

THE EVOLUTION OF NITROGEN CYCLING

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Abstract. Based upon arguments concerning properties of the environment and the energetics of nitrogen transformation reactions, new hypotheses regarding their evolution are presented. These hypotheses are supported by new calculations and observations germane to understanding the evolution of the nitrogen cycle. From calculations of shock production by meteor impact, we suggest that impact produced fixed nitrogen could have resulted in the entire reservoir of Earth's N_2 being converted into fixed nitrogen at the end of accretion. We have significantly improved upon previous calculations of the abiotic fixation rate on the early earth and find a rate of fixation by lightning of $\sim 1-3 \times 10^{16}$ molecules NO/J , which is 2 to 3 times greater than previous estimates. This strengthens the suggestion, corroborated by the predominance of a single nitrogenase enzyme, that biological nitrogen fixation may have been a late evolutionary development, after the development of an aerobic atmosphere. In addition, we show for the first time that HNO , predicted to be the main product of atmospheric photochemical reactions involving NO on the primitive Earth by photochemical models, would eventually become NO_2^- and NO_3^- after reaching the Earth's surface. Based upon microbe-environment interactions on an ecological as well as a biochemical scale we suggest that denitrification arose prior to aerobic respiration and that nitrification arose after the advent of an aerobic atmosphere. We hypothesize the following evolutionary sequence for the biological transformation of nitrogen compounds: Ammonification \rightarrow Denitrification \rightarrow Nitrification \rightarrow Nitrogen fixation.

1. Introduction

Nitrogen is an essential element found in a variety of biologically important molecules including proteins, DNA and RNA. As a consequence, the development and evolution of organisms with the ability to transform nitrogen into the compounds associated with the nitrogen cycle is of major interest in understanding the origin and evolution of life. Generally, fixed nitrogen is defined as NH_3 , NO_x , or N that is chemically bound to either inorganic or organic molecules, and releasable by hydrolysis to NH_3 , or NH_4^+ . Of these, primarily NH_3 , organic-N, and some of the oxides of nitrogen are biologically useful, and readily utilized by a wide variety of organisms. The vast majority of nitrogen on Earth does not exist in the fixed form, but as N_2 in the atmosphere. Without fixed nitrogen, life as we know it could not exist. Biological transformations of nitrogen within the biosphere are primarily carried out by microorganisms and, indeed, some of the key reactions are carried out exclusively by prokaryotes.

In this paper we attempt to combine information regarding the biochemistry of nitrogen metabolism and the history of Earth's atmosphere, to develop a plausible scenario for the evolution of the present day nitrogen cycle. In addition, we have divided the evolution of the biogeochemistry of N-transformations into three major

phases: (1) The prebiotic phase. This phase sets the stage for evolution of the metabolic pathways of the first organisms by providing the necessary substrates for energy and biomass production. (2) The anaerobic phase. During this phase microbe-environment interactions occurred under anaerobic conditions, and ended with the advent of an aerobic atmosphere; and (3) The aerobic phase. This phase is characterized by those microbe-environment interactions that occurred after the advent of an aerobic atmosphere.

2. Prebiotic Nitrogen Cycle

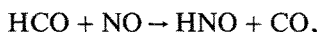
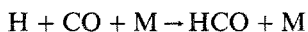
It is useful to consider the nitrogen cycle in the absence of biological processes as a guide to what might have characterized the prebiotic Earth. This can also be used as an indicator of the processes that may have been operative on other planets with earth-like primordial atmospheres, but without life.

The total amount of nitrogen contained in the Earth's crust, atmosphere, oceans and sedimentary rocks, is $\sim 4.9 \times 10^{18}$ kg-N (Walker, 1977). Of this 3.87×10^{18} kg-N, *i.e.*, about 80% of the nitrogen endowment of the planet, resides at present in the atmosphere. Since nitrogen does not readily escape to space, the total amount is preserved through time. Nitrogen outgasses from volcanos as dinitrogen, and as a consequence dinitrogen was the initial form of nitrogen in the prebiotic atmosphere. If there was early outgassing of nitrogen, there would have been an ~ 1 atm of N_2 on early Earth.

As the planet developed through time several abiological processes produced other forms of nitrogen. Currently, for example, the production of NO by lightning is the major non-biological source of fixed nitrogen, contributing about 3% of the total amount of fixed nitrogen (Walker, 1977). In a recent review of lightning, Borucki and Chameides (1984) concluded that the production of NO from N_2 in the present atmosphere is 2.6×10^9 kg-N yr⁻¹. This corresponds to a production efficiency in a lightning stroke of $9 \pm 2 \times 10^{16}$ molecules-NO/J, and a total lightning energy dissipation in the present atmosphere of $\sim 4 \times 10^{10}$ W. In the present atmosphere this rate of fixation by lightning would consume all of the atmospheric N_2 in 1.5×10^9 yr. However, due to the absence of free O_2 on primitive Earth, before the development of an oxygen rich atmosphere, the lightning fixation rate was most likely lower, as described below.

