Nitrogen Isotopic Fractionation and $^{18}$O Exchange in Relation to the Mechanism of Denitrification of Nitrite by Pseudomonas stutzeri* 

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Two types of mechanisms for the enzymatic reduction of NO$_3^-$ to N$_2$O have been proposed. In one, two NO$_3^-$ ions are reduced in parallel, with the nitrogen–nitrogen bond formed from reduced intermediates. In the second, the two NO$_3^-$ ions enter the reaction sequentially, with the nitrogen of at least one of the two having a valence of 3+ when the nitrogen–nitrogen bond is formed. Our objective was to distinguish between these two types of mechanism. Toward that end, the exchange of $^{18}$O from H$_2$O to NO$_2^-$, resulting from a protonation-dehydration step preceding reductive events in both mechanisms, was less than 10% of the rate of denitrification at both low and high [NO$_3^-$]. The value of $\beta_{obs}$ was 1.010 ± 0.001 and 1.020 ± 0.001 at low and high [NO$_3^-$], respectively. Expressions for $\beta_{obs}$ as a function of the measured rate of entry of oxygen from H$_2$O into NO$_2^-$, were derived for both types of mechanism. The measured dependence of $\beta_{obs}$ on substrate concentration, as constrained by the $^{18}$O exchange data, is inconsistent with the first type of mechanism, but consistent with the second type. Thus, by combining nitrogen isotopic fractionation and $^{18}$O exchange data, we rule out any mechanism in Pseudomonas stutzeri in which NO$_3^-$ ions are reduced in parallel, with the nitrogen–nitrogen bond being formed from reduced intermediates.

Denitrification is the process by which nitrogen atoms of NO$_3^-$ and NO$_2^-$ are returned to the atmosphere as N$_2$O or N$_2$. The process stands to N$_2$ fixation as respiration stands to photosynthesis. Despite its crucial position in the nitrogen cycle, the mechanism of the first committed reaction of denitrification, dissimilatory NO$_3^-$ reduction, is still a matter of controversy. Garber and Hollocher (1) presented persuasive evidence that the first step of the enzymatic reduction of NO$_3^-$ by Pseudomonas stutzeri is a reversible protonation-dehydration of enzyme-bound NO$_3^-$, yielding the intermediate, enzyme-bound nitrosyl (E-N$O^+$). There is presently no agreement concerning the further reaction of E-N$O^+$ to produce N$_2$O.

Two types of schemes have been proposed. In the first, two NO$_3^-$ ions are reduced in parallel to the 2+ (2) or 1+ valence state (1). Following this reduction, the two reduced species are combined to form the nitrogen–nitrogen bond needed for N$_2$O production. In one version, NO is an enzyme-bound intermediate (1); and in another, NO is a free intermediate (2, 3). The first version was proposed by Garber and Hollocher (1) and is shown diagrammatically as Mechanism I in Fig. 1. In this proposed mechanism, NO$_3^-$ is activated by protonation-dehydration to form E-N$O_3^+$, and the nitrosyl is reduced by two electrons to E-N$O^-$. Nitrosyl is then released from the enzyme and reacts with another free nitrosyl and hydrogen ions to form N$_2$O and H$_2$O nonenzymatically.

In the second type of mechanism, nitrogen–nitrogen bond formation and reduction occur in a linear, rather than parallel, path from NO$_3^-$ to N$_2$O. At least one of the nitrogen atoms of NO$_3^-$ has a 3+ valence when the nitrogen–nitrogen bond is made. An example is the mechanism proposed by Averill and Tiedje (4) (shown as Mechanism II in Fig. 2). In this mechanism, as in Mechanism I, the enzyme-bound NO$_3^-$ is dehydrated to form E-N$O^+$. At this point, the mechanisms diverge. In Mechanism II, a second NO$_3^-$ makes a nucleophilic attack on E-N$O^+$, resulting in the formation of E-N$_2$O$_3$, which is ultimately reduced to N$_2$O. Whereas Averill and co-workers (5) have subsequently modified their original proposal (see Averill and Tiedje (4)), to take into account more recent experimental results (6, 7), the essential feature (from the point of view of our paper) remains, namely, two NO$_3^-$ ions are added to the reaction sequentially, rather than in parallel, with at least one nitrogen atom having a 3+ valence. In this mechanism, the nitrogen atoms of both NO$_3^-$ ions have a 3+ valence when the nitrogen–nitrogen bond is made.

