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STUDIES ON DISSIMILATORY REDUCTION
OF NITRATE TO AMMONIUM BY SOIL CLOSTRIDIA

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William Horton Caskey

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James M. Tiedje
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STUDIES ON DISSIMILATORY REDUCTION OF NITRATE TO AMMONIUM BY SOIL CLOSTRIDIA

By

William Horton Caskey

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ABSTRACT

STUDIES ON DISSIMILATORY REDUCTION ON
NITRATE TO AMMONIA BY SOIL CLOSTRIDIA

By

William H. Caskey

In some soils incubated anaerobically, reduction of NO_3^- to NH_4^+ in excess of amounts attributable to assimilatory reduction has been observed. Fresh soil and soil which had been air-dried and stored for several years were amended with NO_3^- and glucose, acetate, or water and incubated anaerobically. Soils were either untreated or heat-shocked at 68° C for 1 hour prior to amendment. Ammonium appeared to be produced rapidly after the onset of anaerobiosis, and thereafter was incorporated into organic matter. $^{15}NH_4^+$ plus ^{15}N-organic matter production was observed in glucose-amended fresh soil in quantities up to 23.0% of added $^{15}NO_3^-$ in untreated samples and 32.0% in heat-shocked samples after 24 hours incubation. Extending incubation to 5 days resulted in NO_3^- reduction to NH_4^+ equivalent to 42.9% and 54.9% of added N, respectively. The amount of NH_4^+ produced in the air-dried soil was essentially the same as in the heat-shocked fresh soils. Heat treatment had no effect on NH_4^+ production in the air-dried soil. The activity of clostridia during the NO_3^- reduction was indicated by the absence of any effect exerted by heat-treatment, the production of H_2 and CO_2, and higher numbers of anaerobic sporeforming bacteria relative to denitrifying bacteria in the air-dried soil. Also, the most common isolate

capable of reducing NO_3^- to NH_4^+ was a Clostridium spp. The addition of washed spores of a NO_3^--reducing bacterium isolated from the air-dried Kranzburg soil (Clostridium KDHS2) to glucose-amended Conover soil increased the formation of $^{15}NH_4^+$-N plus ^{15}N-organic N four-fold, an accumulation equivalent to 83% of added $^{15}NO_3^-$-N.

The reduction of NO_3^- to NH_4^+ in soils was not inhibited by NH_4^+ or glutamine, which suggested the mechanism of reduction was dissimilatory. This conclusion is supported by studies with several Clostridium spp. isolated from the soils. The isolates were capable of reducing NO_3^- to NH_4^+ and exhibited increased cell yields when NO_3^- was included in the growth medium.

In pure culture, Clostridium KDHS2 reduced NO_3^- forming NH_4^+ as the product. A 13% increase in cell yield was observed when the organism was cultured in NO_3^--containing media. The reduction of $^{13}NO_3^-$ to $^{13}NH_4^+$ by resting cells was not inhibited by NH_4^+, glutamate, or glutamine. The formation of $^{13}NH_4^+$ from $^{13}NO_3^-$ was also unaffected by methionine sulfoximine (0.01 mM and 10 mM) and azaserine (1 mM). $SO_4^=$ did not inhibit the reduction of $^{13}NO_3^-$, but $SO_3^=$ inhibited the reaction, apparently at the level of NO_2^- reduction. Conversely, NO_3^- exerted no influence on the reduction of $^{35}SO_3^=$ to $^{35}S^=$. In fact, growth in NO_3^--containing media caused a 10-fold increase in the ability of the cells to reduce $SO_3^=$ to $S^=$. Growth in the presence of $SO_4^=$ only mildly enhanced this $SO_3^=$ reducing capacity. $SO_4^=$ was not reduced to $S^=$. Clostridium KDHS2 exhibited a K_s for NO_3^- of 0.5 mM and reduced NO_3^- to NH_4^+ maximally at a rate of 1.5 μg N/hr·mg cells. Partially purified nitrate reductase from the organism catalyzed the reduction of NO_3^- to NO_2^-. The nitrate reductase has a K_m for NO_3^- of 0.15 mM. Glutamine

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synthetase was produced by the bacterium. The activity of the glutamine synthetase was only slightly inhibited by 0.01 mM methionine sulfoximine, but was inhibited 94% by 10 mM methionine sulfoximine and 60% by 10 mM glutamine. Glutamine synthetase exerted no control over either the activity or synthesis of the enzymes involved in the reduction of NO_3^- to NH_4^+. The data presented are consistent with the hypothesis that NO_3^- reduction to NH_4^+ in Clostridium KDHS2 occurs via a dissimilatory pathway.

To furry little Magic, because Dr. Pace said I should.

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Chapter I

INTRODUCTION AND EXPERIMENTAL OBJECTIVES

Nitrates in soil face three possible fates; and, of these, only plant uptake is desirable. In a 10-year study with citrus, Pratt, et al (31) found that NO_3^- not used by the plant either leached through the soil or was denitrified. The drainage characteristics of the soil are especially important in determining whether NO_3^- will be lost through leaching or through denitrification (15). The former fate is characteristic of sandy, more porous soils, while the latter fate is generally encountered in finer-textured soils. Considerable nitrogen can be lost through leaching of nitrates effected by rainfall or irrigation, since the anions are mobile in soils. As much as 55 to 60% of the nitrate in drainage waters may originate from applied fertilizers (23).

Denitrification has long been recognized as an agriculturally undesirable fate of nitrogen from soil (29). The amount of denitrification is generally obtained by subtracting the amounts of nitrogen removed by leaching, plant uptake, and immobilization from the fertilizer inputs. These values are at best no more reliable than the reliability of the other measurements, and errors of all the measurements are summed in the difference estimate. Allison (1) observed a nitrogen deficit that averaged 15% in a number of lysimeter studies and Broadbent and Clark (3) reported denitrification losses ranged from 10-30%. Measuring denitrification directly using N_2 and N_2O fluxes, Ralston, et al. (32) calculated 45-65% of the fertilizer nitrogen in an irrigated soil column was lost via this pathway. From an analysis of a number of nitrogen balance studies, a value of 25% appears to be emerging as an average

estimate of nitrogen losses resulting from denitrification in agricultural soils. Even in the absence of accurate measurements, it is apparent that denitrification impoverishes the soil of the limiting nutrient for productivity and minimizing denitrification is an important agronomic concern.

Denitrification is generally considered to be the major route of NO_3^- reduction under anaerobic conditions. However, several reported cases of NO_3^- reduction differing from the established pathway have appeared. Koike and Hattori (24) and Sørensen (35) observed significant production of NH_4^+ from NO_3^- in coastal sediments, leading Sørensen (35) to conclude the rate of reduction of NO_3^- to NH_4^+ was of the same magnitude as denitrification. Since sediments are rich in reduced nitrogenous compounds, the formation of NH_4^+ appeared to be the product of an alternate dissimilatory pathway for the reduction of NO_3^-. Recent reports by Stanford, et al (36,37) presented evidence for the existence of a similar pathway in soil. Jones (22) found NH_4^+ was the dominant product of NO_3^- reduction by a mixed culture of bovine rumen bacteria and concluded the quantitative significance of denitrification as a pathway of NO_3^- dissimilation in the rumen is small.

The thermodynamic feasibility of the reduction of NO_3^- to NH_4^+ is evidenced by the change in free energy for the reaction at pH 7 (Table 1). The free energy data have been calculated using the free energies of formation from the elements as tabulated by Thauer, et al (38), and do not include the formation or consumption of ATP. The equivalent value for the reduction of NO_3^- to N_2 is given for comparison. Delwiche (11) constructed a similar table, but the values for the free energies were reported inconsistently. Considering only net free energy change

Table 1. The standard free energy change at pH 7 for the reduction of NO_3^- to N_2 and to NH_4^+

Reaction	$\Delta G^{\circ\prime}$ (kcal/mole)	
	H_2	NO_3^-
$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O$	-35.8	-143.3
$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	-53.6	-133.9

Calculated from Gibbs free energies of formation from the elements as tabulated by Thauer, et al (38).

for the reduction of NO_3^-, he concluded the most efficient reaction under conditions of limiting carbon source is denitrification. Conversely, when NO_3^- was limiting and carbon was abundant, the reduction of NO_3^- to NH_4^+ would be more efficient. These predictions, of course, ignore the biological factors, but may be of use in predicting the environments in which NO_3^- reduction to NH_4^+ vs. N_2 might occur. Indeed, evidence has been presented that both carbon availability and concentration of NO_3^- control selection of denitrifying and nitrate-reducing flora (see Focht and Verstraeta (15) for a detailed discussion). Carbon-rich environments which contain limiting quantities of NO_3^- include marine and deep freshwater sediments, the lower portions of the water column of eutrophic lakes, the rumen, silage, sewage sludge digesters, and stream beds below sewage plant discharge sites. In addition to these habitats, some soils which receive pulses of NO_3^- and which become transiently anaerobic might also be expected to harbor significant numbers of bacteria capable of reducing NO_3^- to NH_4^+. Certain agricultural soils and river deltas are examples.

Primitive Earth was also a habitat that was rich in carbonaceous compounds and contained NO_3^- (34). It seems logical that nitrate respiration (linked to oxidative phosphorylation) evolved from fermentative "prokaryotes" which had developed the ability to use NO_3^- as an electron sink, producing NH_4^+ as the product. Such a theory has been proposed by Egami (13,14). His concept of the evolution of energy-yielding metabolism in its general form is: fermentation → fermentation with H_2 release → inorganic types of fermentation → anaerobic respirations → oxygen respiration. Broda (4,5) has argued that NO_3^- reduction to NH_4^+ occurred merely as an evolutionary sideline and that NO_3^-

respiration evolved from aerobic respiration. He cites the absence of NO_3^- on primitive Earth prior to the existence of O_2 in the atmosphere and the omission of photosynthesis in the evolutionary sequence as the weakest points in Egami's scheme. Thermochemical calculations (34) predicted that NO_3^- could be formed in the absence of O_2, and, indeed, NO_3^- and NO_2^- have been found in aqueous solutions in anoxic cavities of rocks [Sugawara, 1949, as cited by Egami (14)]. Although Egami did not consider photosynthesis in his scheme, it seems plausible that a photoautotroph could have evolved from an organism that had developed the capacity for anaerobic respiration. The sequence proposed by Broda (5) did not allow for the presence of cytochromes in fermentative bacteria, a fact now established (16). An evolutionary branch for development of photosynthesis at this point would allow the similarities in electron transport involved in photosynthesis and oxygen respiration to be conserved.

Observations of NH_4^+ as the product of NO_3^- reduction under conditions suggesting a dissimilatory mechanism are listed in Table 2. In addition to the pure cultures listed, Staphyloccus aureus (38) and a number of enteric bacteria including Escherichia coli (10,38), Proteus mirabilis, Citrobacter freundii, and Klebsiella aerogenes (38) reduce NO_2^- to NH_4^+.

These observations of NH_4^+ production from NO_3^- are particularly interesting, since NH_4^+, as a cation, is adsorbed to soil particles and, is therefore, less mobile than NO_3^-. It is also readily assimilated by the soil organisms. The production of NH_4^+ under anaerobic conditions represents a portion of NO_3^- which has been rendered unavailable as a substrate for denitrification. Therefore, NO_3^- reduction to NH_4^+

Table 2. Observations of NH_4^+ as a product of NO_3^- reduction in excess of amounts attributable to assimilatory processes.

System	Reference
Natural Systems	
Flooded soil	MacRae, et al (25)
Well-drained soils	Stanford, et al (36,37), Buresh, et al (6)
Lake sediments	Chen, et al (7)
Marine sediments	Koike and Hattori (24), Sørensen (35)
Rumen	Jones (22)
Pure culture	
Clostridium perfringens	Woods (47), Hasan and Hall (18)
Clostridium tertium	Hasan and Hall (17)
Photobacterium fischeri	Prakash and Sadana (30)
Veillonella alcalescens	Inderlied and Delwiche (20)
Vibrio succinogenes	Wolin, et al (46)
Bacillus licheniformis	Verhoeven (42), Woldendorp (45)
Bacillus filaris and *B. polymyxa*	Illina and Khodakova (21)
Selenomonas ruminantium	deVries, et al (43)
Spirillum itersonii	Thauer, et al (38)
Candida boydinii	Middelhoven, et al (26)

represents a nitrogen-conserving process. An understanding of the etiology of the NH_4^+ production from NO_3^- and the factors controlling the pathway may lead to ultimate exploitation of the reaction to improve nitrogen economy in agriculture.

Although considerable information has accumulated concerning NO_3^- reduction to NH_4^+ by pure cultures, little use of the knowledge has been made to explain NH_4^+ production in natural systems. This is not surprising for soils, since most investigators have failed to detect NH_4^+ as a product of NO_3^- reduction (2,28,44). Also, most of the observations of NO_3^- reduction to NH_4^+ by pure cultures arose incidentally during studies done for other purposes. To this author's knowledge, the only attempt to correlate pure culture experiments with soil studies was by Woldendorp (45) who introduced Bacillus licheniformis into soil after observing the organism reduced NO_3^- to NH_4^+ in laboratory culture. However, the organism failed to reduce NO_3^- in soil.