On early Earth CO_2 and N_2 were the major atmospheric constituents with the partial pressure of N_2 equal to approximately one atm (Walker, 1977). As mentioned above, lightning in such an atmosphere could have been an important source of NO. Theoretical and experimental studies give information regarding fixation rates for an early Earth CO_2 atmosphere. For example, Yung and McElroy (1979) used a freeze-out equilibrium model in conjunction with a photochemical kinetic model to determine the production of NO in an early atmosphere composed primarily of N_2 , with water vapor and CO_2 similar to their present atmospheric levels, and varying amounts of O_2 . They concluded that significant amounts of NO

($\sim 10^9$ kg-N yr⁻¹) would be produced and deposited in the ocean primarily as HNO₃ with some HNO₂. However, Kasting and Walker (1981) studied this problem further and pointed out that the Yung and McElroy analysis failed to consider the reactions of H with NO and NO₂ for the postulated atmospheres. These reactions result in keeping the NO₂ concentration low, leading to the formation and deposition of HNO into the oceans, and not HNO₃ as suggested by Yung and McElroy (1979). In contrast, photochemical reactions in Earth's current atmosphere lead to the production of HNO₃ (Walker, 1977). In an atmosphere with an increased amount of CO₂ the concentration of H atoms is very low ($\sim 10^6$ cm⁻³ at CO₂ = 0.2 atm) (Kasting, 1985), compared to the H concentration in a N₂ atmosphere with present levels of CO₂ (H $\sim 10^9$ cm⁻³) (Yung and McElroy, 1979; Kasting and Walker, 1981). However, HNO is still produced rather than HNO₃ (Kasting, personal communication) through the reactions:



where M is any other atmospheric constituent.

The fate of the resulting nitrosyl hydride (HNO) has not previously been defined. The HNO that is formed in the atmosphere is a reactive N¹⁺ species (Gratzel *et al.*, 1970). In the gas phase it dimerizes and dehydrates to form N₂O and H₂O, at a rate of 4×10^{-15} cm³ s⁻¹ (Hampson and Garvin, 1977). Since this is rather slow, most of the HNO produced in an early atmosphere would fall into the ocean where it would form H⁺ and NO⁻. In aqueous solution NO⁻ reacts with itself and/or solubilized NO to form N₂O₂⁻, and N₃O₃⁻, and their conjugate acids. These species decay rapidly into, N₂O, NO₂⁻, and NO₃⁻ (Gratzel *et al.*, 1970; Doyle and Mahapatro, 1984; Espinosa *et al.*, 1985). Any HNO₂ formed falls into the ocean and forms NO and HNO₃. The HNO₃ dissociates into H⁺ and NO₃⁻. All of these reactions lead to a NO₂⁻ and NO₃⁻ nitrogen pool on the planet's surface.

It seems clear that, independent of the CO₂ content of the primitive atmosphere, the final product of lightning activity is the production of NO₃⁻ and NO₂⁻ in the oceans. Thus, the net effect of lightning fixation of N in the early atmosphere, as in the present atmosphere, was the conversion of atmospheric N₂ to dissolved NO₂⁻ and NO₃⁻, albeit at a slower rate. The capacity of the oceans to hold NO₂⁻ and NO₃⁻ at the present pH, exceeds the amount of N in the ocean-atmosphere-sediment system. Hence, in the absence of a recycling mechanism dissolved NO₂⁻ and NO₃⁻ would accumulate.

2.1. CALCULATIONS OF LIGHTNING PRODUCTION OF NO_x

Theoretical studies of lightning production of NO have typically treated the process as occurring in two steps. In the first step, characterized by temperatures within the lightning channel in excess of a specified 'freeze-out' temperature, the chemical reactions are assumed to be extremely rapid and chemical equilibrium is achieved.

In the second step, characterized by temperatures within the channel below the freeze-out temperature, chemical reaction rates are assumed to be zero (Yung and McElroy, 1979; Chameides and Walker, 1980). Thus the problem simplifies to determining the freeze-out temperature for a given mixture of gases and specific lightning geometry, followed by determining the thermodynamic equilibrium distribution of gases at that temperature.

Previous calculations of NO production by lightning in a primitive earth-type atmosphere composed of CO₂ and N₂ (Kasting *et al.*, 1979; Chameides and Walker, 1980; Kasting and Walker, 1981) were based upon theoretical models with no experimental verification. Unfortunately, this resulted in basing results upon a freeze-out temperature of 3500 K. By comparing theoretical calculations to experimental results, we show that the freeze-out temperature is probably closer to 2000 K, and we use this value in our improved calculations.

The only experimental work on abiotic dinitrogen fixation in a predominantly CO₂ atmosphere is that of Levine *et al.* (1982), who performed experiments in which lightning was simulated by an electrical spark in an atmosphere composed of 95.9% CO₂, 3.97% N₂, 180 ppm SO₂, 50 ppm Ar, and 30 ppm CO, at a total pressure of 1 bar. They obtained an NO production rate of $3.7 \pm 0.7 \times 10^{15}$ molecules/J. Their experimental result for NO production by lightning in air was $5 \pm 2 \times 10^{16}$ molecules/J. Any modeling study of NO production on the early earth must be consistent with these important results.

