produced varied directly with time but amounted to only 0.069 mg by day 13. Since the soil was sterile and the reaction appeared linear, it is possible that this N_2O was the result of chemical rather than biological processes.

When the Keld soil was used N_2O production was found to be low. This was probably due to the low soil pH of 5.5. Oxygen consumption was quite low and by day 13 45% of the original O_2 in the system was still present indicating low microbial activity. Nitrous oxide formation levelled off as nitrite levels in the soil decreased. Loss as N_2O amounted to 0.080 mg by the termination of the experiment.

Trends in N_2[1] formation were similar to those of the 200 ppm-N as NO_2^- experiments (Fig. 11a-c) except on a decreased scale. The Wellwood soil treated with glucose showed a rapid rise in N_2[1] production on day 4 reaching a peak of 1.94 mg (Fig. 12a). Oxygen levels had decreased greatly by this time due to the high biological oxygen demand. This resulted in further reduction of N_2O to N_2. Continued incubation would probably have resulted in a decrease in N_2O found and the presence of N_2[1] only. However, all sample containers leaked after 4 days due to the high amounts of gas produced. With the decrease in N_2O after day 11 in the untreated control sample came a dramatic increase in N_2[1]. Amounts of this gas rose from 0.037 mg over 9 days to 1.108 mg by day 13. As with the glucose-treated sample, this was probably the result of a very actively

respiring microbial population.

In sterilized Wellwood and untreated Keld, microbial activity remained low throughout the experiment. Thus N_2^1 was never produced to any great extent reaching levels of 0.025 mg and 0.020 mg in Wellwood and Keld respectively. It seemed evident that N_2^1 production was the result of microbial reduction of N_2O. This occurred only after most of the O_2 and NO_2^- had been consumed by soil organisms and an alternate O_2 source was required.

Formation of van Slyke-like nitrogen seems to be generally unaffected by sterilization or glucose addition. The maxima of this gas were 0.109, 0.275 and 0.443 mg for glucose-amended, unamended and sterile soils respectively (Figs. 12a,b,c). Nitrogen gas, derived via the van Slyke-like reaction from the Keld soil was approximately 0.50 mg. The fact that Keld produced more N_2^2 than Wellwood under the same conditions was probably due to

a) lower pH of the Keld soil (5.5 vs 6.1)

b) more organic matter in Keld (8.1% vs 4.6%).

The van Slyke-like reaction was pH-dependent and was found to increase with decreasing pH. More organic matter would presumably tend to increase the N_2^2 formation rate as more soil N atoms would be available to combine with the soil NO_2^- to complete the van Slyke-like reaction.

Experiment 7 The Effect of pH on the van Slyke Reaction in Buffered Solutions.

Less than 10% of the added 2 mg N was recovered as N_2 in each atmosphere at pH 5 (Fig. 13). This percentage recovery dropped to approximately 1% by pH 6 and approached zero as the pH continued to rise.

When air was the incubation atmosphere, nitrogen evolution amounted to 0.267 and 0.286 mg at pH 2.3 and 3.0, respectively. Complete recovery of amino acid-N did not occur in air as inorganic N analysis showed that much of the added nitrite (80%) was oxidized to nitrate leaving little HNO_2 to react with the amino N. Slightly less nitrite was oxidized at pH 3 (78%) accounting, in part, for the slight increase in N_2 at this point.

When argon was the incubation atmosphere, recovery of added N as N_2 was found to be higher than that observed when air was used. Slightly in excess of 0.4 mg of N_2 were recovered at pH 2.3 and 3.0, representing almost 21% recovery. As pH rose, recovery as N_2 dropped greatly to 9.4% at pH 4, 1.4% at pH 5, and 0.3% at pH 6 (Table 9).

The results of this study seem to indicate that, in the pH range of soils used in this study, the classical van Slyke reaction would not be expected to be a major loss mechanism.

However, work by Harter and Ahlrichs (1967) has shown that the suspension hydrogen ion concentration may be up to 100 times lower than that at the soil colloid surface. Thus, it is