The observations of Stanford, et al (36) that up to 55% of added NO_3^--N is reduced to NH_4^+-N emphasized the need for studies to identify the agents catalyzing the NO_3^- reduction. The soils used by Stanford, et al (36,37) were air-dried and had been stored for several years. This suggested a shift may have occurred in the microflora favoring sporeforming bacteria over vegetative cells. The first major objective of this research was to test the hypothesis that Clostridium species and/or Bacillus species were responsible for the reduction of NO_3^- to NH_4^+ in soils.

Clostridium species appeared to be largely responsible for the NO_3^- reduction to NH_4^+ observed in the air-dried soils. One isolate, Clostridium KDHS2, was particularly active and evidence indicated the

NO_3^- was reduced to NH_4^+ by a dissimilatory, rather than assimilatory process. The amounts of NH_4^+ produced were in excess of those needed for assimilation, and the formation of NH_4^+ occurred in presence of reduced nitrogenous compounds. The second objective of this study was to provide evidence that the pathway of NO_3^- reduction was a dissimilatory route.

Considerable information is available regarding NO_3^- reduction to NH_4^+ by pure cultures of bacteria. Among the enteric bacteria, ATP generation is coupled to NO_3^- reduction to NO_2^-, but not to the reduction of NO_2^- to NH_4^+ (38). The Clostridium species reported to reduce NO_3^- to NH_4^+, do so in conjunction with their energy metabolism, but the increased ATP yield is attributed to the shift in the fermentation end products toward increased acetate production in the presence of NO_3^-, thereby increasing the amount of substrate-level phosphorylation (17,18). An interesting discovery is the possession, by the majority of NO_3^--reducing anaerobes, of cytochromes, some of which are functional in NO_3^- reduction (16,43). Electron transport appears to be mediated by ferredoxin in Clostridium perfringens (9). The nitrate reductase of Clostridium perfringens has been purified to near homogeneity and was characterized as an iron-sulfur-molybdenum protein (8).

Information regarding the regulation of the NO_3^--reducing pathway in Clostridium species is conspicuously lacking. Inhibition by oxygen is a trait generally attributed to dissimilatory enzymes, although considerable variance in the tolerance of O_2 exists for enzymes from different organisms. Use of this criterion, as well as the typical experiments involving inhibition of electron transport, would provide no useful information, since Clostridium spp. are obligately anaerobic and

the presence of cytochromes has been established for only two species. Furthermore, both the dissimilatory and assimilatory nitrate reductases share common structures and kinetic parameters. The only clear distinction is based on function. The best approach, it was decided, was to demonstrate the absence of regulatory features commonly attributed to the enzymes involved in assimilatory NO_3^- reduction. These investigations formed the third objective of this research.

In assessing the regulatory characteristics of assimilatory nitrate reduction, the question of regulation by glutamine synthetase arose. Enzymes subject to nitrogen catabolite repression in Klebsiella aerogenes have been shown to be under positive control by glutamine synthetase (41). A review of the literature describing glutamine synthetase, its regulation, and its regulatory role, in enteric bacteria is not within the scope of this dissertation. This information has been critically examined in excellent reviews by Tyler (41) and Shapiro and Stadtman (33). Of interest relative to this study is the report by Tubb (39) that the assimilatory NO_2^- reductase in Klebsiella pneumoniae may be controlled by glutamine synthetase. Newman and Cole (27), however, observed glutamine synthetase exerted no regulatory control over nitrite reductase in Escherichia coli. The glutamine synthetase of Gram positive bacteria appears to be different, both immunologically and biochemically, from the Gram negative (or enteric-type) enzyme (40). Rather than its activity controlled by an adenylation-deadenylation reaction, the activity of the glutamine synthetase from Bacillus subtilis is regulated by alkylation of sulfhydryl groups on the surface of the enzyme (12). No involvement of Bacillus glutamine synthetase in regulation of other enzymes involved in nitrogen metabolism has been

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reported, but synthesis of the B. subtilis enzyme is apparently autogenously regulated (41). Only a single reference to the existence of glutamine synthetase in a Clostridium (Clostridium pasteurianum) and its similarity to the enzyme found in Bacillus has been published (19). Therefore, the presence of glutamine synthetase in Clostridium KDHS2 was sought. A rudimentary characterization of the enzyme and an examination of possible regulatory effects exerted by it on the NO_3^--reducing system was to be accomplished.

Some kinetic parameters describing NO_3^- reduction by Clostridium KDHS2 were estimated. A thorough investigation of the competitive ability of this organism, relative to denitrifying bacteria is needed. It is hoped the information about the ecology and physiology of NO_3^- reduction to NH_4^+ by Clostridium KDHS2 presented in this study will be helpful in understanding the reaction in nature and, perhaps, provide the foundation for research which will reveal approaches permitting exploitation of this nitrogen-conserving reaction.

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CHAPTER II

EVIDENCE FOR CLOSTRIDIA AS AGENTS OF DISSIMILATORY REDUCTION OF NITRATE IN SOILS

The putative concept that denitrification results in essentially quantitative conversion of the NO_3^- removed to nitrogenous gases (N_2 plus N_2O) has been challenged by Stanford, et al. (24,25). They reported substantial amounts of $^{15}NH_4^+$ production from $^{15}NO_3^-$ in six widely differing soils after 4 hours of anaerobic incubation (24), and observed that the proportion of NO_3^- reduced to NH_4^+ and organic N was correlated with available carbon (25). Although other investigators (2,19,27) using $^{15}NO_3^-$ failed to detect NH_4^+ as a reduction product in soils, MacRae, et al. (16) observed that 4.5-39% of the added $^{15}NO_3^-$ was converted to organic N after 6 weeks in rice soils. In marine sediments, Koike and Hattori (11) reported 20-70% of the added NO_3^- was converted to ammonia and particulate organic N. Sørensen (23) demonstrated in marine sediments that the capacity for NO_3^- reduction to NH_4^+ was of the same magnitude as denitrification and suggested the process is quantitatively important in marine sediments. These observations suggest that reduction of NO_3^- to NH_4^+ in excess of amounts attributable to assimilatory reduction may be a significant fate of NO_3^- in some soils under denitrifying conditions. Moreover, NO_3^- reduction to NH_4^+ is thermodynamically favorable. The $\Delta G^{\circ\prime}$ for the reduction of NO_3^- to NH_4^+ is -143.3 kcal/mole of NO_3^-; for the reduction of NO_3^- to N_2, -133.9 kcal/ mole. Considering only net free energy change for the reduction of NO_3^-, Delwiche (7) concluded the most efficient reaction under conditions of limiting carbon source is denitrification. However, when

NO_3^- was limiting and carbon was more abundant, the reduction of NO_3^- to NH_4^+ would be more biologically advantageous.

The dissimilatory reduction of NO_3^- to NH_4^+ has been observed in lake sediments (15), in marine sediments (11,23), in the rumen (14), and in pure culture by bacteria (13,22,28) and by yeast (18). Among soil bacteria, the capacity for the reduction of NO_3^- to NH_4^+ seems to reside primarily with sporeforming bacteria (12,30).

The respiratory nature of this process was demonstrated with pure cultures of Clostridium perfringens by Hasan and Hall (10). The inclusion of NO_3^- in the culture medium permitted increased growth yield of the bacterium and resulted in increased production of more-oxidized metabolites. The yield of acetate per mole of glucose was doubled, while the amounts of ethanol, butyrate, and hydrogen were reduced. The increase in acetate production reflected an increase in ATP synthesis from acetyl phosphate made possible by NO_3^- replacing acetyl phosphate as the terminal electron acceptor. This increased capacity for substrate level phosphorylation accounted entirely for the increased cell yields observed in the presence of NO_3^-. A similar phenomenon has been described in Escherichia coli with NO_2^- serving as electron acceptor (6). In both cases, the flow of electrons to NO_3^- (or NO_2^-) is not coupled to phosphorylation, but merely serves as an electron sink increasing the proportion of metabolite molecules which can participate in substrate level phosphorylation.

The reduction of NO_3^- to NH_4^+ in soils is particularly interesting because it conserves N. Because the high levels of NH_4^+ accumulation observed by Stanford, et al. (25) occurred in air-dried soils, increased activity of sporeforming bacteria was considered the likely reason.

This study was designed to estimate the relative importance of this pathway in fresh and air-dried soils, to identify the organisms responsible for the reduction of NO_3^- to NH_4^+, and to provide evidence for the dissimilatory rather than assimilatory nature of the reduction.

MATERIALS AND METHODS

Soil Studies

Two agricultural soils, a Kranzburg silt loam (udic haploboroll) from South Dakota and a Conover loam (udollic ochraqualf) from Michigan were used in this investigation. The Conover soil was freshly collected and had a pH of 6.8 and an organic carbon and Kjeldahl N content of 3.1% and 0.18%, respectively. The air-dried Kranzburg soil was one of the six studied by Stanford, et al. (25) and has been characterized by Stanford and Smith (26). One Kranzburg sample had been air-dried and stored for several years. Another Kranzburg sample was freshly collected at the same site as the air-dried sample.

Soil (5 g) was placed into Hungate tubes (Bellco Glass Co., Vineland, New Jersey), wetted with 0.5 ml sterile water and incubated aerobically overnight at 28° C. Duplicate series of tubes for each soil were prepared, one of which was heat-shocked (68° C for 1 hour) following pre-incubation. The adequacy of this treatment for significant killing of vegetative cells was verified (Table 1). Both series received 10 ml sterile water containing 400 µg NO_3^--N (as KNO_3) and carbon source as indicated. Carbon sources were glucose and acetate (40 mg C per 10 ml NO_3^- solution) which were filter-sterilized before use. For each series, duplicate samples were prepared and chemical analyses were performed on

Table 1. Effect of exposure of *Pseudomonas fluorescens* (rif^R) in soil to 68 °C for 1 hr

Treatment	Population (#cells/g soil)
None	3.60×10^9
1 hr heat-shock	1.00×10^4
2 hr heat-shock	1.10×10^4
1 hr heat-shock, incubated aerobically	1.60×10^4

Washed resting cells, starved for 8 hour, were added to Conover soil. Standard plate counts were made immediately and after 1 hour and 2 hour heat-shock using tryptic soy agar (Difco) supplemented with 0.1 % (w/v) KNO_3 and 50 µg/ml each of rifampicin and cyclohexamide. The plates were incubated anaerobically in a Fretertype anaerobic glovebox for 48 hours. The origin and characterization of the rifampicin-resistant mutant is described by Smith (M. S. Smith, Ph.D. Thesis, Mich. State Univ., 1978).

the combined samples. All treatments, except additions of inhibitors, were repeated and results are reported as means of the two replicates. The tubes were capped under an atmosphere of O_2-free Ar and incubated at 28° C for up to 5 days. Following incubation, the soil slurry was centrifuged, and the soils were extracted with 10 ml 1N KCl and centrifuged again. The supernatants were combined and NH_4^+-N was assayed by steam distillation as described by Bremner (1). Soil organic N, where determined, was assayed as NH_4^+-N after Kjeldahl digestion of the extracted soil. No attempt was made to directly measure gaseous losses. Samples containing low concentrations of N were diluted with unenriched NH_4Cl prior to final distillation to yield samples containing at least 5 mg N for analysis in the mass spectrometer. Gas analyses for CO_2 were accomplished using a Carle Model 8515 gas chromatograph equipped with a microthermister detector and He as the carrier gas. Hydrogen was similarly determined, but Ar was the carrier gas.

Nitrate was added as $K^{15}NO_3$ enriched to 56.75 atom percent ^{15}N. Samples were prepared for ^{15}N analysis by redistilling into HCl the distillates which had been collected and titrated as described above. These samples were evaporated to dryness, converted to N_2 using the method of Porter and O'Deen (21), and analyzed by mass spectrometry in the laboratory of J. O. Legg, USDA, Beltsville, Md. All NO_3^- not reduced to NH_4^+ or organic matter presumably was denitrified as essentially no residual NO_3^- was observed after 5 days incubation. In subsequent experiments using ^{15}N, a single determination of combined samples was judged adequate because of the very low standard error observed in earlier replicated experiments.

The effect of various compounds on the reduction of NO_3^- to NH_4^+ was determined by including the compound in the added NO_3^--glucose solution. The influence of general assimilatory inhibitors was determined using heat-shocked, freshly collected Kranzburg soil. Inhibitors used were NH_4^+ (as NH_4Cl, 2.9 mM) and glutamine (1.5 mM) and L-methionine-DL-sulfoximine (0.02 mM and 20 mM) obtained from Sigma Chemical Co., St. Louis, MO.