We have calculated nitrogen fixation by lightning in a broad range of CO₂ – N₂ mixtures overlapping the experimental work of Levine *et al.* (1982). Figure 1 depicts the theoretical NO production rate for shock pressures of 1, 10, and 100 bars over a range of temperatures for the mixture of Levine *et al.* (1982). Their experimental result is shown for comparison. These theoretical results were obtained using a thermodynamic equilibrium model to calculate the expected production rates of NO and other nitrogen-oxygen compounds. The theoretical production rate P_i of a species in molecules/J is determined by the method of Borucki *et al.* (1984), which assumes an isothermal shock and neglects radiative processes:

$$P_i = \frac{\eta_i(T_f) A}{\mu(T_f) [H(T_f) - H(T_0)]} \quad (1)$$

where $\eta_i(T_f)$ is the mole fraction of the species under consideration at T_f , the freeze-out temperature, A is Avogadro's number, $\mu(T_f)$ is the molecular weight of the gas mixture at T_f , and H is the enthalpy. T_0 is the initial gas temperature.

Comparison of the theoretical results with the experimental values suggests that the freeze-out temperature for NO production is between 2000 K and 2250 K (Figure 1). Thus, we have direct experimental determination of the key adjustable parameter in our model. The freeze-out temperature for NO production previously used has varied from 2000 K (e.g. Yung and McElroy, 1979) to as high as 3500 K (e.g.

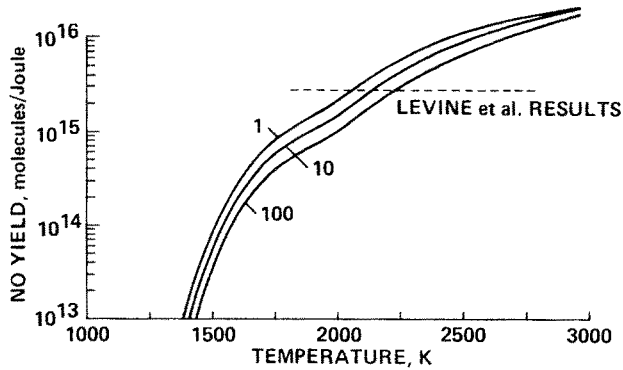


Fig. 1. The theoretical NO production rate by lightning for pressures of 1, 10 and 100 bars over a range of temperatures for mixture of Levine *et al.* (1982), whose experimental result is shown for comparison. These theoretical results were obtained using a thermodynamic equilibrium model based upon a 'freeze-out' temperature, the temperature above which the chemical reactions are assumed to be extremely rapid and chemical equilibrium is achieved. Below the freeze-out temperature, chemical reaction rates are assumed to be zero. Comparison of the theoretical results with the experimental values suggests that the freeze out temperature for NO production is between 2000 K and 2250 K.

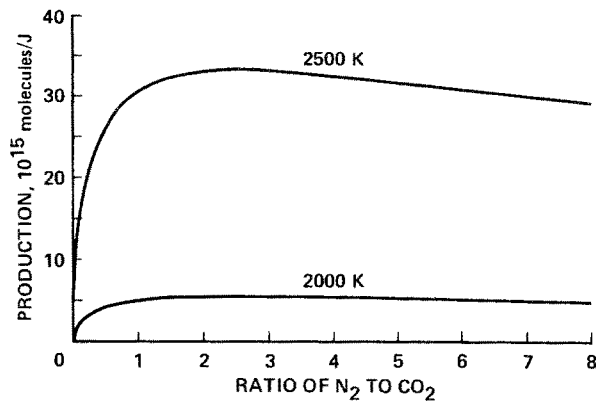


Fig. 2. Theoretical production rates by lightning in molecules/J of NO in N_2 - CO_2 atmospheres for varying values of the ratio of N_2/CO_2 . The results are based on the 'freeze-out' model of shock chemical production. The lower curve corresponds to a temperature of 2000 K (the nominal NO freeze-out temperature) and the upper curve to a value of 2500 K.

Chameides and Walker, 1980). Our results indicate that for the mixture of Levine *et al.* (1982) the production rate is not strongly dependent on shock pressure, consistent with the predictions of Lewis *et al.* (1982).

Based upon the previous discussion we have adopted a nominal freeze-out temperature of 2000 K, but we also consider a range of freeze-out temperatures from 2000 to 2500 K for computation of the NO production rate on the early Earth. Figure 2 depicts the results for theoretical production rates by lightning in molecules/J of NO, at a shock pressure of 10 bars, in N_2 - CO_2 atmospheres for varying values of the ratio of N_2/CO_2 . The method of Borucki *et al.* (1984) and Gordon and McBride

(1971) was used to compute η_i for each species over a wide range of temperatures and pressures. The lower curve corresponds to a temperature of 2000 K (the nominal NO freeze-out temperature) and the upper curve to a value of 2500 K. The difference between these curves shows that the results are very sensitive to the choice of freeze-out temperature.

Using a freeze-out temperature of 2000 K, our model calculations predict a yield of NO in the present atmosphere of 8×10^{16} molecules NO/J which is in very good agreement with the values of Borucki and Chameides (1984) of $9 \pm 2 \times 10^{16}$. This is an indication that our estimate of the freeze-out temperature is valid. (For a freeze-out temperature of 2500 K our theoretical result is 17×10^{16} molecules NO/J). We find that N fixation by lightning in a N_2 - CO_2 atmosphere ($\sim 1 - 3 \times 10^{16}$ molecules NO/J) is only a factor of 3–10 less efficient than production in the current N_2 - O_2 atmosphere, not 17 times less as suggested by Kasting and Walker (1981).