The first reductive step in both mechanisms (the step with rate constant $k_1$ in Mechanism I and $k_2$ in Mechanism II) and steps beyond are represented as unidirectional. The consequences, for the interpretation of the data reported here, of the assumption of unidirectionality of these steps being partially or wholly incorrect is addressed under “Discussion.”

The reversible dehydration step(s) (see, for example, the second step of both mechanisms in Figs. 1 and 2) allows for enzymatically catalyzed exchange of $^{18}$O between water and an intermediate of the reaction (e.g. E-N$O^+$), leading to the possibility of $^{18}$O incorporation into product, N$_2$O, and substrate, NO$_3^-$.

Such $^{18}$O incorporation into N$_2$O by three species of denitrifying microorganisms and into NO$_3^-$ by two species

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1 Garber and Hollocher (6) showed positional equivalence of nitrogen in $^{15}$N$_2$O produced by concomitant reduction of $^{15}$NO$_3^-$ and $^{15}$NO. This was interpreted as favoring NO$^-$ as precursor of N$_2$O. However, Aerssens et al. (5) pointed out that cis-hyponitrite (an intermediate not explicitly included in the originally proposed mechanism of Averill and Tiedje (4)) is an equally plausible intermediate if the nitrogen atom attached to the metal center of the enzyme exchanges rapidly with the other nitrogen atom, as they consider likely based on an analogous system (8). In the original proposal of Averill and Tiedje (4), the step from E-N$O^+$ + NO$_3^-$ → E-N$_2$O$_3$ was irreversible. The nitrogen isotopic fractionation data of Bryan et al. (7) require that such a step, if it exists, be reversible (see “Discussion”). In the latest published version of their mechanism, Aerssens et al. (5) show this step as reversible.
was experimentally demonstrated by Garber and Hollocher (1). Aerssens et al. (5) showed that the extent of enzymatic exchange of $^{18}$O from H$_2$O to N$_2$O decreased with increasing N$_2$O concentration. The investigators interpreted this finding as favoring Mechanism II over I. On the other hand, Garber et al. (9) observed isotopic equilibrium between $^{15}$N and $^{14}$N in N$_2$O produced in the presence of denitrifying bacteria and chemical decomposition of N$_2$O, plus unlabeled N$_2$O$^+$, one of the intermediates of Mechanism II. This was interpreted as showing that N$_2$O$^+$ cannot be an intermediate of denitrification. We regard this interpretation as inconclusive because of the high background of nonenzymatic evolution of N$_2$O in cell-free controls which resulted from decomposition of N$_2$O$^+$.

In addition, their data are not completely internally consistent.\(^2\) If the finding of Garber et al. (9) and its interpretation are verified, it would appear that Mechanism II, as proposed, cannot be correct. However, it does not rule out all mechanisms of the second type, namely, sequential addition of two N$_2$O ions to the reaction.

Chien et al. (11) and Mariotti et al. (12) found that the overall nitrogen isotope effect ($\beta_{obs}$) associated with denitrification of N$_2$O in soils decreased when carbonaceous materials were added. Bryan et al. (7) reported a similar finding using pure cultures and cell-free extracts of P. stutzeri in these competitive experiments.\(^3\) Bryan et al. found that $\beta_{obs}$ decreased with the concentration of the ultimate electron donor (succinate in these experiments) and increased with the concentration of N$_2$O. Whether the concentration of N$_2$O or the ultimate electron donor was varied, the relationship between $\beta_{obs}$ and the velocity of the reaction was linear.