Population Densities and Pure Culture Studies

The numbers of denitrifying bacteria and NO_3^--reducing sporeforming bacteria were determined using a modified MPN procedure. The medium used was tryptic soy broth (Difco) prepared in 0.5% (w/v) concentration, supplemented with 3.5 mM KNO_3 and 0.05% sodium thioglycollate. The medium (TSBN) was dispensed in 9 ml aliquots into Hungate tubes flushed with Ar using the anaerobic procedure of Bryant and Robinson (5). The tubes were sterilized at 121° C for 15 min and allowed to stand overnight before inoculation. A series of 10-fold dilutions of the soil incubation mixture was prepared in Hungate tubes containing sterile 0.85% NaCl. Suspended inocula of 1.0 ml of each dilution were transferred using syringes to each of 5 tubes of TSBN. The tubes were incubated for 1 week at 28° C and scored for the presence of denitrifying bacteria by analyzing for N_2O (20). The tubes were then heat-shocked (68° C for 1 hour) and 0.1 ml from each tube was inoculated into a fresh tube containing the same medium. Growth, as evidenced by turbidity, supplemented by microscopic examination to determine cell morphology, was used as the criterion for the presence of sporeforming bacteria.

Pure cultures of sporeforming bacteria were obtained from TSBN Agar plates which had been streaked with aliquots of positive tubes from the MPN assay. TSBN Agar was prepared by adding 1.5% agar to TSBN and substituting 10^{-3}M Ti(III) citrate (31) for sodium thioglycollate as the reductant. The isolates were examined for their ability to grow aerobically and for catalase production. The ability of each of the isolates to reduce NO_3^- to NH_4^+ was determined by inoculating TSBN with 0.1 ml of an actively growing culture. Aliquots of the medium were assayed for NH_4^+ and NO_3^- after maximum growth had occurred. Growth was monitored spectrophotometrically at 640 nm, and absorbance was converted to dry weight values using experimentally determined conversion factors.

A spore suspension of Clostridium KDHS2, isolated from the air-dried Kranzburg soil as described above, was prepared by growing the organism on the sporulation medium of Duncan and Strong (8). The presence of spores was confirmed microscopically. The spores were harvested by centrifugation, washed three times with distilled water, and resuspended in a small volume of water. The spore suspension was heat-shocked (68° C for 30 min), diluted and added to 5 g Conover soil in Hungate tubes at a concentration of approximately 2×10^7 spores/g soil. $K^{15}NO_3$ and glucose were added as before. Incubation was under O_2-free Ar. For comparison, two similar samples of Conover soil were prepared and amended with the glucose-NO_3^- solution containing no spores. One of these soils was heat-shocked (68° C for 1 hour) before amendment. All treatments were prepared in duplicate and incubation was at 28° C for 24 hours. The incubation mixtures were extracted and analyzed for $^{15}NH_4^+$ and ^{15}N-organic N as described.

RESULTS

The accumulation of $^{15}NH_4^+$-N and ^{15}N-organic matter as products of $^{15}NO_3^-$ reduction in the fresh Conover and the fresh and air-dried Kranzburg soils amended with glucose is shown in Table 2. The proportion of NO_3^- reduced to free NH_4^+ relative to that present as organic N is much higher after 24 hours of incubation when compared to that observed after 5 days incubation. After 5 days, most of the $^{15}NO_3^-$ reduced to NH_4^+ had been incorporated into organic N. This suggests that NO_3^- was rapidly reduced to NH_4^+, then converted to organic N. (Thus, reference to NH_4^+ as the product of NO_3^- reduction should be understood to mean both free NH_4^+ and organic N unless otherwise stated.) Heat-shocking caused greater accumulation of NH_4^+ and organic N in both the Conover and fresh Kranzburg soils after 24 hours incubation. The heat treatment had no effect on the accumulation of NH_4^+ plus organic N in the Conover soil and in the air-dried Kranzburg soil after 5 days incubation, but resulted in a higher concentration in the fresh Kranzburg soil. Since air-drying has the same effect as heat-shock, these data suggest that sporeforming bacteria are responsible for the reactions.

The effect of energy source on the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ is illustrated in Table 3. Glucose, which is readily utilized by many clostridia, greatly stimulated the amount of $^{15}NH_4^+$ produced as compared to the unamended soils. Acetate, which cannot be utilized as electron donor by clostridia, resulted in only a mild increase in the levels of $^{15}NH_4^+$ observed.

The amounts of CO_2 and H_2 produced during the incubation correlated well with enhanced NH_4^+ production. Both gases are produced copiously

Table 2--Accumulation of $^{15}NH_4^+$-N and ^{15}N-organic matter in glucose-amended soils†

Soil	Treatment	1 Day			5 Days		
		NH_4^+-N	Organic N	% of Added ^{15}N	NH_4^+-N	Organic N	% of Added ^{15}N
		-----μg ^{15}N/g soil§-----			-----μg ^{15}N/g soil≠-----		
Conover (Fresh)	None	3.3	7.8	22.0	0.6	19.9	40.5
	Heat-shocked	3.3	10.2	26.8	0.8	20.5	42.1
Kranzburg (Fresh)	None	1.6	10.1	23.0	0.4	21.5	42.9
	Heat-shocked	5.4	11.0	32.2	4.5	23.5	54.9
Kranzburg (Air-dry)	None	-	-	-	0.9	18.1	42.1
	Heat-shocked	-	-	-	1.6	18.2	43.9

† Soils were amended with 8 mg C/g soil as glucose and incubated anaerobically for 1 day and 5 days at 28° C following addition of 80 mg $^{15}NO_3^-$-N (56.75 atom %) per gram of soil (not dried). Soils were either untreated or heat-shocked (68° C for 1 hr.) prior to amendment.

≠ Total N is sum of NH_4^+-N and organic-N.

§ Values are means of repeated experiments. Standard error calculated from analysis of variance is 0.2 for each mean for NH_4^+-N and 0.3 for organic N.

Table 3--Effect of carbon source[†] on the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ and ^{15}N-organic matter and on the concurrent production of H_2 and CO_2 in soils incubated anaerobically for 5 days.

Soil & treatment	Carbon source			Gas production					
				Unamended		Glucose		Acetate	
	Unamended	Glucose	Acetate	CO_2	H_2	CO_2	H_2	CO_2	H_2
	μg ^{15}N/g soil[‡]			total μl					
Conover									
None	0.9	20.4	3.5	63	39	-	-	-	-
Heat-shocked	0.8	20.4	6.4	250	156	-	-	-	-
Kranzburg (fresh)									
None	2.2	21.8	1.3	30	19	693	189	42	0
Heat-shocked	3.9	28.0	3.5	115	89	1595	439	84	0
Kranzburg (air-dry)									
None	0.9	18.9	2.3	712	252	2473	845	530	0
Heat-shocked	1.4	19.8	4.6	765	261	2044	1100	277	0

[†] Soils were either untreated or heat-shocked (68° C for 1 hr.) prior to amendment with 80 μg $^{15}NO_3^-$ and 8 mg C as glucose or acetate per g soil (not dried).

[‡] Values for ^{15}N are means of repeated experiments. Standard error calculated from analysis of variance is 0.2 for each mean.

during the growth of Clostridium species, but not of Bacillus species. The addition of glucose to the soils tremendously increased the amounts of both CO_2 and H_2 produced, whereas the addition of acetate did not increase CO_2 or H_2 production. Again, heat treatment increased the quantity of both gases in the glucose-amended fresh Kranzburg soil, but no effect was observed in the air-dried Kranzburg.

The above data suggested that the reduction of NO_3^- to NH_4^+ observed was the result of the activity of Clostridium spp. Further evidence that clostridia were responsible for the NH_4^+ production is reflected in the population densities of denitrifying and sporeforming-NO_3^- reducing bacteria shown in Table 4. The freshly collected Kranzburg soil, which produced more NH_4^+ than the Conover soil in the first day of incubation, and the Conover soil contained essentially the same number of sporeforming NO_3^--reducing bacteria. However, the ratio of the sporeforming bacteria to denitrifying bacteria was greater in the Kranzburg sample. These population differences are also reflected in the responses to heat-shock in the fresh soils. Assuming the heat-treatment affected the non-sporeforming population of each soil similarly, the differences between the ratios would be amplified. Thus, a greater response to heat-treatment would be expected for the fresh Kranzburg soil. The lack of a response to heat-treatment in the air-dried Kranzburg sample suggested the entire population was shifted in favor of sporeforming bacteria, many of which were denitrifying bacteria.

A total of 22 sporeforming NO_3^--reducing bacteria were isolated and the characteristics of representative isolates are shown in Table 5. Nine of the organisms were Bacillus spp., based on their ability to grow aerobically. The three Bacillus isolates listed are representative of

Table 4--Population densities of denitrifying bacteria and sporeforming NO_3^--reducing bacteria in three soil samples.

Soil & treatment	Sporeforming NO_3^--reducers	Denitrifiers	Ratio[†]
	---------log number/g soil---------		
Conover	3.95[‡]	6.43	0.003
Kranzburg (fresh)	4.43	5.70	0.054
Kranzburg (air-dry)	4.86	5.96	0.080

[†] Ratio is (sporeforming NO_3^--reducers:denitrifiers).

[‡] Statistically significant differences at the 95% confidence level occur for values differing by at least 0.52.

Table 5--Reduction of NO_3^- to NH_4^+ by pure cultures of bacteria isolated from soils and growth yields in the presence and absence of NO_3^-.

Isolate	Aerobic Growth	Presence of Catalase	Type of anaerobic metabolism[¶]	Growth yields		Nitrogen[†] in medium as		% recovery of N
				No NO_3^-	NO_3^-	NO_3^-	NH_4^+	
				---mg cells---		---µg N/ml---[‡]		
Bacillus CON3B	+	-	Ferm.	0.26	0.27	43.3	1.2	90.8
Clostridium KFHS1B	-	-	Ferm.	0.24	0.23	40.9	0	83.5
Clostridium CONHS1	-	-	Nit. Red.	0.41	0.69	25.4	19.4	91.4
Bacillus KFH56	+	-	Nit. Red.	0.36	0.45	18.6	21.9	82.6
Clostridium KDHS1A	-	-	Nit. Red.	0.39	0.64	21.4	16.2	76.7
Clostridium KDHS1B	-	-	Nit. Red.	0.46	0.54	23.2	16.8	81.6
Clostridium KDHS2	-	-	Nit. Red.	0.31	0.50	17.3	21.8	79.8
Clostridium KDHS3	-	-	Nit. Red.	0.33	0.50	20.0	21.8	85.3
Bacillus KDHS4[§]	+	+	Denit.	0.28	0.45	22.3	14.4	74.9

[†] No NO_2^- was detected in any of the cultures.

[‡] NH_4^+ values corrected for free NH_4^+ measured in uninoculated medium.

[§] N_2O detected in atmosphere above medium.

[¶] Ferm., fermentation; Nit. Red., nitrate reduction to ammonium; Denit., denitrification.

those encountered. One had a fermentative metabolism under anaerobic conditions and did not reduce NO_3^-. A second was a classical denitrifying organism, and the third reduced NO_3^- to NH_4^+. Both Bacillus spp. capable of reducing NO_3^- exhibited increased cell yields when NO_3^- was included in the medium. Twelve of the 13 Clostridium spp. isolated were capable of reducing NO_3^- to NH_4^+ and the presence of NO_3^- permitted increased growth yields of these organisms. The Clostridium isolate unable to reduce NO_3^- exhibited no increase in cell yield in the presence of NO_3^-. The most active NH_4^+-producing isolates were Clostridium KDHS2 and Bacillus KDHS3.

Clostridium KDHS2 was capable of reducing NO_3^- to NH_4^+ in a soil system while in competition with other bacteria for both NO_3^- and carbon source (Table 6). The addition of approximately 2×10^7 washed spores of the organism per g of soil resulted in a four-fold increase in the amount of NO_3^- reduced to NH_4^+, relative to the untreated soil. This accumulation of 41.8 µg of N as NH_4^+ and organic N represents about 83% of the added NO_3^-. The population of denitrifying bacteria in the untreated soil was 2.7×10^6 organisms per g of soil.

The nature of the reductive mechanism observed in soil was examined by studying the effect of inhibitors of ammonia assimilation and assimilatory NO_3^- reduction on the reduction of NO_3^- to NH_4^+ (Table 7). Glutamine and NH_4^+, inhibitors of assimilatory NO_3^- reduction, had no inhibitory effect on the formation of NH_4^+. The apparent stimulation of activity observed in the presence of NH_4^+ probably resulted from the $^{15}NH_4^+$ produced being trapped in the large pool of free NH_4^+, but it may reflect increased electron flow to NO_3^- allowed because of additional

Table 6. Reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ by Clostridium spores added to glucose-amended soil.

Sample	NH_4^+-N	Organic N	% Of Added N
	----- μg ^{15}N/g soil[†] -----		
Untreated soil	3.3	7.8	22.0
Heat-shocked soil	3.3	10.2	26.8
Untreated soil + spores[‡]	17.5	24.3	82.7

[†] Values are reported for a single determination of combined duplicate samples.

[‡] Approximately 2 x 10^7 spores added per g soil.

Table 7--Effect of inhibitors on the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ and ^{15}N-organic matter in heat-shocked (68° C for 1 hour) Kranzburg soil (freshly collected)†.