We do not know the frequency of occurrence of lightning in the primitive atmosphere. Most workers have assumed it to be equal to the present rate of 4×10^{10} W (Borucki and Chameides, 1984). To compute the total production of NO in the early atmosphere the production efficiency from Figure 2 must be multiplied by the total lightning energy dissipation rate. Using the present rate, a primordial atmosphere composed of equal amounts of N_2 and CO_2 gives a NO production rate of $\sim 10^9$ kg-N yr⁻¹.

2.2. OTHER SOURCES OF FIXED NITROGEN

Other mechanisms have been suggested that could have played a role in abiotic production of fixed N on early Earth. Zahnle (1986) proposed a mechanism by which N atoms produced in the upper atmosphere by extreme-ultraviolet solar radiation would be converted to HCN and NO. Hydrogen cyanide produced by this mechanism requires a source of CH_4 , which may not be plausible. For a CO_2 atmosphere the flux of N atoms at the top of this model (100 km) is $\sim 5 \times 10^9$ molecules $cm^{-2} s^{-1}$, which is equal to 2×10^{10} kg-N yr⁻¹, many times the lightning source. However, without a strong source of CH_4 most of the N that is produced remains as atomic N and recombines to form N_2 (Zahnle, 1986).

Schrauzer *et al.* (1983) reported that sterile sands containing TiO_2 with low levels of Fe, from various geographic locations, are capable of photochemically reducing N_2 to NH_3 using wavelengths of light between 390 nm and 420 nm. These reactions occur in the presence of O_2 , and would certainly have been operational on prebiotic Earth under an anaerobic atmosphere, after the formation of dry land. However, ammonia produced by this mechanism would be rapidly destroyed ($\tau \leq 10$ yr) by photolysis and reactions with OH radicals (Walker, 1977).

Another small but possibly important form of nitrogen on the prebiotic earth was organic nitrogen. Organic compounds containing nitrogen are important basic constituents of living systems. If the standard paradigm of the origin of life is correct then these compounds had to be present before life arose on Earth. Initially, organic molecules could have formed from volatiles present in the atmosphere. However this is difficult in a CO_2 dominated atmosphere (Folsome *et al.*, 1981; Chang *et al.*,

1983). Alternatively, these biochemical compounds (including, amino acids, peptides, carbohydrates, purines, pyrimidines, and porphyrins) could have been imported to Earth by comets and meteorites.

An additional source of fixed nitrogen on the early Earth may have been from shock formation due to impacts during the late stages of accretion. Impact shock formation has been considered by Fegley *et al.* (1986) for the formation of HCN in early atmospheres in which the C/O ratio was greater than unity. For a CO₂ atmosphere (C/O = 0.5) HCN is not produced but NO is. We have estimated the total production of NO following the approach of McKay *et al.* (1988). They have estimated the shock production of N₂ and organics from an NH₃-CH₄ atmosphere on the primordial Titan.

We consider the accretion of the last 1% of the Earth's mass, presumably after the formation of the atmosphere. Since our results saturate, the exact value of the impacting mass we use is not important. The infall velocity of such impactors would be $\sim 10 \text{ km s}^{-1}$. Hence, they would have been highly supersonic and generated high temperature shocks ($> 2000 \text{ K}$) as they passed through the atmosphere. Only a small portion of the initial kinetic energy of such an impactor is lost to the atmosphere, and of this portion $\sim 30\%$ is converted to shocks (see McKay *et al.*, 1988). If we consider the accretion of the last 1% of Earth's mass, and assume a planetesimal size distribution that varies as r^{-3} with densities of about 2 g cm^{-3} impacting into a 1 bar atmosphere, we deduce an energy released as atmospheric shocks of $\sim 4 \times 10^{29} \text{ J}$.

This corresponds to an enormous amount of shock energy. It is equivalent to the energy dissipated in lightning shock production integrated over a period of about 320×10^9 years. The formation of NO by accretional impactors is not a temporarily distributed source, however. Models of accretion suggest that the timescale of accretion were comparable to, or less than, 10^8 years (see Holland, 1984 for a review). If the yield of NO per Joule is taken to be $\sim 10^{17}$ molecule, the total fixed nitrogen could exceed the total amount of nitrogen in the crust-atmosphere system. The NO that was produced in the atmosphere would rapidly (rainout times ~ 10 days) be removed from the atmosphere and accumulate in the ocean, as described above. Hence, essentially all of the Earth's nitrogen ($3.87 \times 10^{18} \text{ kg-N}$) could have been in the form of fixed nitrogen at the end of accretion. At the *current* rate of biological demand for fixed nitrogen this supply would have lasted only about 20 million years. After accretion, individual impactors could have been an important occasional source of NO as late as 3.8×10^9 years ago, based upon considerations similar to those of Fegley *et al.*, (1986) for HCN.