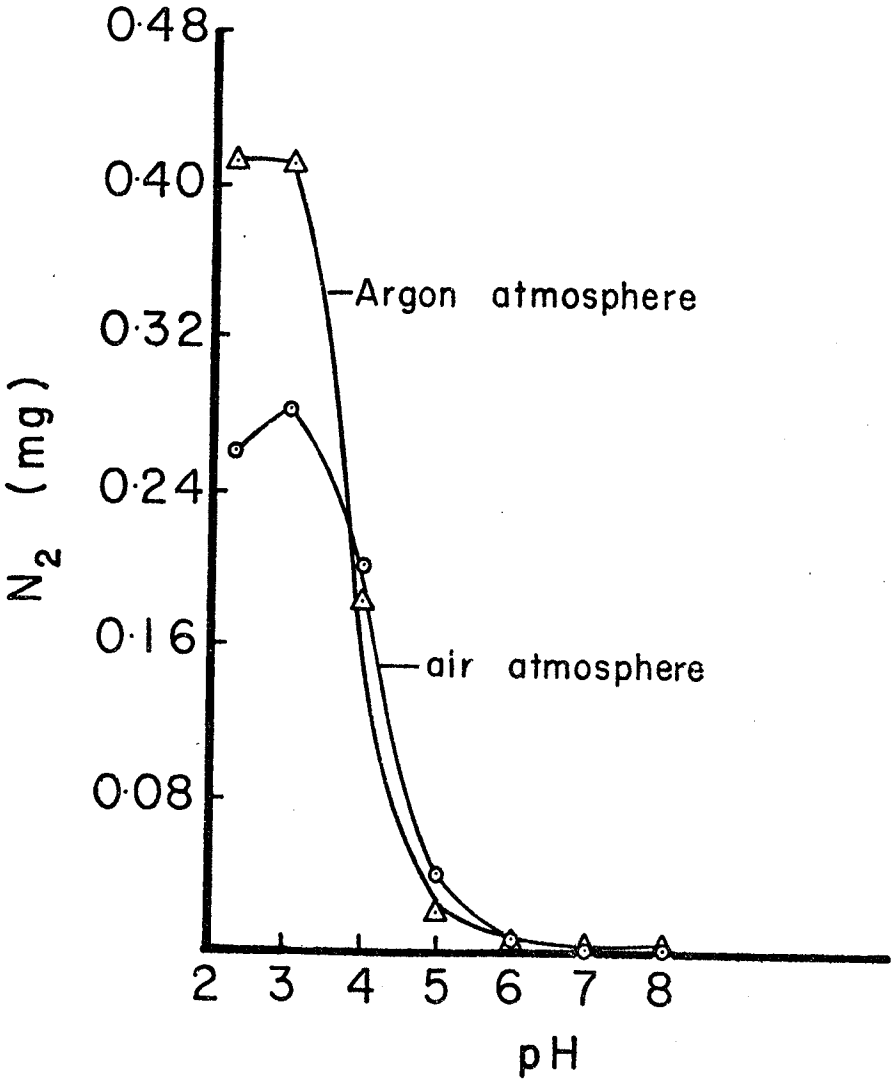

Fig 13. The Effect of pH on formation of van Slyke N_2 in Buffered Solutions

conceivable that while the pH values measured in many soil samples were over pH 6, the actual pH in the area of reaction near the colloid could have been very much lower, thereby allowing the classical van Slyke-type of reaction to occur.

TABLE 9

Amount of N_2 produced in air and argon between pH 2.4 and pH 8.0 in 3 days.

pH	N_2 mg. Air Atmosphere	Argon Atmosphere
2.43	0.267	0.418
3.0	0.286	0.416
4.0	0.203	0.187
5.0	0.043	0.027
6.0	0.003	0.005
7.0	0.004	0.004
8.0	0.007	0

SUMMARY AND CONCLUSIONS

Urea nitrogen was mixed with samples of Wellwood and Neuenberg soils and incubated for 2 months. Gaseous evolution rates were determined every 3 days and losses of N were measured mass spectrometrically. The concentration of urea in these samples was quite high (up to 100 ppm-N on a dry soil basis) in order to simulate conditions at the pellet site in soil.

With the Wellwood soil, gaseous losses as N_2O and N_2 amounted to approximately 25% of the added N. At pH 6.1 Wellwood samples were shown to accumulate NO_2^-, the appearance of NO_2^- coinciding with the appearance of gaseous N products in the soil atmosphere. As the NO_2^- was further oxidized to NO_3^-, gaseous production declined. The majority of the N_2 gas which accounted for 40% of the nitrogen gas produced was the result of a van Slyke-type reaction in which one atom of N came from a soil source and one came from the fertilizer. The remaining 60% of the nitrogen lost was recovered as N_2O. When the pH of the Wellwood soil was increased to 7.6 by the addition of 10% $CaCO_3$, the accumulation of NO_2^- occurred to a greater extent and lasted for the duration of the study. Losses of nitrogenous gases were slightly greater in this case. The rate of gaseous evolution slowly declined with time even though NO_2^- persisted.

Using another N source, $Ca(NO_3)$, in similar concentrations and similar conditions as were employed in the urea experiment, it was found that nitrate was stable when incubated aerobically. Slight

losses were recorded when the concentration of nitrate was lowered from 1000 to 100 ppm -N possibly due to increased biological activity with lower concentrations of nitrate added.

In the Wellwood soil without added $CaCO_3$ but with 1000 ppm-N as urea, the pH rose from 6.1 to 8.3 and then slowly decreased to 5.7 by the end of the experiment. When the carbonated soil was used, the pH rose to 8.3 and slowly decreased to 7.6 where it remained for the duration of the study.