Inhibitor	Concentration	Mode of Action	NH_4^+-N	Organic N	Total N	% of Added N
			----------μg ^{15}N/g soil‡----------			
None			5.4	11.0	16.4	32.2
NH_4Cl	2.9 mM	Suppresses assimilatory nitrate reduction	13.6	5.8	19.4	38.0
Glutamine	1.5 mM	Suppresses assimilatory nitrate reduction	10.0	5.5	15.5	30.4
Methionine sulfoxime	0.02 mM	Inhibits ammonia assimilation	13.9	9.4	23.3	45.8
Methionine sulfoximine	20.0 mM	Inhibits ammonia assimilation	14.1	10.4	24.5	47.9

† Soil was amended with 3.2 mg C/g soil (not dried) and incubated anaerobically for 1 day at 28° C.

‡ Values are reported for a single determination of combined duplicate samples.

reduced N available to the bacteria. Methionine sulfoximine, an inhibitor of ammonia assimilation (3), stimulated NH_4^+ production at both concentrations used (0.02 mM or 20 mM).

DISCUSSION

The present study confirms earlier observations (24,25) that NH_4^+ may be produced in significant amounts from NO_3^- in air-dried soil placed under anaerobic conditions. The amounts of NH_4^+ produced varied significantly in the two unamended fresh soils, but the potential for NH_4^+ production was essentially equal in both soils as demonstrated by the similar quantities of NH_4^+ produced in the glucose-amended samples after 5 days incubation (Table 3).

Free NH_4^+ generally has not been detected as a product of NO_3^- reduction in soils. MacRae, et al. (16) observed a significant portion of NO_3^- incorporation into the organic N fraction without observing NH_4^+ as an intermediate. However, the analyses were performed after 6 weeks incubation and the present study demonstrates that NH_4^+ was produced during the first 24 hours of anaerobiosis and by 5 days it had been virtually completely converted to organic N (Table 2). Stanford, et al. (24,25) observed the same sequence of events. Ammonia appeared after 4 hours incubation and increased for the first 24 hours. During this period there was a simultaneous increase of ^{15}N in organic matter, suggesting NH_4^+ was an intermediate. Keeney, et al. (15) observed a relatively constant level of $^{15}NH_4^+$ during incubation of lake sediments following addition of $^{15}NO_3^-$. Although termed assimilatory reduction by the authors, the fate of the NO_3^- in this sediment is not unlike that observed in soils.

Several genera of bacteria have been reported capable of reducing NO_3^- to NH_4^+ (Chapter I). More recently, an Alcaligenes and a Micrococcus have been observed to produce NH_4^+ (M. K. Firestone, personal communication). Although this study does not obviate the involvement of these bacteria, reduction of NO_3^- to NH_4^+ in the soils studies appeared primarily to be the result of the activity of sporeforming NO_3^--reducing bacteria, principally Clostridium spp. Five facts support this thesis. First, heat-treatment (68° C for 1 hour) did not reduce the quantity of NH_4^+ produced in the Conover soil and the air-dried Kranzburg soil. In the fresh Kranzburg soil, heat treatment stimulated the rate of NH_4^+ accumulation. Bacterial spores are not damaged by exposure to these temperatures; in fact, spore dormancy is broken and germination is induced. Vegetative cells, on the other hand, are generally destroyed by such harsh treatment (Table 1). Second, the amount of NH_4^+ produced was greatly increased by glucose, while acetate produced only a slight increase. Clostridium spp. are able to metabolize glucose, but not acetate, as an energy and carbon source. Third, copious production of CO_2 and H_2 in the glucose-amended samples also indicated a high level of activity by Clostridium spp., since Bacillus spp. do not produce large amounts of H_2. There was no H_2 detected in the acetate-amended samples, and only a small increase in CO_2 evolution was observed. Fourth, the initial ratio of sporeforming NO_3^--reducing bacteria to denitrifying bacteria was greater in the soil which produced larger quantities of NH_4^+ in the first 24 hours. Finally, the most common isolate encountered was Clostridium, all but one of which reduced NO_3^- to NH_4^+.

A number of organisms have been isolated previously which were capable of reducing NO_3^- to NH_4^+ under laboratory conditions. Some of

these could reduce NO_3^- only as resting cells (29), whereas others reduced NO_3^- to NH_4^+ during growth (9,10,13,22,30). But, none were shown to be active in a soil system. Woldendorp (28) observed that Bacillus licheniformis reduced NO_3^- to NH_4^+ in pure culture, but the organism failed to reduce NO_3^- when introduced into the rhizosphere of pea plants. Clostridium KDHS2, however, did produce NH_4^+ from NO_3^- when added back to soil (Table 6), indicating it can effectively compete with denitrifying bacteria for NO_3^- in anaerobic soils if carbon is available. This observation also provides additional support for the thesis that clostridia are responsible for the reduction of NO_3^- to NH_4^+ in soils.

The process by which NO_3^- was reduced to NH_4^+ appeared to be a dissimilatory mechanism. Available carbon did limit the amount of NO_3^- reduced to NH_4^+ (Table 3), and the increase in NH_4^+ produced following amendment with glucose could imply an assimilatory mechanism. However, all of the Clostridium strains isolated exhibited increased cell yields when NO_3^- was present in the growth medium (Table 5), demonstrating the dissimilatory function of the reduction. As shown in Table 7, neither glutamine nor NH_4^+, both inhibitors of assimilatory NO_3^- reduction suppressed the reduction of NO_3^- to NH_4^+.

When the extracellular concentration of NH_4^+ is less than 1 mM, NH_4^+ is assimilated by enteric bacteria via the combined activity of glutamine synthetase and glutamate synthase (4) as shown in Equations 1 and 2.

$$\text{Glutamate} + NH_4^+ + ATP \xrightarrow{(a)} \text{Glutamine} + ADP + P_i \qquad (1)$$

$$\text{Glutamine} + \alpha\text{-Ketoglutarate} + NADPH + H^+ \xrightarrow{(b)} 2\ \text{Glutamate} + NADP^+ \qquad (2)$$

(a) glutamine synthetase

(b) glutamate synthase

In addition to this function, glutamine synthetase has been shown to regulate the synthesis of a number of enzymes responsible for supplying nitrogen to cells (17). Brenchley (3) observed with Klebsiella aerogenes that 0.01 mM methionine sulfoximine caused approximately 70% inhibition of glutamine synthetase activity. Increasing the concentration to 10 mM produced a complete inhibition of both glutamine synthetase and glutamate synthase. Therefore, if this assimilatory process were involved in the observed formation of NH_4^+, the quantity of NH_4^+ produced would be much greater in the presence of methionine sulfoximine. The observed increase suggests that regulation of the enzymes reducing NO_3^- to NH_4^+ may be linked to this assimilatory mechanism, contradicting the evidence obtained using glutamine and NH_4^+ as effectors. However, glutamine synthetase has been shown to exert no effect on the reduction of NO_3^- to NH_4^+ by pure cultures of Clostridium KDHS2 (Chapt. III). Thus, the presence of methionine sulfoximine was probably inhibiting the growth of other microorganisms in the soil, thereby reducing competition for both electron donor and acceptor. The increase in NH_4^+ accumulation, then, was the result of greater activity of the NH_4^+-producing bacteria and was unrelated to the character of the enzymes involved.

All of the evidence presented above indicated the reduction of NO_3^- to NH_4^+ occurred via a true dissimilatory process. This theory is supported additionally by previous reports that two Clostridium spp. reduce NO_3^- to NH_4^+ by a primitive form of anaerobic respiration (9,10).

Also, none of the effectors above exert any influence on the reduction of NO_3^- to NH_4^+ by pure cultures of Clostridium KDHS2 (Chapt. III).

Because significant amounts of NO_3^- can be reduced to NH_4^+ by Clostridium spp., caution must be exercised in studies of denitrification using air-dried soils in which the ratio of sporeforming to non-sporeforming NO_3^--reducing bacteria may have shifted. But, as indicated by the data in Table 2, the potential for NH_4^+ formation from NO_3^- exists in fresh soils, too. Although other investigators (19,27) using $^{15}NO_3^-$ have not detected NH_4^+, the lengthy incubation periods, as pointed out by Stanford, et al. (25), could have made detection of the process difficult. Therefore, any short-term study of anaerobic nitrogen transformations in soil systems should include analysis for this fate of NO_3^-.

The observation that Clostridium KDHS2, when supplied with an appropriate electron donor, successfully compete for NO_3^- with denitrifying bacteria is encouraging. A greater understanding of the biochemical and ecological controls exerted on the competition for NO_3^- between Clostridium spp. and denitrifying bacteria could lead to development of management practices which conserve nitrogen in soils under denitrifying conditions.

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Chapter III

THE DISSIMILATORY REDUCTION OF NITRATE TO AMMONIUM BY A *CLOSTRIDIUM* SPP. ISOLATED FROM SOIL

In anaerobic environments, denitrification is generally considered to be the principal pathway of NO_3^- reduction. Although evidence is sparse, flooded soils (22) and sediments (18,20,29) have shown significant quantities of NO_3^- reduced to NH_4^+. While agricultural soils have typically shown no NH_4^+ production from NO_3^- when incubated anaerobically (3,25,34), recent reports correlating increased NO_3^- reduction to NH_4^+ with increased amounts of available carbon (30,31) suggested the potential for NH_4^+ formation exists in such soils. Elsewhere (Chapt. II) evidence was presented that NH_4^+ was produced through the activity of sporeforming bacteria, principally *Clostridium* species, via a dissimilatory pathway. A clearer understanding of the physiology of these NO_3^--reducing bacteria may reveal approaches which can be used to predict the occurrence of NO_3^- reduction to NH_4^+, or possibly to enhance this N-conserving process in agricultural soils.

Clostridium perfringens (15), *C. tertium* (14), and a number of unidentified *Clostridium* spp. (Chapt. II) have been shown to reduce NO_3^- to NH_4^+. All strains capable of this reduction showed increased growth yields. Hasan and Hall (15) have shown the additional ATP production results from a shift in the oxidation state of the fermentation end products in the direction of increased acetate formation which allows increased substrate-level phosphorylation. Cytochromes are involved in anaerobic electron transport to nitrate in *Veillonella alcalescens* and *Selenomonas ruminantium* (33), but ferredoxin was reported to mediate

NO_3^- reduction in C. perfringens (8). The nitrate reductase of C. perfringens has been purified to near homeogeneity (7). It is an inducible, soluble molybdo-iron-sulfur protein.

Comparative fermentation balances and measurement of Y_{ATP} with and without NO_3^- have provided evidence for the dissimilatory function of NO_3^- reduction in Clostridium spp. However, reliable measurements of the extent of NO_3^- reduction by these bacteria are lacking. Furthermore, little information is available on the regulation of NO_3^- reduction by Clostridium spp. This study, in addition to providing definitive balances of NO_3^- reduction to NH_4^+, presents evidence that the NO_3^--reducing system of Clostridium KDHS2 shares no regulatory features with assimilatory nitrate reductases.

MATERIALS AND METHODS

Bacterial strain and culture media. Clostridium KDHS2 was isolated from a Kranzburg silt loam (Chapt. II) which had been air-dried and stored for several years after collection in South Dakota. Cultures were maintained on a basal medium (R.N. Costilow, personal communication) that contained (per liter): vitamin-free casamino acids (Difco), 5.0 g; trypticase (BBL) and yeast extract (Difco), 1.0 g each; and sodium thioglycollate, 0.5 g. Glucose (0.2 % w/v) was supplied as an energy source, and KNO_3 was added where indicated at 3.5 mM. Monosodium glutamate (0.2 % w/v) was added to the medium for studies involving glutamine synthetase.

The response of Clostridum KDHS2 to the addition of NO_3^- to the medium was determined in batch culture using the basal medium. Cultures were grown in Hungate tubes (Bellco Glass, Vineland, N.J.) under O_2-free

Ar according to the procedure of Bryant and Robinson (6). Growth was monitored at 640 nm and absorbance was converted to dry weight using an experimentally determined conversion factor. Aliquots were analyzed at the beginning and end of exponential growth for glucose, NO_3^-, NO_2^-, and NH_4^+.

Chemical analyses. Glucose was determined colorimetrically using the ortho-toluidine reagent (Sigma Chemical Co., St. Louis, MO) (28). NH_4^+-N was determined by titration following steam distillation as described by Bremner (2). NO_3^--N was similarly determined following reduction to NH_4^+ using Devarda's alloy (2). NO_2^--N was measured colorimetrically by the diazo-coupling method (1).

Resting cell experiments. Clostridium KDHS2 was cultured in basal medium containing NO_3^- and harvested in mid-exponential growth (8-10 h) by centrifugation. The cells were washed three times in 0.01 M Tris (hydroxymethyl) aminomethane (Tris)-maleate buffer, pH 7.0, and resuspended in the same buffer. The suspension was introduced into Hungate tubes containing sufficient pre-reduced, sterile sodium thioglycollate to yield a 0.05 % (w/v) concentration of the reductant. The atmosphere over the suspension was O_2-free Ar. The cell suspensions were stored at 4° C until used, the time of storage not exceeding 18 h. Final concentration of cells was approximately 2 mg/ml (dry weight).