The relationship between velocity (normalized to $V_{max}$) and $\beta_{obs}$ was seen in whole cells and cell-free extracts.

These isotopic fractionation results bear on the mechanism of denitrification in the following way. The value of $\beta_{obs}$ is established by intrinsic isotopic fractionation factors associated with individual steps within the overall reaction, by the relative rates of steps in the forward versus reverse directions, and by the mechanism of the overall reaction. Since the magnitude of intrinsic isotope effects is very nearly constant,\(^4\) the only way in which $\beta_{obs}$ can vary is for the relative rates of forward and reverse reactions to vary. It can therefore be concluded that in P. stutzeri the concentrations of both N$_2$O and the ultimate electron donor affect the relative rates of forward and reverse reactions. With additional information concerning the magnitude and direction of changes in the relative rates of forward and reverse steps of the reaction in response to changes in N$_2$O and succinate concentration, the concomitant variation in $\beta_{obs}$ can be used to provide information about the reaction mechanism.

This paper reports enzymatically catalyzed exchange of $^{18}$O between H$_2$O and N$_2$O and the nitrogen isotope effect in denitrifying cultures of P. stutzeri measured in the same experiment. The objective was to determine the rate of return of enzyme-bound nitrogen intermediates to the free N$_2$O pool compared to the overall rate of disappearance of N$_2$O in order to determine whether one or both types of mechanism described above are consistent with the nitrogen isotopic fractionation data, as constrained by the $^{18}$O exchange data.

**MATERIALS AND METHODS**

**Preparation of Resting Cultures—** Cultures of P. stutzeri (JM300) were grown anaerobically in defined medium (100 mM NaN$_3$, 25 mM sodium succinate, 18.7 mM NH$_4$Cl, 0.36 mM FeSO$_4$, and 2 ml/liter trace elements). The trace element solution consisted of 125 mg of EDTA, 154 mg of MnSO$_4$·H$_2$O, 10 mg of CuSO$_4$·5H$_2$O, 24.5 mg of Co(NO$_3$)$_2$ · 6H$_2$O, 17.7 mg of Na$_2$B$_4$O$_7$·10H$_2$O, and 100 mg of Na$_2$MoO$_4$·2H$_2$O in 100 ml of H$_2$O. The pH of the growth medium was 6.8. Cells were harvested at midlog phase by centrifugation; washed three times with

\(^2\) For example, the $^{18}$N abundance of N$_2$O produced when P. stutzeri was incubated with $^{15}$NO$_2$ and $^{18}$NO$_2$ was lower than would be expected from the amount of N$_2$O produced by biological denitrification (as opposed to the amount produced by chemical decomposition of N$_2$O$^+$). This requires a higher rate of N$_2$O$^+$ decomposition in the presence of P. stutzeri than in its absence. However, when P. stutzeri was incubated with N$_2$O$^+$ (no NO$_2$ present), less N$_2$O was formed than in controls without cells. Thus, decomposition of N$_2$O$^+$ does not seem to be enhanced by the presence of the bacteria. Despite this and other inconsistencies, the data of Garber et al. (9) cast enough doubt on N$_2$O$^+$ as an intermediate in the denitrification of NO$_2$ that we consider the burden now to rest on those who propose enzyme-bound N$_2$O$^+$ as an intermediate in biological denitrification to demonstrate that it is a suitable substrate for the enzyme.

\(^3\) In competitive isotopic fractionation experiments, both heavy and light isotopes (usually at natural abundance) are present in the same reaction vessel and are thus exposed to the same enzyme concentration. This type of experiment is the method of choice when measuring isotopic fractionation involving heavy atoms (atoms heavier than hydrogen).

\(^4\) Isotopic fractionation factors are a function of temperature. However, in the physiological temperature range, this variation is quite small.
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}O$ Exchange

100 mM MOPS, 0.8 mM MgSO$_4$, 5 mM KH$_2$PO$_4$, 5 mM K$_2$HPO$_4$ (pH 6.8); and resuspended in washing medium to a concentration 10 times that at harvest.