If Earth's early atmosphere was reducing, N is fixed in lightning as HCN at rates that are also $\sim 10^{16}-10^{17}$ molecules/J (Chameides and Walker, 1980). Essentially, the total lightning production of N is invariant but the HCN/NO ratio varies with the reducing state of the atmosphere.

To summarize, the major source of fixed nitrogen on prebiotic Earth probably resulted from lightning. The time scale for fixation of the entire N₂ inventory would have been enormous, $\sim 10^{10} \text{ yr}$ for the current lightning rate. Hence, lightning

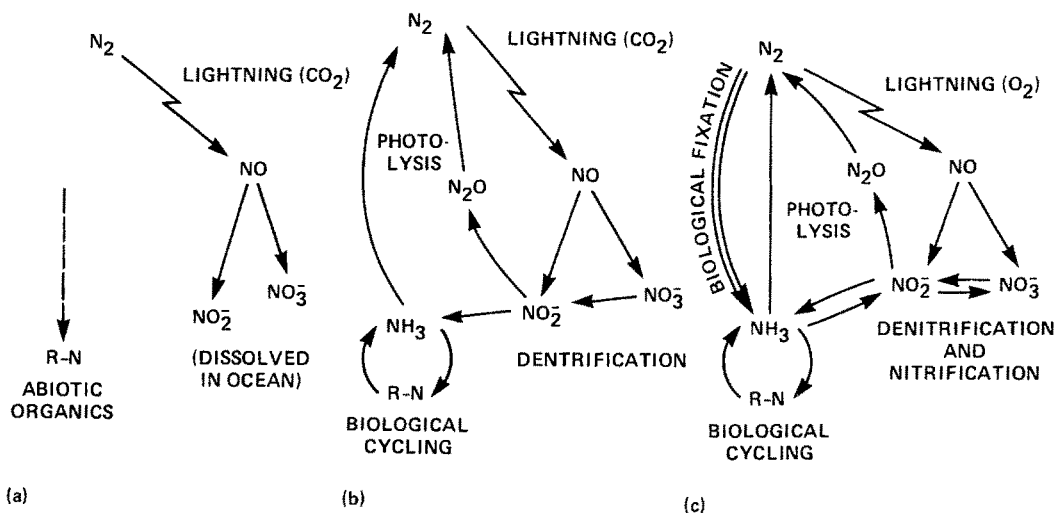


Fig. 3. Three stages illustrating the major events in the evolution of the nitrogen cycle. A. The prebiotic N-cycle where NO , produced from N_2 and CO_2 in the atmosphere by lightning, reacts to form dissolved NO_3^- and NO_2^- in the oceans. R-N indicates the possible deposition of organic nitrogen containing compounds in the ocean. B. The N-cycle after the origin of living systems, under an anaerobic atmosphere. C. The major pathways of the N-cycle after the rise of O_2 in the atmosphere and the rise of multicellular terrestrial life.

would have had little effect on the amount of N_2 in the early atmosphere. However, the fixed nitrogen produced could have been a significant reservoir of material for the earliest organisms. The NO produced in the lightning channel would almost certainly have undergone further reactions in the atmosphere and in the oceans. These reactions would result in the formation of NO_3^- and NO_2^- , which would have remained dissolved in the ocean until the evolution of organisms capable of using them as resources.

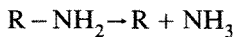
3. Nitrogen Cycling in an Anaerobic Environment

Once living cells became established, the formation of organisms with complex enzyme systems became possible. Our present understanding of the origin and evolution of life on Earth suggests that life arose during the early stages of planetary development, when surface conditions were dominated by crust formation and out-gassing of the initial atmosphere (Schopf, 1983; Pollack and Yung, 1980). However, the record of the origin and early evolution of life has been obscured by extensive surface activity. The oldest fossil evidence of life, stromatolites and microfossils, date back 3.5 billion years and by that time, a fair degree of biological complexity had already evolved (Schopf, 1983; Awramik, 1983; Schopf and Packer, 1987). These ancient stromatolites appeared to have been comprised of an ordered, complex community, inhabited by a diverse array of organisms capable of carrying out a variety of metabolic processes.

3.1. AMMONIFICATION

Since organisms ultimately incorporate NH_3 and not NO_3^- , or NO_2^- it would have

been more efficient for the very first organisms to utilize the small available supply of NH_3 . Soon all of this N-source would have been converted to biomass as the organisms readily consumed all available nutrients. To allow for the production of new biomass would have required a mechanism to recycle this biological N. At this point either organisms capable of reducing NO_3^- to NH_3 and assimilating it must develop, or the organic ammonia must be recycled. The fact that it is more efficient for an organism to utilize NH_3 directly for producing amino acids and nucleic acids, than to convert NO_3^- to NH_3 and then synthesizing these cellular constituents (see *e.g.*, Gutschick, 1981) suggests there would have been an early advantage for the development of metabolic pathways capable of deaminating organic compounds yielding free ammonia:



where R represents an organic group. One can then envision ammonification *via* simple fermentation reactions as the first biological step to evolve in the nitrogen biogeochemical cycle. Further, it has also been suggested by Walker (1977), that because abilities to both assimilate NH_3 as well as release it through anaerobic fermentation reactions are more widespread among the microbial community than nitrate metabolism, that the metabolic pathways involving NH_3 assimilation and release by fermentation evolved prior to those involving NO_3^- . In this case widespread ability can support antiquity because it involves two separate complex systems involving several enzymes not found on readily transferable plasmids.