An estimate of the K_s for NO_3^- for Clostridium KDHS2 was made by measuring NH_4^+ production from NO_3^- during a 1 hour incubation at room temperature. The reaction mixture contained in 10 ml: 2.0 ml resting cell suspension, 2.0 mM KNO_3, 0.2% (w/v) glucose, and 0.05% (w/v) sodium thioglycollate. Aliquots were removed at 10 min intervals and analyzed as described for NO_3^-, NO_2^-, and NH_4^+. No NO_2^- was detected in any of

these samples. The data were analyzed as a progress curve (9) to determine the K_s for NO_3^-.

The production and purification of $^{13}NO_3^-$ and the ^{13}N detection methods are described in detail elsewhere (12). The $^{13}NO_3^-$ substrate used usually contained some $^{13}NO_2^-$ (5-10%), hence the $^{13}NH_4^+$ produced was from both compounds. The reaction mixture contained in 4.5 ml: 1.0 ml resting cell suspension, glucose (0.2% w/v), 1 µg N as KNO_3, and sodium thioglycollate (0.05%). Approximately 0.4 mCi $^{13}NO_3^-$ was added in 0.5 ml and the mixture was incubated in Hungate tubes at room temperature for 20 min. The reaction was stopped by filtering through a 0.45 µm pore size membrane filter. Aliquots of the filtrate were analyzed for $^{13}NO_3^-$, $^{13}NO_2^-$, and $^{13}NH_4^+$ using a high pressure liquid chromatograph (HPLC) equipped with an anion exchange column (Partisil SAX, Whatman, Inc., Clifton, N.J.). The column was eluted at the rate of 6 ml/min with 0.05 M phosphate buffer, pH 3.0. The effluent was monitored by dual 2" x 2" NaI(Tl) coincidence detectors, computer-linked for data collection and correction for half-life and background.

The effect of a variety of compounds on NO_3^- reduction to NH_4^+ was measured. NH_4Cl (3.5 mM), glutamate (3.5 mM). and glutamine (1.75 mM) were studied as inhibitors of assimilatory nitrate reduction. L-Methionine-D,L-sulfoximine (Sigma Chemical Co., St. Louis, MO) (0.01 mM and 10 mM) was included as an inhibitor of ammonia assimilation (4,5). Azaserine (1.0 mM) was used as an analog of glutamine (36,37). Also, Na_2SO_4 and Na_2SO_3 (3.5 mM) were studied as possible alternate substrates for the enzyme system reducing NO_3^- to NH_4^+.

Similar experiments were done using resting cells grown in basal medium supplemented with glutamate and 0.01 mM and 10 mM methionine

sulfoximine. The $^{13}NO_3^-$-reducing activity of these cells was measured in the presence of methionine sulfoximine at the concentration in which they were grown and in the absence of the compound.

^{35}S-sulfate and -sulfite were used to directly determine whether the NO_3^- reducing enzyme system in Clostridium KDHS2 could also reduce these electron acceptors. Resting cells were prepared from cultures grown in basal medium supplemented with KNO_3 (3.5 mM) or Na_2SO_4 (3.5 mM) for these experiments. Sulfite-grown cells could not be used because Clostridium KDHS2 would not grow in media containing 3.5 mM $SO_3^=$. The reaction mixture contained in 5.0 ml: 1.0 ml of cell suspension, 0.2% glucose, 0.3 mCi $^{35}SO_3^=$ (or $^{35}SO_4^=$) plus unlabelled $SO_3^=$ (or $SO_4^=$) to 3.5 mM, 0.05% sodium thioglycollate, and either 0, 1.0, or 3.5 mM KNO_3. The production of $S^=$ after 2 hours incubation in Hungate tubes was measured by adding concentrated H_2SO_4 to the reaction mixture to a final concentration of 1.7 M (D. E. Caldwell, Ph.D. Thesis, Mich. State Univ., 1974) and sparging with air for 10 min into a trap containing 5.0 ml of a saturated solution of lead acetate. One ml of the trapping solution was added to 15 ml of aqueous scintillation counting solution (ACS, Amersham, Arlington Heights, Ill.) and the amount of $^{35}S^=$ measured in a Packard Model 3310 Tri Carb Scintillation Spectrometer.

Preparation of cell free extracts. Cultures of Clostridium KDHS2 were allowed to grow into late exponential phase ($\sim$ 10 hours) in the basal medium containing KNO_3. Cells were collected by centrifugation, washed with 0.01 M Tris-HCl buffer, pH 8.0, and resuspended in the same buffer. The organisms were ruptured by passage through a French pressure cell at 16,000 psi. Debris was removed by centrifugation at 9,000 x g for 30 min and the supernatant was used as crude extract. Nitrate

reductase was partially purified by $(NH_4)_2SO_4$ precipitation. The precipitate at 40-80% saturation (7) was collected by centrifugation at 19,000 x g for 20 min and was immediately dissolved in 0.01 Tris-HCl buffer, pH 8.0, then dialyzed against the same buffer.

Clostridium KDHS2 grown on the basal medium supplemented with glutamate and KNO_3 and harvested 4-6 hours into stationary phase. The cells were washed with and resuspended in 0.01 M morpholinopropane sulfonic acid (MOPS) buffer, pH 7.0, containing 0.01 M $MnCl_2$ and 0.001 M mercaptoethanol. Crude extracts were prepared as above and glutamine synthetase was partially purified by $(NH_4)_2SO_4$ precipitation. The fraction precipitating at 50-70% saturation (17) was collected by centrifugation at 19,000 x g and immediately dissolved in the MOPS buffer described above, then dialyzed against the same buffer. Protein was determined by the method of Lowry, et al (21) using bovine serum albumin as the standard.

Enzyme assays. Nitrate reductase activity was measured using a modification of the method described by Chiba and Ishimoto (7). The reaction mixture contained in a final volume of 0.5 ml: 95 µmol of Tris-HCl buffer, pH 9.0, 10 µmol of KNO_3, 1 µmol of methyl viologen, 30 µmol of $Na_2S_2O_4$, 20 µmol of Na_2CO_3, and approximately 0.7 mg of the partially purified enzyme. The reaction was started by adding $Na_2S_2O_4$-Na_2CO_3 and stopped after incubation for 15 min at 37° C by vigorous agitation on a vibrator until the violet color of the reduced dye disappeared. One ml of 95% ethanol was added and the mixture was centrifuged at 3000 x g for 5 min. An aliquot of the supernatant was assayed for NO_2^- by the method described above. The amount of NO_2^- formed was proportional to the amount of enzyme preparation used. An

estimate of the K_m for NO_3^- was made by increasing the volumes of all reagents 10-fold and using progress curves (9). The assay was demonstrated valid for 5 μmol and 20 μmol KNO_3.

Glutamine synthetase activity was monitored by measuring the glutamate-dependent liberation of ortho-phosphate (P_i) in the biosynthetic reaction (17). The extract would not catalyze the reverse, or γ-glutamyl transfer, reaction. The assay mixture contained in 0.4 ml: 0.1 mg protein, 0.0075 M ATP, 0.1 M glutamate, 0.05 M NH_4Cl, 0.005 M $MnCl_2$, and 0.01 M MOPS buffer, pH 7.0. Incubations were carried out in open tubes at 37° C for 15 min. Reactions were initiated by the addition of enzyme. Controls lacking glutamate were included in each incubation, and corrections were made for the phosphate liberated in the absence of substrate. Measured activity of the enzyme was proportional to the amount of enzyme preparation added and was linear with time over 15 min when 0.1 mg of enzyme was used. Activity was expressed as μmol P_i formed per min per mg protein.

The inhibition of glutamine synthetase activity by methionine sulfoximine and glutamine was measured by including the appropriate amount of each inhibitor in the ATP solution. Methionine sulfoximine was added to the assay mixture at concentrations of 0.01 mM and 10 mM, and glutamine was added at 10 mM. The apparent K_m of the enzyme for NH_4^+ was estimated using initial velocity reactions and the data were analyzed using direct linear plots (11).

RESULTS

The addition of NO_3^- to the culture medium resulted in more total growth and in a more rapid growth rate for Clostridium KDHS2 (Fig. 1).

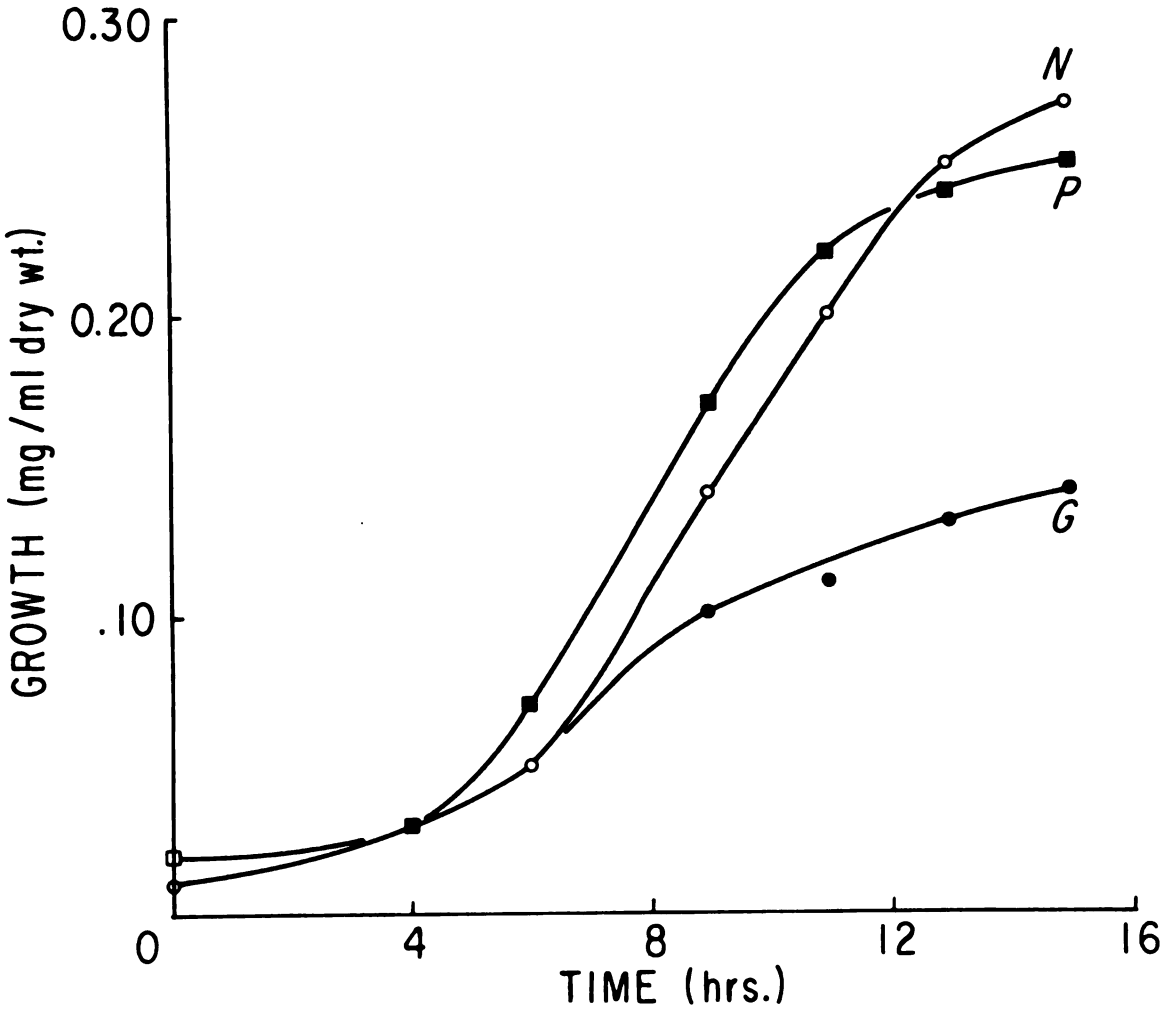

Fig. 1. Growth response of Clostridium KDHS2 to NO_3^- in the medium. Growth in presence (O—O) and absence (●—●) of 3.5 mM NO_3^- for inoculum grown without NO_3^-. Growth in the presence of NO_3^- (■—■) for inoculum grown in NO_3^--containing medium.

Curves labelled P and N represent the growth of the organism in the basal medium containing NO_3^- after inocula were grown in media with and without NO_3^-, respectively. Curve G depicts the growth of the organism in the absence of NO_3^-. There was a consistent lag of about 4 hours before initiation of exponential growth. The molar growth yields of the bacterium in the presence of NO_3^- were about 13% higher when compared to those obtained in media without NO_3^- (Table 1). The molar growth yields were linearly related to glucose concentration up to 0.3% (w/v) glucose.

The extent of NO_3^- reduction to NH_4^+ was measured concurrently with the molar growth yields (Table 1). Significant amounts of NH_4^+ were produced in the medium in the absence of NO_3^-, and this value was used as a background to correct the amounts measured in the NO_3-containing medium. A similar phenomenon was observed by Hasan and Hall (14,15) and corrections were made using this approach. However, this approach was valid only when applied to NO_3^- reduction during exponential growth. The calculated amounts of NH_4^+ produced agree quite well with the measured amounts of NO_3^- reduction, these amounts representing approximately 20% of the added NO_3^-. No NO_2^- was observed and NH_4^+ was apparently the only product of NO_3^- reduction.