Incubation Conditions—Cells were incubated anaerobically (under helium) in a medium consisting of 5 mM K$_2$HPO$_4$, 5 mM KH$_2$PO$_4$ (pH 6.8), 0.3 M NaCl, 25 mM sodium succinate, and a concentration of NO$_3^-$ (at natural abundance) in $^{18}O$-enriched (200%$^{18}O$ or 0.0408 atom % excess $^{18}O$) H$_2$O. This low $^{18}O$ enrichment was selected in order that $^{15}N$ and $^{18}O$ analyses could be done on identical samples incubated in parallel. (About 0.8 mg of nitrogen is required for analysis. Therefore, for incubations at low NO$_3^-$ concentrations, a large volume was used. For example, when the concentration of NO$_3^-$ was 0.107 mM, the volume of the incubation mixtures was 1000 ml. High $^{18}O$ enrichments would have been prohibitively expensive.) The time course of the reaction was followed by measuring the concentration of NO$_3^-$ colorimetrically (13). The reaction was stopped when about half of the added NO$_3^-$ had been consumed by adding 5 ml of 50% (w/w) NaOH/liter. NaOH was added at time 0 to obtain control samples. The initial concentration of NO$_3^-$ in control samples was half that in experimental samples, so that the final NO$_3^-$ concentration was approximately the same in control and experimental samples.

Isotope Abundance of Azide from Residual NO$_3^-$ for Analysis—The incubation mixture was concentrated to a volume of about 100 ml by heating on a hot plate in a fume hood, care being taken to avoid bringing the samples to dryness. NH$_4^+$, part of which arises from the breakdown of compounds such as amidases as a result of heating under alkaline conditions, was removed by steam distillation (14). The nitrogen of the residual NO$_3^-$ was then reduced to NH$_4^+$ and collected by steam distillation (14). After titration to determine the quantity of nitrogen, acidification with H$_2$SO$_4$, and concentration to about 1 ml, the (NH$_4$)$_2$SO$_4$ was placed into a Sprinson-Rittenberg tube, frozen in liquid N$_2$, and evacuated three times prior to generation of Nz by syringe. The flask was further evacuated (briefly) prior to exhaustively evacuating because under acidic conditions, substantial concentrations of NO$_3^-$ (at natural abundance) in 100%$^{18}O$-enriched (200%$^{18}O$) N$_2$O were then cryogenically transferred to a second tube containing 2 ml of 50% (w/w) NaOH allowed to equilibrate overnight at room temperature. This step is necessary to ensure removal of the last trace of CO$_2$, which grossly interferes with analysis of $^{18}O$ in N$_2$O, when using an isotope ratio mass spectrometer (as required for precise measurement of the low $^{18}O$ enrichment we used). After three successive cryogenic transfers, the N$_2$O was analyzed for $^{18}O$ abundance.

Isotope Analysis—The $^{15}N$ abundance of NH$_3$ generated from NH$_4^+$ and the $^{18}O$ abundance of N$_2$O produced by reduction of NO$_3^-$ by N$_2$ was measured in a dual collector, dual inlet mass spectrometer (VG Micromass 602E). $^{18}O$ abundances were determined by comparing the ratio 46 m/e + 44 m/e to that of an $^{18}O$ working standard. The working standard for $^{18}O$ measurements was tank N$_2$O whose $^{18}O$ abundance relative to atmospheric N$_2$ was measured by A. Mariotti (Institut National de la Recherche Agronomique, Versailles, France). $^{18}O$ abundances were measured by comparing the ratio 46 m/e + 44 m/e to that of an $^{18}O$ working standard. The working standard for $^{18}O$ measurements was tank N$_2$O whose $^{18}O$ abundance compared to standard mean ocean water was measured by comparing it to N$_2$O generated from NO$_3^-$ with N$_2$O in standard H$_2$O of known $^{18}O$ abundance under conditions in which complete $^{18}O$ exchange between H$_2$O and NO$_3^-$ was achieved. Conditions for complete $^{18}O$ exchange were to adjust the NO$_3^-$ solution to pH 11 and then to return the pH to 4.0 with H$_2$SO$_4$ and to carry out the reaction as described above, but in the absence of halides. The $^{18}O$ abundance of the standard H$_2$O was measured by Tom Anderson (University of Illinois). The $^{18}O$ abundance of H$_2$O in the medium was determined by comparing the $^{18}O$ abundance of CO$_2$ equilibrated with medium versus standard H$_2$O.