3.2. NITRATE METABOLISM: ASSIMILATION AND DENITRIFICATION

When the biosphere became depleted in available NH_3 , due to increases in demand as a result of increases in biomass and 'leakiness' in the cycling of the limited biological nitrogen that had accumulated thus far, organisms capable of assimilating the abiotically produced available NO_3^- and NO_2^- as nitrogen sources would have been favored. Several species of obligate and facultative anaerobes contain a nitrite reductase that catalyzes the reduction of NO_2^- to NH_3 (assimilation), rather than nitrous oxide, *via* a *c*-type cytochrome. The universality and anaerobicity associated with these reactions supports our hypothesis that N assimilation was the next development in the evolution of the nitrogen cycle.

It is possible that the abundance of NO_3^- accumulating in the environment would have also allowed organisms to use it as an energy source *via* anaerobic respiration. The current hypothesis regarding the evolution of the reduction of NO_3^- to N_2 *via* anaerobic respiration (denitrification) is that denitrification arose after aerobic respiration. For example, it has been postulated that dissimilatory nitrate reduction, and the subsequent reduction of nitrite, nitric oxide and nitrous oxide to yield dinitrogen, marked the last major development during the evolution of bacterial respiration and that denitrification arose after aerobic respiration (Jones, 1985). This is thought to be primarily due to the unavailability of NO_3^- in the early, highly reducing at-

mosphere (e.g., Jones, 1985). The atmospheric environmental scenario upon which this hypothesis is based was that of a reducing one containing CH_4 , NH_3 and H_2O , with no CO_2 , or N_2 . Under these conditions there would be no abiotic production of NO_3^- or NO_2^- , and as a consequence no substrate for denitrification. In contrast, as stated in the previous sections of this paper the ready availability of abiotically produced NO_3^- and NO_2^- in a $\text{CO}_2\text{-N}_2$ atmosphere, which is the type of atmosphere that is thought to have existed before the advent of an aerobic environment, would give denitrifiers a selective advantage, because the primary source of nitrogen would be NO_3^- and NO_2^- . This strongly argues in favor of denitrification arising in an anaerobic environment prior to aerobic respiration.

The individual biochemical reactions involved in denitrification also suggest that denitrification evolved prior to aerobic respiration. The key enzyme involved in dissimilatory nitrite reduction (anaerobic respiration) is the cytochrome *cd* nitrite reductase. The corresponding enzyme for aerobic respiration is cytochrome oxidase. It has been suggested that since cytochrome *cd* nitrite reductase preferentially binds O_2 , rather than NO_2^- , and reduces it to water, that it evolved under aerobic conditions (e.g., Jones, 1985). However, O_2 reduction by cytochrome *cd* nitrite reductase does not result in cellular energy production (as it does with cytochrome oxidase), and as a consequence the cytochrome *cd* nitrite reductase is essentially being poisoned by the presence of O_2 . This argues for evolution of cytochrome *cd* in a pre-oxygenic atmosphere. A further observation suggesting that the cytochrome *cd* nitrite reductase evolved under anaerobic conditions is the fact that some denitrifiers can use substances, such as H_2 and sulfur compounds, as the terminal electron donor in their cytochrome system (e.g., Jones, 1985) indicating that they evolved under anaerobic conditions. Because the cytochrome *cd* nitrite reductase has a strong affinity for O_2 and because all organisms that possess cytochrome *cd* nitrite reductase also possess cytochrome oxidase, one may hypothesize that the cytochrome *cd* was the predecessor to the oxygen cytochrome oxidase *i.e.*, when oxygen became available in the atmosphere the cytochrome *cd* nitrite reductase evolved into the O_2 cytochrome oxidase.

A copper containing nitrite reductase has been isolated from some denitrifying organisms (Miyata and Mori, 1968; Iwasaki and Matsubara, 1972; Sawadah and Satoh, 1980). However, it most likely evolved separately, at a much later date, due to its rare occurrence.

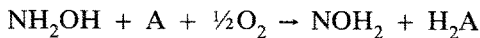
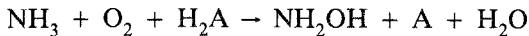
The antiquity of nitrate metabolism is further evidenced by the fact that NO_3^- reduction leading to assimilation *via* ammonia, as well as dissimilatory NO_3^- reduction *via* anaerobic respiration occur in such metabolically diverse organisms as facultative heterotrophs and chemoautotrophs; as part of a complex enzyme system that would not be readily obtained through lateral gene transfer in nature. These arguments also suggest that denitrification evolved prior to aerobic conditions, and therefore prior to aerobic respiration. We conclude that denitrification was the third major development in the evolution of the nitrogen cycle, after the evolution of ammonification and nitrate assimilation.