The kinetics of NO_3^- reduction by Clostridium KDHS2 were studied using intact cells and partially purified NO_3^- reductase. The organism was observed to reduce NO_3^- maximally at a rate of 1.5 μg N/hr mg cells and the concentration of NO_3^- producing one-half this velocity was estimated as 0.5 mM. For the partially purified NO_3^- reductase, the K_m for NO_3^- was observed to be 0.15 mM.

Compounds which are known inhibitors of assimilatory NO_3^- reduction and ammonia assimilation were examined for possible effects on NH_4^+

Table 1. The reduction of NO_3^- to NH_4^+ by Clostridium KDHS2 in axenic culture and its effect on cell yield.

	Without NO_3^-	With NO_3^-	Preinduced cells with NO_3^-
Nitrogen Balance (μg N/ml)[†]			
NO_3^- reduced	-	10.0	9.6
NH_4^+ in medium			
Initial	8.2	8.2	8.2
After growth	33.7[‡]	42.7	42.9
From NO_3^-	-	9.0	9.2
Glucose used (μmol/ml)	6.5	9.8	11.4
Cell yield (dry wt mg/ml)	0.10	0.17	0.20
Molar growth yield (Dry wt gram/mol glucose)	15.4	17.4 (+13.0%)	17.5 (+13.6%)

[†]Media containing NO_3^- received 49.9 μg/ml NO_3^--N.

[‡]Media with no NO_3^- contained considerable NH_4^+. This value was used as a background to correct the values determined in the NO_3^- containing media.

production by resting cells of Clostridium KDHS2 (Table 2). None of these compounds, including NH_4^+, glutamate, glutamine, 0.01 mM and 10 mM methionine sulfoximine, and azaserine, had any effect on the reduction of $^{13}NO_3$ to $^{13}NH_4^+$. Sulfate and sulfite were tested as possible alternate substrates for the enzyme system. Sulfate was without effect, but sulfite inhibited $^{13}NO_3^-$ reduction. Figure 2 illustrates the reduction of $^{13}NO_3$ + $^{13}NO_2$ during the 20 min incubation period. The disappearance of the NO_2^- peak in the samples incubated with no inhibitor indicated NO_2^- is an intermediate in NO_3^- reduction to NH_4^+, and that the bacterium is capable of reducing NO_2^-. Note, however, that the NO_2^- peak persisted in the $SO_3^=$ inhibited sample, suggesting the inhibition occurs at the level of nitrite reductase. Although not as apparent because of the large amount of $^{13}NO_3^-$ present, $^{13}NO_3^-$ was reduced to $^{13}NH_4^+$ along with the $^{13}NO_2^-$.

To provide more information on the apparent $SO_3^=$ inhibition of NO_3^- reduction by Clostridium KDHS2, the reduction of $SO_4^=$ and $SO_3^=$ to $S^=$ in the presence of two concentrations of NO_3^- was measured (Table 3). Significant amounts of $SO_4^=$ were not reduced to $S^=$ by Clostridium KDHS2 grown in basal medium containing either NO_3^- or $SO_4^=$. A low level of $SO_3^=$ reducing activity was observed in cells grown in the absence of electron acceptors. Slightly higher levels of activity were observed when Clostridium KDHS2 was grown in the $SO_4^=$-containing medium. A 10-fold increase in activity relative to fermentatively-grown cells was observed for cells grown in the NO_3^--containing medium, indicating that the enzymes induced by NO_3^- also reduce $SO_3^=$. There was no inhibition of $SO_3^=$ reducing activity by either concentration of NO_3^-.

Table 2. Effect of various compounds on the reduction of $^{13}NO_3^-$ to $^{13}NH_4^+$ by resting cells of Clostridium KDHS2 grown in basal medium containing NO_3^-.

Inhibitor	$^{13}NH_4^+$ Produced (log dpm/mg cells)† Expt. 1	Expt. 2
None	5.39	5.22
NH_4^+	4.99	5.01
Glutamate	5.47	5.31
Glutamine	5.05	5.26
Methionine sulfoximine, 0.01 mM	5.02	4.99
Methionine sulfoximine, 10 mM	5.56	5.30
Azaserine, 1.0 mM	5.53	5.09
$SO_3^=$, 3.5 mM	4.43**	4.34**
$SO_4^=$, 3.5 mM	5.14	5.10
Autoclaved cells	--None detected--	

†Incubated at room temperature for 20 min with 14.3 nM unlabeled NO_3^--N.

**Significantly different at 95% confidence level.

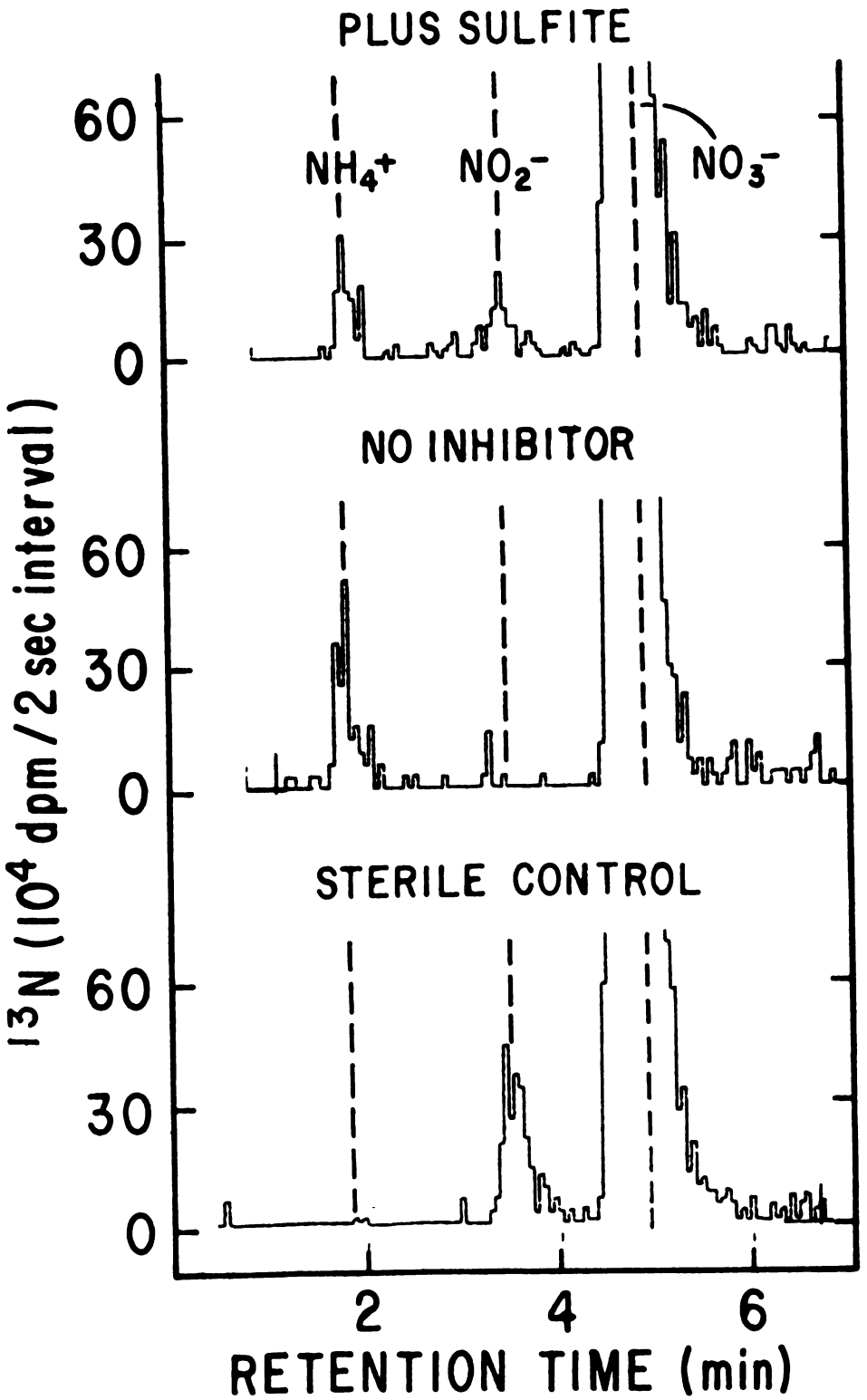

Fig. 2. The reduction of $^{13}NO_3^-$ and $^{13}NO_2^-$ to $^{13}NH_4^+$ by resting cells of Clostridium KDHS2, by resting cells in the presence of 3.5 mM $SO_3^=$, and by autoclaved cells.

Table 3. Effect of NO_3^- on the reduction of $^{35}SO_4^=$ † and $^{35}SO_3^=$ to $^{35}S^=$ after growth with different electron acceptors.

Electron acceptor in growth medium	$^{35}S^=$ Produced from $SO_3^=$ (10^2 dpm/mg cells)			$^{35}S^=$ Produced from $SO_4^=$ (10^2 dpm/mg cells)		
	No NO_3^-	1.0 mM NO_3^-	3.5 mM NO_3^-	No NO_3^-	1.0 mM NO_3^-	3.5 mM NO_3^-
None	3410	3210	2620	42	19	20
NO_3^-	25800	30900	32100	21	13	40
$SO_4^=$	5880	6030	6680	10	16	30

† Reaction mixture contained 0.3 mCi ^{35}S as $SO_4^=$ (or $SO_3^=$) in final concentration of 3.5 mM $SO_4^=$ (or $SO_3^=$). Incubation period was 2 hours.

Glutamine synthetase was produced by Clostridium KDHS2 (Table 4). Glutamine at 10 mM inhibited the enzyme by 60%. Methionine sulfoximine caused 3% inhibition when added at 0.01 mM, but resulted in 94% inhibition when the concentration was increased to 10 mM. The K_m for NH_4^+ for Clostridium KDHS2 glutamine synthetase was calculated to be 4.0×10^{-4} M (Fig. 3).

To ascertain whether glutamine synthetase exerted any regulatory influence on the synthesis or activity of the enzyme system reducing NO_3^- to NH_4^+, Clostridium KDHS2 was grown in the basal medium containing 0, 0.01, and 10 mM methionine sulfoximine. The data in Table 5 depict the ability of these cells to reduce NO_3^- to NH_4^+ in various concentrations of methionine sulfoximine. No effect was observed on activity or synthesis of the NO_3^--reducing pathway.

DISCUSSION

Clostridium KDHS2 grew more rapidly in the presence of NO_3^- and also exhibited a greater yield of cells per mole of glucose utilized. This increase in cell yield suggested that more efficient utilization of the energy derived from glucose catabolism was permitted when NO_3^- was present. Hasan and Hall (14) observed the same dual effect on C. tertium, but with C. perfringens only an increase in growth yield was observed (15). With C. tertium the observed increase in Y_{ATP} in the presence of NO_3^- was attributable to the increased growth rate, but the increase in cell yield could only be explained if the NO_3^- was being reduced by some type of dissimilatory mechanism.

Nitrate was reduced solely to NH_4^+ by Clostridium KDHS2, with NO_2^- the only detectable intermediate. Because the concentration of NO_3^- in

Table 4. Effect of inhibitors on activity of glutamine synthetase prepared from cell-free extracts of *Clostridium* KDHS2.

Inhibitor	Activity[†] (μmol P_i min^{-1} mg $protein^{-1}$)	Inhibition (%)
None	0.31	-
Methionine sulfoximine, 0.01 mM	0.30	3
Methionine sulfoximine, 10 mM	0.02	94
Glutamine, 10 mM	0.12	61

[†] Values reported are means from three separate experiments.