Isotope abundances were expressed as $\delta$ values (% enrichment of the heavy isotope), i.e.

$$
\delta ^{18}O = \frac{^{18}O_{cont}}{^{18}O_{standard}} - 1 \times 1000 \%
$$

where H and L refer to the concentration of molecules bearing the heavier and lighter isotope, respectively, and spl and std refer to experimental sample and standard, respectively.

The overall nitrogen isotopic fractionation factor ($\delta_{N_{tot}}$) was calculated from an approximate relationship derived by Mariotti et al. (17):

$$
\delta_{N_{tot}} = 1 - \frac{\delta ^{15}N_{cont} - \delta ^{15}N_{standard}}{\delta ^{15}N_{N_2}} \times 1000 \text{ln } f
$$

where the subscripts s,t = 0 and s,t = t refer to the substrate at zero time and at the time the incubation was stopped, respectively, and $f$ is the fraction of substrate remaining at t = t.

The percent enzymatically catalyzed $^{18}O$ exchange between H$_2$O and NO$_3^-$ was calculated as

$$
\% ^{18}O \text{ exchanged} = \frac{\delta ^{18}O_{H_2O}}{\delta ^{18}O_{cont}} \times 100
$$

where the subscripts spl, cont, and H$_2$O refer to the experimental sample, the control, and H$_2$O in the incubation medium, respectively.

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The abbreviation used is: MOPS, 4-morpholinepropane sulfonic acid.
RESULTS

Fig. 3 shows the time course of disappearance of NO₂ during incubation with resting *P. stutzeri* cells as measured by col-

orimetric assay. Four replicate incubations were done using two initial concentrations of NO₂ (0.107 and 2.29 mM). The con-

centration of cells was adjusted so that the time required for disappearance of half of the NO₂ was approximately the same for the two NO₂ concentrations. (For the high NO₂ concentration, the cell concentration was 6.4 times higher than for the low NO₂ concentration). In two replicate incu-

bations at each initial NO₂ concentration, the water in the incubation medium contained ¹⁸O. After stopping the reaction, these four samples were used to determine the ¹⁸O abundance of the residual NO₂, whereas the other four samples were used to determine the abundance of the residual NO₂. NO₂ disappearance was linear during the period of the incubation. Velocities, calculated from the slope of the regressions, were 88.1 and 242 nmol (min·mg of cell, dry weight)⁻¹ for the low and high NO₂ concentrations, respectively.

The amount of NO₂ consumed between time 0 and the time that the reaction was stopped was determined by distillation techniques. Since NO₂ consumption was linear with time, it was possible to calculate the velocity of the reaction from this measurement as well (see Table I). These calculated velocities are close to the values calculated from the slope of the regressions of Fig. 3. Bryan et al. (7) previously reported that denitrification of NO₂ by *P. stutzeri* followed Michaelis-

Menten kinetics. The velocities calculated from the data shown in Table I are most consistent with values for apparent kₘ and Vₘₐₓ of 0.204 mM and 248 nmol (min·mg of cell)⁻¹, respectively, in reasonable agreement with values found by Bryan et al. (7) (kₘ = 0.16 mM and Vₘₐₓ = 294 nmol (min·mg of cell)⁻¹).