Gaseous losses of N from Neuenberg soils were minimal in similar experiments, possibly due to the lack of nitrite accumulation and higher initial pH.

When 100 ppm-N as $NaNO_2$ was added to the Wellwood soil, losses during incubation were very substantial, even though the soil atmosphere contained some O_2. This seems to indicate that nitrite is a better electron acceptor than nitrate and soil microbes will preferentially use nitrite instead of nitrate as an O_2 source during respiration. Nitrite was not stable in Wellwood soil.

Further studies were carried out to determine the effects of concentration of nitrite and pH of the soil on the rate of denitrification. It was hoped to be able to relate nitrite concentration and pH found during incubation with added urea to the values of those variables of this study. At 50 and 100 ppm-N as nitrite the rate of N_2O formation decreased with increasing pH. At higher nitrite concentrations, inhibition of the microbial population by this ion resulted in decreased rates of N_2O formation over 3 days. As pH rose, the inhibition was overcome although at the

highest pH used, the rate of N_2O formation had again decreased. Nitrogen formed via the reduction of N_2O was not a significant loss mechanism. The rate of formation of this gas tended to decrease as pH rose with a given concentration of NO_2^- added. At a given pH, the rate of formation of van Slyke-like N_2 rose with an increase in concentration of added nitrite. At all concentrations of nitrite studied, the rate of van Slyke-like nitrogen formation decreased as pH increased.

When 2 pH values, 6.1 and 7.6, were chosen and nitrite concentration increased over the range of 50 to 500 ppm N, a direct relationship between concentration of added NO_2^- and rate of van Slyke-like N_2 producton was shown to exist. The rate of loss was much less at pH 7.6 than at pH 6.1 although a linear relationship was still apparent.

In an attempt to assess the effect of microbial activity on the rate of denitrification, the soil population was stimulated by addition of glucose and retarded by steam sterilization. It was found that when glucose was added, the majority of N lost was lost as N_2O or N_2 reduced from N_2O. Little loss was recorded as van Slyke N_2, probably because the actively respiring soil population had used most of the soil nitrite before it could react with soil N to yield N_2[2]. The opposite was found when the soil was sterilized. Loss as N_2O was then very low. On the other hand the rate of formation of van Slyke nitrogen was unaffected by treatments which affected biological activity.

An acidic soil, Keld, was also used in the study and was shown to produce van Slyke-like nitrogen but little N_2O. This lack of N_2O production could have been due to low microbial activity induced by the low soil pH.

In summation, the work of this thesis has shown that gaseous losses of N can occur when nitrite accumulates during the oxidation of urea in slightly acid soil. The van Slyke reaction, which had previously been thought to be unimportant in soil accounted for 40% of the N lost from the system, losses which occurred under seemingly aerobic conditions.

Losses of van Slyke nitrogen varied directly with concentration of nitrite and inversely with pH when $NaNO_2$ was the nitrite source. The maximum calculated pH at which this reaction was expected to occur in Wellwood soil with NO_2^- as the N source was pH 7.57. This reaction appears to be chemical as its rate is unaffected by soil sterilization. Substantial van Slyke N_2 was produced in the Wellwood soil to which 10% $CaCO_3$ and 1000 ppm urea N were added even though the pH was 7.6 or higher throughout the course of the incubation. It was not possible to readily correlate losses of nitrogen in NO_2^- studies with losses obtained with urea, possibly due to the fact that the pH measured in the urea experiments was quite probably not the pH near the site of the soil-nitrite interactions.

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APPENDIX I

Percent of oxygen remaining in incubation container at various sampling periods of Experiment 1.

3 day interval ending on day	Percent O_2 remaining*	
	Wellwood pH 6.1	Wellwood pH 7.6
3	34	30
6	6	8.6
9	11	2.3
12	22	.05
15	38	23.5
18	37	11.9
21	44	23.6
24	46	19.9
27	42	38.6
30	48	46.7
33	33	42.3
36	-	48.6
39	15	-
42	50	56.4
45	26	50.3
48	55	65.6

* where 100% indicates O_2 levels in incubation vessels equal to those of atmosphere.

APPENDIX 2

Percent of oxygen remaining in incubation container after 3 days in Experiment 4.

Nitrite concentration (ppm-N)	Percent O_2 remaining*	
	Wellwood pH 6.1	Wellwood pH 7.6
50	42	66
100	66	20
150	62	-
200	70	27
250	74	-
400	65	51
500	51	50

*where 100% indicates O_2 levels in incubation vessels equal to those of atmosphere.

APPENDIX 3

Percent of oxygen remaining in incubation vessel after 3 days in Experiment 5.*

Nitrite concentration (ppm-N)	pH			
	6.1	6.8	7.2	7.6
50	57	42	23	13
100	60	53	29	19
200	72	50	29	15
400	65	63	41	25

*where 100% indicates O_2 levels in incubation vessels equal to those of the atmosphere.