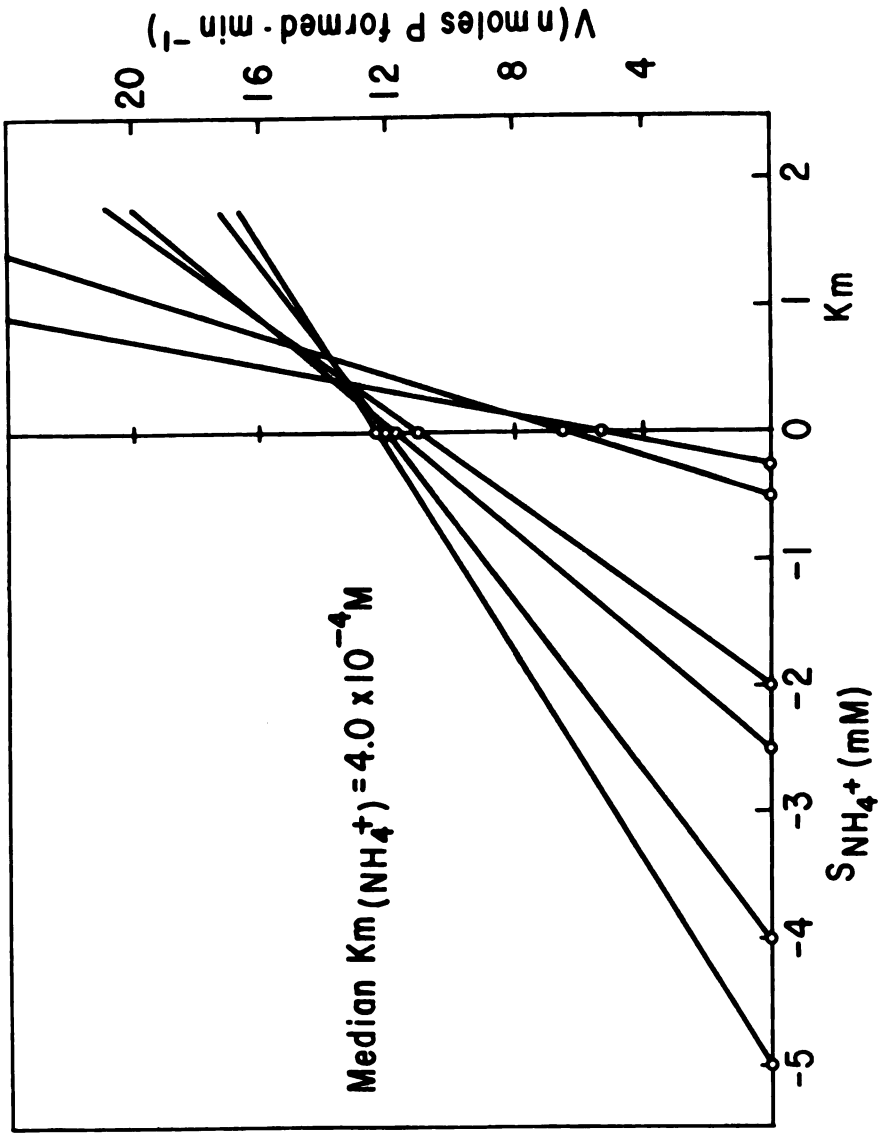

Fig. 3. Direct linear plot of K_m estimation for NH_4^+ for glutamine synthetase of Clostridium KDHS2.

Table 5. Effect of methionine sulfoximine on the reduction of $^{13}NO_3^-$ to $^{13}NH_4^+$ by resting cells of Clostridium KDHS2.

Methionine sulfoximine in growth medium (mM)	$^{13}NH_3^+$ Produced (log dpm/mg cells)		
	Methionine sulfoximine in resting cell suspension (mM)		
	0	0.01	10
0	5.39†	5.01	5.43
0.01	5.21	5.02	-
10	5.48	-	5.33

† No significant difference occurs between any of the values at the 95% confidence level.

the growth medium was selected for minimal NO_2^- accumulation, no NO_2^- was observed in any of the cultures, however. NO_2^- is toxic to many bacteria and in C. perfringens the ability of NO_3^- to support enhanced growth yields is limited to 3.5 mM NO_3^- or less, because of NO_2^- accumulation (15). A similar pattern was observed for Clostridium KDHS2. The disappearance of NO_2^- in the $^{13}NO_3^-$ experiments and the accumulation of NO_2^- as the product of the partially purified nitrate reductase provide evidence for NO_2^- being an intermediate.

The nitrogen balances obtained are reasonable and consistent with those observed for both C. perfringens and C. tertium. As mentioned earlier, the amount of NH_4^+ produced from NO_3^- could only be calculated during exponential growth. The difficulty in balancing the nitrogen species after the organism began to enter stationary phase was most likely the result of a physiological change in the NO_3^--reducing system. Chiba and Ishimoto (7) observed the nitrate reductase of C. perfringens was produced at high specific activity only during exponential growth.

The data presented thus far confirms earlier reports of NO_3^- reduction by Clostridium species, and is consistent with a dissimilatory reductive process. Additional evidence supporting the dissimilative function of the NO_3^- reduction is supplied by the lack of inhibition by NH_4^+, glutamate, and glutamine (Table 2). NH_4^+ has been demonstrated to inhibit activity of assimilatory NO_3^- reduction (13,27), whereas dissimilatory NO_3^- reduction is unaffected in facultative bacteria (13,24,35). Glutamate and glutamine are readily utilized as nitrogen sources by bacteria and would be expected to inhibit assimilatory NO_3^- reduction.

One proposed mechanism for NH_4^+ production is via decomposition of organic nitrogenous compounds formed by assimilation of NO_3^-. Brenchley,

et al (4) observed that 0.01 mM methionine sulfoximine caused a 70% inhibition of activity of glutamine synthetase, but had no effect on the activity of glutamate synthase. Increasing the concentration of methionine sulfoximine to 10 mM completely inhibited both enzymes. Thus, if the NH_4^+ was being produced according to the proposed mechanism, the presence of methionine sulfoximine would enhance the amount of NH_4^+ produced. However, no such effect was observed (Table 2). Additional evidence disproving this assimilation-release theory is provided by the absence of any effect exerted by azaserine on the amount of NH_4^+ produced (Table 2). Azaserine is a competitive inhibitor of glutamine in reactions involving amino group transfer (37). Therefore, if the proposed hypothesis were valid, the presence of azaserine would decrease the amount of NH_4^+ produced. These data further emphasize the non-assimilatory features of the Clostridium KDHS2 NO_3^--reducing enzymes.

Enzyme preparations from Escherichia coli and baker's yeast that possess the capacity for NO_2^- reduction have been shown to be primarily $SO_3^=$ reductases in vivo (19,26). If this were the case for the enzyme system of Clostridium KDHS2, $SO_4^=$ and/or $SO_3^=$ would be expected to suppress the production of NH_4^+ from NO_3^-. Sulfate exerted no influence on the production of NH_4^+, but $SO_3^=$ did inhibit the reaction (Table 2). The data in Table 3 suggest that $SO_3^=$ reducing activity was induced by growth on NO_3^-. But, surprisingly, the presence of up to equimolar amounts of NO_3^- had no effect on $^{35}SO_3^=$ reduction to $^{35}S^=$. Since $SO_3^=$ inhibition of NH_4^+ production from NO_3^- occurred at the level of NO_2^- reduction (Fig. 2), this apparent anomaly can be explained. Nitrate has no effect on $SO_3^=$ reduction, unless some critical level of NO_2^- accumulates.

It appears the NO_2^--reducing enzyme of Clostridium KDHS2 is inducible and can reduce both NO_2^- and $SO_3^=$.

Two possible explanations exist for the inability of the organism to reduce $SO_4^=$ to $S^=$. The cell may lack a dissimilatory sulfate reductase. Harrison and Laishley (Abstr. Ann. Meet. Amer. Soc. Microbiol, 1978) found an inducible, dissimilatory $SO_3^=$-reducing system in C. pasteurianum, which was distinct from the $SO_4^=$ reduction accomplished by this organism, which was restricted to the assimilatory pathway. Alternatively, Clostridium KDHS2 may not readily transport $SO_4^=$. The two-fold stimulation of $SO_3^=$-reducing activity by cells grown in the presence of $SO_4^=$ suggests, however, that $SO_4^=$ does enter the cell.

The Clostridium KDHS2 glutamine synthetase is similar to the glutamine synthetase from C. pasteurianum with respect to inhibition by glutamine (17). Glutamine synthetases from Bacillus species have also been reported to be inhibited by glutamine (10,16). The K_m of 4.0×10^{-4} M for NH_4^+ is the same as reported for the enzyme from B. subtilis (16). This low K_m suggests the existence of a low-NH_4^+ assimilating pathway similar to that found in Klebsiella (5).

The absence of any regulatory effect by glutamine synthetase on the reduction of NO_3^- to NH_4^+ is shown in Table 5. Newman and Cole (23) observed no influence of glutamine synthetase on nitrite reductase in E. coli. The product of NO_2^- reduction by this organism is NH_4^+ and the reaction is similar to the reduction of NO_3^- to NH_4^+ accomplished by Clostridium KDHS2. On the other hand, the NO_2^- reductase of Klebsiella pneumonia may be regulated by glutamine synthetase (32). Therefore, this lack of regulatory control by glutamine synthetase on NO_3^-

reduction in Clostridium KDHS2 is consistent with the thesis that NO_3^--reducing enzymes catalyze a dissimilatory reaction.

Thus, it appears that the NO_3^- reduction to NH_4^+ carried out by Clostridium KDHS2 is linked to the energy metabolism of the cell and is truly a dissimilatory process. The competitiveness of this pathway with denitrification remains a question. In a previous report (Chapt. II), evidence was presented that this organism, when introduced into soil, could effectively compete with denitrifying bacteria for NO_3^- under anaerobic conditions if an appropriate carbon source is supplied. A thorough investigation of the interaction of denitrifying bacteria and organisms reducing NO_3^- to NH_4^+ is needed before the natural significance of this pathway can be understood. Such knowledge may permit exploitation of the bacteria involved to conserve nitrogen in agricultural soils.

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APPENDICES

APPENDIX A

THE ABSENCE OF A REGULATORY FUNCTION FOR GLUTAMINE SYNTHETASE IN THE SYNTHESIS OF ENZYMES INVOLVED IN DENITRIFICATION

Glutamine synthetase (GS) has been shown to possess, in addition to its biosynthetic role, a regulatory function controlling the synthesis of several proteins involved in nitrogen metabolism. In the enteric bacteria, GS regulates the synthesis of enzymes subject to nitrogen catabolite repression (10,15), in addition to autogenously regulating its own synthesis. In organisms other than the enteric bacteria, the regulation of gene expression by GS has not been extensively studied. Nitrogen fixation in Rhizobium (9) may be regulated by GS, and, in Bacillus subtilis, the synthesis of GS is apparently autogenously regulated (2).

Since GS regulates the expression of the enzymes involved in assimilation of nitrogen from a number of nitrogenous compounds, the question arises whether the enzymes catalyzing assimilatory nitrate reduction are subject to similar control. This question has not been addressed experimentally but synthesis of nitrite reductase in Klebsiella aerogenes is repressed by NH_4^+ (13), suggesting GS may play a regulatory role.

Regulation by GS of the assimilatory enzymes, but not of the enzymes of the dissimilatory NO_3^--reducing system, is easily postulated. However, the differences between the assimilatory and dissimilatory nitrate reductases have not been well-delineated. In fact, van't Riet, et al (13) have suggested that the catalytic subunits of the enzymes are

identical, the distinction lying in assembly with different regulatory subunits. The dissimilarity became less clear with the observation that nitrate reductase B may serve either assimilatory or dissimilatory functions, depending upon the bacterium from which it is obtained (12). Studies with a series of five nitrate reductase mutants of Pseudomonas aeruginosa led van Hartingsveldt and Stouthamer (6) to suggest the existence of a molybdenum-containing cofactor common to both the assimilatory and dissimilatory systems. The purpose of this study was to ascertain the presence of GS in Pseudomonas fluorescens, to partially characterize the enzyme, and to examine the effect of GS on the synthesis and activity of the denitrifying enzymes in this organism.

Pseudomonas fluorescens, strain 72, isolated by Gamble, et al (5), was cultured in a basal mineral salts medium (M. R. Betlach, personal communication) that contained (per liter): glucose, 9.0 g; $NaH_2PO_4 \cdot H_2O$, 2.7 g; $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; vitamin-free casamino acids (Difco) and yeast extract (Difco), 0.1 g each; KNO_3, 2.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.15 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $MnCl_2 \cdot 4H_2O$, 1.0 mg; $FeSO_4 \cdot 7H_2O$, 0.55 mg, and citric acid, 0.52 mg. Monosodium glutamate was added at 0.2% (w/v) to the medium as a nitrogen source. P. fluorescens cells were harvested by centrifugation 4-6 h after entry into stationary phase during anaerobic incubation. and cell-free extracts were prepared by passage through a French pressure cell. Glutamine synthetase was partially purified by collecting the precipitate at 50-70% saturation with $(NH_4)_2SO_4$ (7). The precipitate was dissolved in 0.01 M morpholinopropane sulfonic acid (MOPS) buffer, pH 7.0, containing 0.01 M $MnCl_2$ and 0.001 M mercaptoethanol. Glutamine synthetase activity was monitored using the biosynthetic reaction described by Hubbard and Stadtman (7),

except the MOPS buffer above was substituted for imidazole buffer. Controls lacking glutamate were included in each incubation, and corrections were made for the ATP hydrolysis that occurred in the absence of substrate. Measured activity of the enzyme was proportional to the amount of enzyme preparation used and was linear with time up to 15 min.

The activity of glutamine synthetase in the partially-purified enzyme preparation from P. fluorescens and by glutamine and methionine sulfoximine are shown in Table 1. The apparent K_m for NH_4^+ was 4.5 x 10^{-4} M (Fig. 1). The data in Table 2 indicate GS exerted no positive control on the activity or the synthesis of denitrifying enzymes in P. fluorescens.