4. Nitrogen Cycling After the Appearance of O₂

4.1. NITRIFICATION

The appearance of O₂ in the atmosphere, approximately 2×10^9 years ago (Schidlowski, 1976), could have led to the evolution of organisms capable of oxidizing NH₄⁺ (derived from degradation of biomass) to NO₂⁻ and NO₃⁻ (nitrification). Geochemically, the appearance of this metabolic capability was important because it provided a potential source of nitrate in the biosphere greater than that produced abiotically. Although today nitrification occurs primarily by chemoautotrophic bacterial metabolism (Schmidt, 1978), a variety of heterotrophic microorganisms (*e.g.*, species of *Arthrobacter*, and the fungus *Aspergillus*) contribute to the process (Verstraete and Alexander, 1972; Focht and Verstraete, 1977; Alexander, 1977). However, not all of these heterotrophs have the ability to couple these oxidation reactions to energy production (Alexander, 1977).

Nitrifying activity occurs in aerobic environments and can be primarily attributed to two metabolically interdependent groups of bacteria. These are species of *Nitrosomonas* and *Nitrosococcus* that oxidize NH₄⁺ to NO₂⁻ (through hydroxylamine *via* ammonia hydroxylase), and *Nitrobacter* species who oxidize NO₂⁻ to NO₃⁻ *via* a cytochrome *c* reductase (Hollocher, *et al.*, 1981; Hooper *et al.*, 1978 and Suzuki, 1974):



where A represents an electron acceptor. The O₂ is reduced by cytochrome oxidase.

This reaction clearly illustrates that O₂ is required for nitrification. Because nitrification requires O₂ it most likely evolved after the rise of oxygen in the atmosphere. This is further supported by the fact that cytochrome oxidase plays a role in the process. We thus hypothesize that nitrification was the fourth major development in the nitrogen cycle.

4.2. BIOLOGICAL NITROGEN FIXATION

We suggest that on the early Earth, primary productivity was low compared to modern ecosystems. Thus, recycling of the available nitrogen, along with abiotic production, probably supplied sufficient fixed nitrogen to the system so that biological nitrogen fixation (microbial conversion of N₂ → NH₃) was not necessary. As a consequence, biological N-fixation may not have evolved until the advent of an oxygen containing atmosphere, higher plants, and the concomitant increase in biological demand for fixed nitrogen. This view is supported by the observation that the productivity of certain present-day microbial ecosystems are low enough such that recycling of N in the system as well as the in-fall of NO₃⁻ is sufficient to provide all their requirements for fixed nitrogen (*e.g.*, Friedmann and Kibler, 1980).

Increases in both biological activity and total biomass led to an increase in demand for nitrogen. Undoubtedly, this became substantial after the appearance of O₂ in

the environment and the evolution of higher plants and animals. Biological nitrogen fixation is by far the dominant source of fixed nitrogen in the present biosphere. The importance of biological nitrogen fixation is underscored by the fact that many terrestrial ecosystems are limited by the availability of fixed nitrogen (see *e.g.*, Alexander, 1977; Stevenson, 1982; Mancinelli, 1986). Although many different types of bacteria are able to fix N_2 , they all contain nitrogenase, which is encoded by a single set of genes (*nif* genes). In addition to uptake of dissolved fixed nitrogen through their root system, many plants have developed very sophisticated strategies for obtaining fixed nitrogen, including insectivory, and symbiosis with N-fixing bacteria. Yet, there are no nitrogen fixing organelles in eukaryotes. In light of the endosymbiotic theory of organelle development (Margulis, 1970), had biological nitrogen fixation evolved as early as oxygenic photosynthesis one might expect that a nitrogen fixing organelle would have developed, given the importance of fixed nitrogen and its apparent obligatory bacterial nature. These aspects of nitrogen fixation may be clues to the evolutionary history of biological nitrogen fixation.

Although it has been suggested that biological nitrogen fixation evolved early (*e.g.*, Schopf, 1983; Walker, 1977; Imshenetskii, 1962), no direct evidence exists in support of this hypothesis. The presence of heterocyst-like structures found microfossils dating to 2.8 bya (*e.g.*, Hofmann and Schopf, 1983) are not necessarily analogous to the modern cyanobacterial heterocysts, because modern heterocysts exist to shield nitrogenase from oxygen, which presumably was not present 2.8 bya. In addition, many organisms produce such cyst-like structures that are not associated with nitrogen fixation (*e.g.*, Whittenbury *et al.*, 1970). One may argue that biological nitrogen fixation evolved early because the nitrogenase enzyme is found in a physiologically broad group of microorganisms, and that it is inactivated in the presence of O_2 . However, the enzyme sometimes exists on a readily transferable plasmid. Consequently, one may just as easily hypothesize that it evolved late, and that the reason it is found in a wide variety of organisms is because once nitrogenase evolved it was readily transferred to other organisms through lateral gene transfer *via* the plasmid. In addition, its sensitivity to O_2 is related to the ability of its MoFe and Fe protein reaction centers to transfer electrons, and reduce the substrate. In the presence of O_2 (an oxidant) the reduction of dinitrogen to NH_3 is thermodynamically unfavorable (Smith, 1983). The O_2 sensitivity of the nitrogenase enzyme may be a thermodynamic effect not associated with its evolutionary history. Nitrogen fixation is a very energetically demanding process requiring 15 molecules of ATP per molecule of N_2 reduced, and probably only evolved when demand for fixed nitrogen exceeded the supply. This can readily be seen in extant ecosystems where organisms capable of fixing nitrogen exist in the environment, but only do so when the system is nitrogen limited. We conclude therefore that biological nitrogen fixation arose relatively late, when the demand for fixed nitrogen exceeded the supply, after the development of an aerobic environment.