Table II gives the ¹⁵N enrichment of the residual NO₂ and the fraction of NO₂ remaining after the incubation was stopped. From these data, we calculated the observed nitrogen isotopic fractionation factor (βobs) associated with denitrification of NO₂. This calculation requires the assumption that βobs is constant over the course of the incubation. This as-

sumption is justified by the finding reported by Bryan et al. (7) that, at both high and low initial NO₂ concentrations, velocity and βobs were constant, within experimental error, if the extent of reaction was restricted to 60% or less (see Figs. 1 and 2 of Ref. 7). In agreement with previously reported observations (7), the value of βobs was significantly greater at high than at low initial NO₂ concentration. Bryan et al. reported that βobs increased linearly with velocity. For Mi-

chaelis-Menten kinetics, this is equivalent to βobs decreasing naturally with v/[S]. We find the latter formulation more convenient. The values of βobs shown in Table II may be compared to values calculated from the empirical relationship deduced from the data of Bryan et al. (7), namely,

\[
\beta_{obs} = 1.0245 - 0.00464 \frac{v}{V[S]}
\]

where [S] is substrate concentration (initial) in millimolar, v is velocity, and V is the maximum velocity. The constant, 0.00464, has units of millimolar. This relationship predicts values for βobs of 1.011 and 1.023 for [NO₂] of 0.107 and 2.29

**TABLE I**

| Sample | Initial [NO₂] | Incubation volume | Incubation time | Quantity of cells | Residual NO₂ | Velocity |
|--------|--------------|-------------------|----------------|------------------|-------------|----------|
|        | mM           | ml                | min            | mg               | μmol        | nmol (min·mg cell⁻¹) |
| 1      | 0.107        | 1000              | 61             | 9.98             | 55.7        | 84.3      |
| 2      | 0.107        | 1000              | 62             | 9.98             | 53.6        | 84.3      |
| 3      | 2.290        | 125               | 70             | 8.02             | 161.4       | 222.5     |
| 4      | 2.290        | 125               | 70             | 8.02             | 155.7       | 223.6     |

Mean ± S.E. 827.6 ± 5.1

**TABLE II**

| Sample | Initial [NO₂] | Fraction of substrate remaining (f) | Change in ¹⁵N abundance | βobs |
|--------|--------------|-------------------------------------|-------------------------|------|
|        | mM           |                                      |                         |      |
| 1      | 0.107        | 0.520                                | +7.22                   | 1.011|
| 2      | 0.107        | 0.501                                | +6.31                   | 1.009|
| 3      | 2.290        | 0.564                                | +10.63                  | 1.019|
| 4      | 2.290        | 0.544                                | +11.98                  | 1.020|

Mean ± S.E. 1.020 ± 0.001

*δ¹⁵N difference between control and experimental sample (sample minus control).*
TABLE III

| Sample | Initial [NO₃] | Incubation medium | Control NO₃ | Sample NO₃ | mM | d¹⁸O | Enzyme-catalyzed ¹⁸O exchange |
|--------|---------------|-------------------|-------------|------------|----|------|-----------------------------|
|        |               |                   |             |            |    |      |                             |
|        | 0.107         | 198.3             | 15.1        | 5.8        | 5  | -    |                             |
|        | 0.017         | 198.3             | 13.2        | 4.8        | 5  | -    |                             |
| 7      | 2.290         | 180.0             | 14.2 ± 1.0  | 5.3 ± 1.2  | 7  | -    |                             |
| 8      | 2.290         | 180.0             | 21.1        | 5.4        | 8  | -    |                             |

For the above information, the differential equation given above can be written more explicitly as follows.

\[
\frac{dq(t)}{dt} = v_{sw} - \frac{q(t)}{i - v_{oxs}} v_{oxs}
\]

The integrated form of this equation, found by the method described by Leighton (18), is as follows.

\[
q(t) = \frac{i - v_{oxs}}{v_{oxs}} \ln\left(\frac{i}{i - v_{oxs}}\right)
\]