Previous reports indicate that the biochemical and immunological properties of GS from Gram negative bacteria are similar (14). P. fluorescens was inhibited 60% by 10 mM glutamine, whereas the enzyme of Salmonella typhimurium was reported to be inhibited only 20% (7). Hubbard and Stadtman (7) were unable to measure glutamine inhibition by the P. fluorescens enzyme because of an interfering enzyme activity. Similar problems were not encountered in the extracts used in this study. The P. fluorescens GS also appeared to be less sensitive to 0.01 mM methionine sulfoximine than other Gram negative bacteria. The enteric GS is inhibited 70% by this concentration (1), but only a 30% inhibition was observed for P. fluorescens. Also, the K_m for NH_4^+ for the P. fluorescens enzyme is an order of magnitude lower than the 1.8 x 10^{-3} M value reported for Escherichia coli (16). But, P. fluorescens, a soil bacterium, is likely to encounter lower concentrations of NH_4^+ in its natural habitat. Indeed, this K_m is similar to the value 4.0 x 10^{-4} M reported for the GS of two bacteria found in soil, Bacillus subtilis (3) and a Clostridium spp. (Chapt. III).

Table 1. Effect of inhibitors on activity of glutamine synthetase from Pseudomonas fluorescens

Inhibitor	Activity[†] (μmol P min^{-1} mg protein $^{-1}$)	Inhibition (%)
None	0.28	-
Methionine sulfoximine, 0.01 mM	0.20	29
Methionine sulfoximine, 10 mM	0	100
Glutamine, 10 mM	0.11	61

[†]Assay mixture contained in 0.4 ml: 0.1 mg protein, 0.0075 M ATP, 0.1 M glutamate, 0.05 M NH_4Cl, 0.005 M $MnCl_2$, and 0.01 M MOPS buffer, pH 7.0. Incubation was at 37° C for 15 min. Values are means of three experiments. Protein was measured using the method of Lowry, et al (8) with bovine serum albumin as the standard.

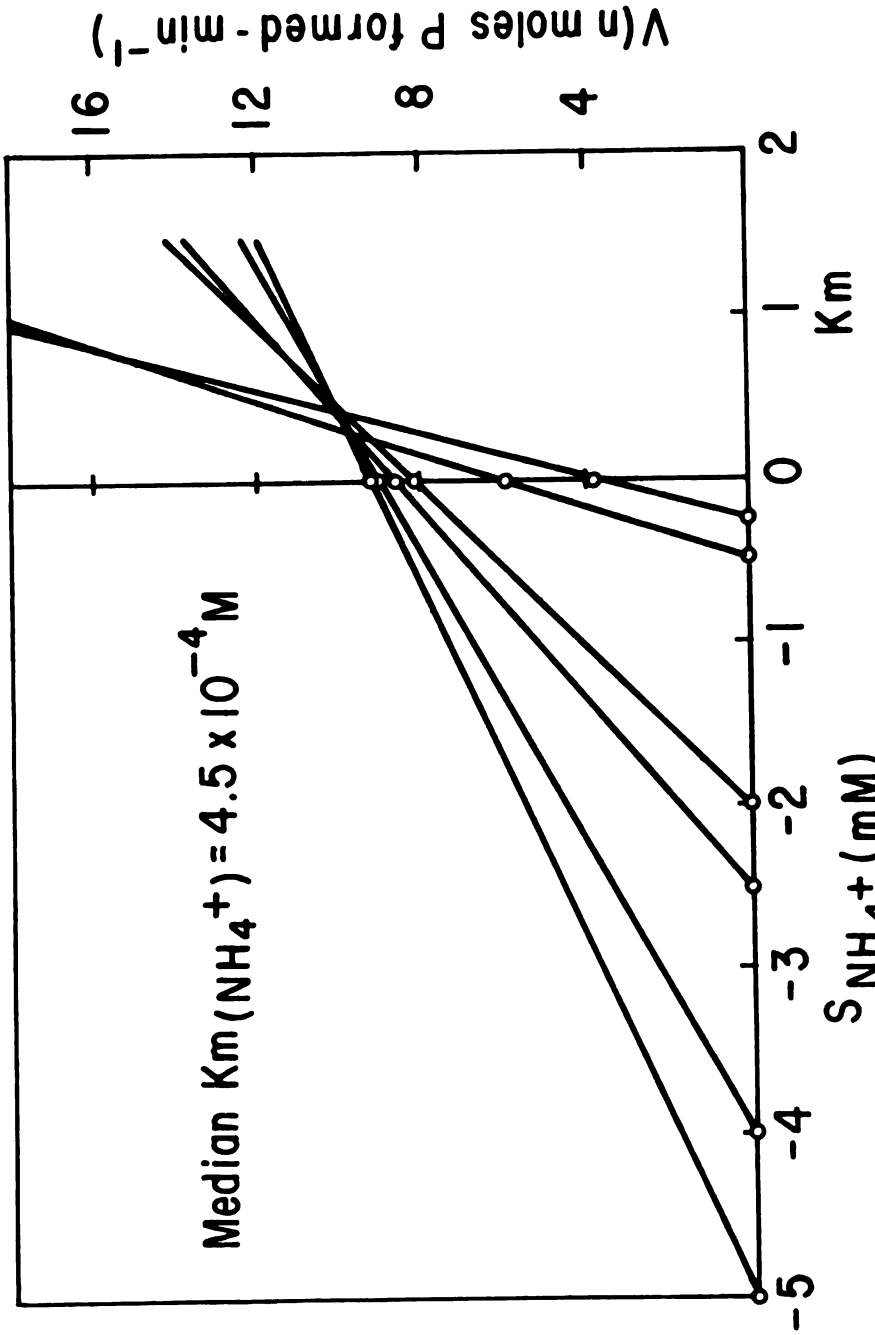

Fig. 1. Direct linear plot (4) of K_m estimation for NH_4^+ for glutamine synthetase of Pseudomonas fluorescens (Strain 72).

Table 2. Effect of methionine sulfoximine on the rate of denitrification[†] by resting cells of Pseudomonas fluorescens.

Concentration of methionine sulfoximine		N_2O production (µl mg cells^{-1})				Rate of N_2O production[‡]
Growth medium	Resting cell suspension	0.5 h	1.0 h	1.5 h	2.0 h	(µl mg cells^{-1} h^{-1})
0	0	4	7	11	15	7.5
	0.01 mM	5	8	12	16	8.5
	10 mM	5	7	12	15	8.1
0.1 mM	0	4	6	-	15	7.2
	0.1 mM	4	7	11	15	7.5
10 mM	0	4	8	-	16	8.0
	10 mM	4	8	11	16	7.8

[†] Denitrification rate measured as N_2O production in presence of 0.1 atm acetylene.

[‡] No significant differences occur among rates at the 95% confidence level.

The absence of any regulatory effect by glutamine synthetase on denitrifying enzymes in P. fluorescens is not surprising. Newman and Cole (11) found no influence of GS on nitrite reductase in E. coli. An earlier report (Chapt. III) presented evidence that the dissimilatory NO_3^--reducing enzymes in a Clostridium species isolated from soil were not subject to regulation by GS. Thus, it appears that the regulatory control exerted by GS on enzymes involved in assimilatory nitrogen metabolism does not extend to the dissimilatory NO_3^--reducing enzymes.

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APPENDIX B

NITRATE-STIMULATED MINERALIZATION OF AMMONIUM IN ANAEROBIC SOILS

The phenomenon referred to as "priming" is defined by Jenkinson (1971) as a change in the decomposition rate of native soil organic matter, either stimulation or retardation. It usually results from the addition of fresh organic matter, but the same events may be triggered by the addition of inorganic nitrogen or fertilizer salts (Broadbent and Nakashima, 1971; Westerman and Tucker, 1974). As a result of priming action, either mineralization or immobilization of soil N occurs (Smith and Douglas, 1971). The quantitative significance of this effect has not been established, and the mechanism remains controversial. Laura (1974) proposed that the decomposition of organic matter is entirely chemical, the result of the protolytic action of water, while Westerman and Tucker (1974) implicated enhanced activity of soil microorganisms. Neither theory adequately explains the phenomenon, although the latter seems more reasonable. In the course of studying dissimilatory reduction of NO_3^- to NH_4^+ in anaerobic soils (Chapter II), NO_3^--stimulated mineralization of organic matter was observed. This note points out the magnitude of this effect and its significance to ecological studies on the nitrogen cycle.

Soil (5 g) was placed in Hungate tubes and amended with 10 ml water containing carbon and/or nitrate as indicated. Tubes were sealed and incubated anaerobically under O_2-free Ar for 5 days at 28° C. After incubation the soils were extracted with 1N KCl and analyzed for NH_4^+ (Bremner, 1965). $^{15}NH_4^+$-N was measured as described elsewhere (Chapter

II) using the ratio mass spectrometer in the laboratory of J. O. Legg, USDA-ARS, Beltsville, Md. Four tubes per treatment were prepared; the extracts of two were pooled and the mean of the duplicate determinations reported.

The accumulation of NH_4^+-N and $^{15}NH_4^+$-N in three soil samples is shown in Table 1. The increase in NH_4^+ accumulation following NO_3^- addition was evidenced by the larger amount of NH_4^+ in the soils to which NO_3^- was added. The origin of the NH_4^+ was not NO_3^-, since reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ was minor. Thus, NO_3^--stimulated mineralization was responsible for the NH_4^+ formation. In the fresh, unamended soils, addition of NO_3^- resulted in an 22-25-fold increase in the amount of mineralization. For glucose-amended soils, the apparent stimulation was only a factor of 14-18, probably because of enhanced immobilization. Parnas (1976) suggested the rate of mineralization was a function of the C/N ratio of the substrate, with mineralization favored when this ratio was low with respect to the optimum C/N ratio for the growth of the carbon-decomposing bacteria present. Immobilization (or assimilation) would be enhanced if the change in C/N ratio resulted in a ratio higher than this optimum. Simultaneous amendment with carbon and NO_3^- does not lower the C/N ratio as much as addition of NO_3^- alone; thus, the observed effect is consistent with this hypothesis. The higher levels of mineralization in the dry Kranzburg soil is significant. Drying of soils, a common practice for many soil studies, apparently alters the organic N fraction of the soil rendering it more susceptible to microbial attack.

The addition of carbon to the soils increased the amount of $^{15}NO_3^-$ converted to ^{15}N-organic N reflecting the increased demand for nitrogen

Table 1. Production of total NH_4^+ and $^{15}NH_4^+$ from $^{15}NO_3^-$ after 5 days anaerobic incubation in response to NO_3^- and carbon additions to three soils.

Amendments			Ammonium produced (μg $^{15}NH_4^+$-N/g soil)								
			Conover-fresh			Kranzburg-fresh			Kranzburg-dry		
Carbon (mg/g)	$^{15}NO_3^-$† (μg N/g)	NH_4^+	$^{15}NH_4^+$	^{15}N-Organic N	NH_4^+	$^{15}NH_4^+$	^{15}N-Organic N	NH_4^+	$^{15}NH_4^+$	^{15}N-Organic N
0	0	0.2	-	-	0.5	-	-	0.6	-	-
0	80	5.0	0.2	0.9	11.2	0.6	1.6	33.4	0.1	0.6
Glucose, 8	80	3.7	0.6	19.9	6.8	0.4	21.5	18.5	0.1	18.1
Acetate, 8	80	5.0	0.1	3.4	4.4	0.9	1.2	37.4	1.3	1.1

† For $^{15}NO_3$ additions, $K^{15}NO_4$ at 56.75 atom % ^{15}N was added. Standard errors for NH_4^+ and $^{15}NH_4^+$ determinations were 0.2.

for growth of bacteria. Note that the addition of glucose tremendously stimulated NO_3^- assimilation, while the addition of acetate, a carbon source utilized by only a limited portion of the bacterial flora, resulted in a considerably lower amount of NO_3^- assimilation.

One mechanism that could account for NO_3^--stimulated priming in anaerobic soils is that NO_3^- would serve as a terminal electron acceptor, allowing more oxidative metabolism to occur (by denitrifying bacteria, for example). Acetate is not readily metabolized by fermentative bacteria, but is used by virtually all denitrifiers. If this mechanism were operable, acetate should be more rapidly used by denitrifying bacteria than soil organic carbon, thereby removing the NO_3^- and reducing the priming effect. Acetate caused reduced priming in only the fresh Kranzburg soil, suggesting that oxidative respiration is not the only mechanism responsible for NO_3^--induced priming.

From the data presented, it is apparent that ecological studies in soil involving nitrogen require use of a tracer such as ^{15}N if quantitative measurement of N cycle processes are to be made. Since carbon and nitrogen are closely related in priming action, carbon tracer studies are also mandated. Priming action raises a potential problem with use of isotope tracers. If a method assumes steady-state rates, for mineralization or immobilization in the case of N, clearly then data measured would be subject to large errors, compounded because priming may result in either a positive or negative effect. Experiments based on isotope dilution require such assumptions.

The soils used in this study were incubated anaerobically which may have enhanced NH_4^+ accumulation relative to that which occurs in the

partially anaerobic soils more commonly encountered in nature. Nevertheless, because of the considerable extent of NO_3^--induced priming, the phenomenon can not be dismissed as being trivial.

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