5. Conclusions

We have examined the possible evolutionary history of the nitrogen cycle on early Earth. We conclude that as a result of shock production by impacts associated with the later stages of accretion of the Earth, the entire reservoir of nitrogen in the crust-atmosphere system could have been converted into fixed nitrogen at the end of accretion. On pre-biotic Earth, lightning was the major continuous source of fixed nitrogen, with the other abiotic mechanisms playing a secondary role. Although in the $\text{CO}_2\text{-N}_2$ dominated early atmosphere, nitrogen fixation by lightning would have been about one third as efficient as in the present atmosphere, it would still have had the effect of allowing NO_3^- and NO_2^- to accumulate in the oceans.

The first organisms to evolve probably assimilated abiotically produced NH_3 and organic nitrogen, and then developed the capability to deaminate organic nitrogen compounds (ammonification). Because of the increase in total biomass and losses in the recycling of nitrogen, the initial endowment of organic nitrogen would certainly have been rapidly depleted. This would have created a niche for the evolution of organisms capable of utilizing the NO_3^- and NO_2^- produced abiotically, through assimilation and anaerobic respiration (denitrification). The appearance of denitrifiers at this time when NO_3^- and NO_2^- were plentiful in the environment seems logical. After the advent of aerobic conditions and the development of terrestrial ecosystems, the supply of fixed nitrogen may have become a limiting factor. Postulating that denitrification evolved under such conditions, when the demand for fixed nitrogen was increasing, would seem to be an evolutionary anomaly.

On the early Earth the abiotic production rate of fixed nitrogen may have exceeded its biological demand, and as a consequence there was no niche for the evolution of such an energetically expensive process as biological nitrogen fixation. An implicit assumption of this hypothesis is that fixed N was not the limiting nutrient in early anaerobic microbial ecosystems. If N was not limiting, what was? Possibly energy as postulated for some modern day microbial systems (see *e.g.*, Gutschick, 1981). The answer to this question is not clear, but the absence of evidence for other limits to growth is not necessarily an indication of nitrogen limitation, and the answer may possibly be found in a careful study of the nitrogen economy of present day microbial ecosystems. It should be noted that the many uncertainties about the primitive atmosphere, its composition, photochemistry, and energy sources make it impossible to state with certainty whether abiotic fixed nitrogen was abundant or rare.

Another implication of the hypothesis that there was no biological fixation of nitrogen on the early Earth, is that the rate of burial of N in sediment is constrained by the abiological sources. Currently, the burial rate of nitrogen in sediment is $\sim 10^{10}$ kg-N yr^{-1} (Walker, 1977), several times the present level of lightning fixation, but much smaller than the total nitrogen fixation rate. If the burial of organic material, organic nitrogen in particular, has been roughly constant through geological time, as some investigators have suggested based on carbon isotopic data (Schidlowski, 1984), then on the early Earth a lightning source such as we have suggested

could not supply the indicated amount. There are three alternatives: (1) The burial rate of N was less than it is today (by at least a factor of 3–10) on the early Earth. (2) The abiotic fixation rate was much higher than we have estimated, either due to higher lightning occurrence rates or the operation of other mechanisms. (3) There was biological nitrogen fixation on the early Earth.

The rise of oxygen in the atmosphere, from oxygenic photosynthesis, first in microsites, followed by macrosites, and then globally, provided the niche for organisms to develop the capability to oxidize NH_3 to NO_2^- and NO_3^- . With the growth of the biosphere from the development of multicellularity, and the rise in atmospheric oxygen, there was a significant increase in the demand for fixed nitrogen. One may speculate that because they would not have had access to dissolved NO_3^- , or NO_2^- , terrestrial ecosystems may have been the trigger which allowed for the evolution of the energy expensive biological nitrogen fixation process. In fact, the limited availability of fixed nitrogen in terrestrial ecosystems compared to the biological demand continues to be a primary driver in nitrogen biogeochemical cycling. Although abiotic production of fixed nitrogen from lightning was increased due to the presence of free O_2 , the evolution of biological fixation was required to meet the demand. Therefore, biological nitrogen fixation may be the most recent evolutionary development in the nitrogen cycle.

The pre-biological Earth's environment defined the first organisms and their metabolic pathways by supplying the building blocks and substrates necessary for their origin (see e.g., Walker, 1980). The evolution of the nitrogen cycle, as outlined in this paper, illustrates that the atmosphere at first guides what type of organism can evolve on a planet, then the organisms change the environment by consuming certain chemical compounds and transforming them. This then changes the environment and allows for other types of organisms to evolve, which are better suited to the new conditions. This process clearly illustrates that the abiotic component of the system is tightly linked to the biota inhabiting the planet. We suggest that this interdependent linkage led to the following evolutionary sequence for biological nitrogen transformation reactions: Ammonification \rightarrow Denitrification \rightarrow Nitrification *Nitrogen fixation*.

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