Our measurement of the exchange of ¹⁸O between H₂O and NO₃ allows us to calculate \( q \) at time \( t' \), the time the incubation was terminated; \( q(t') = \) the fractional exchange of ¹⁸O from H₂O to NO₃ times the total quantity of NO₃ in the medium at time \( t' \). Substituting this value and the measured values of \( i \), \( t' \), and \( v_{oxs} \) into this equation permits us to calculate \( v_{oxs} \) at \( t' \). But since \( v_{oxs} \) is constant with time, the value at \( t' \) is its value at all times. Using values in Tables I and III, we calculated \( v_{oxs} \) and \( v_{sw}/v_{oxs} \). Table IV shows that the rate of enzymatically catalyzed entry of oxygen from H₂O to the NO₃ pool is about 5–8% of the overall rate of oxygen atom loss. The work of other investigators (1, 5) with P. stutzeri has shown that the initial protonation-dehydration step is highly reversible. It is therefore reasonable to conclude that enzyme-substrate dissociation is extremely slow in this organism.

**DISCUSSION**

One possible explanation for the observation that \( \beta_{obs} \) increases with substrate concentration is that entry of substrate into the cell is diffusion-limited at low substrate concentration and by an isotopically sensitive enzymatic reaction at high concentration. In this case, as NO₃ concentration approached 0, \( \beta_{obs} \) would approach approximately 1.00 since there is little isotopic fractionation associated with the diffusion of an ion in aqueous solution. As substrate concentration approached infinity, the isotope effect associated with the rate-limiting enzymatic reaction would be fully expressed. Such a set of circumstances would result in the observed variation of \( \beta_{obs} \) with NO₃ concentration. This simple explanation does not account for the variation in \( \beta_{obs} \) associated with denitrification of NO₃ in P. stutzeri because the variation in \( \beta_{obs} \) was also observed in cell-free extracts, and the relationship between \( \beta_{obs} \) and velocity (normalized to maximum velocity) was the same in whole cells and cell-free extracts (7), that is, the rate of entry of NO₃ into the cell was fast compared to the enzymatic reaction even at low substrate concentrations. It can be concluded that the isotope effect for the enzymatic reaction varies with substrate concentration in the case of NO₃ denitrification by P. stutzeri. The mechanistic significance of this variable isotope effect is discussed below.

**Mechanism I**—The small degree of incorporation of oxygen from H₂O into NO₃ at both high and low [NO₃] means that either (or both) step 2 (dissociation of the enzyme-substrate complex) or (and) step 4 (hydration-deprotonation) must be very slow compared to the forward reactions with which they compete, irrespective of [NO₃]. Because of the substantial degree of incorporation of oxygen atoms from H₂O into NO₃ in P. stutzeri observed by others (1, 5), it seems most likely that the rate of enzyme substrate dissociation rather than hydration-deprotonation limits the rate of entry of oxygen atoms into NO₃. However, it seems prudent to examine both cases. If the low rate of entry of oxygen into NO₃ from H₂O resulted from rate limitation by enzyme-substrate dissociation...
(step 2), then the observed isotope effect would reflect predominantly the (virtually nonexistent) isotopic preference of enzyme-nitrite association. The result would be a value of $\beta_{\text{obs}}$ quite close to 1.00 for all NO$_3^-$ concentrations, in contrast to the experimental result reported here and in Ref. 7; namely, that $\beta_{\text{obs}}$ increases with NO$_3^-$ concentration. If, on the other hand, the rate of entry of oxygen into NO$_2^-$ were limited by the rate of hydration-deprotonation (step 4), any isotope effect associated with step 3 (protonation-dehydration of E-NO$_2^-$) would be almost fully expressed. However, this cannot explain the variation of $\beta_{\text{obs}}$ with [NO$_3^-$] because the ratio of rates of the forward to the reverse reaction of E-NO$_2^-$ ($k_3[E$-NO$_2^-$]/$k_4[E$-NO$_2^-]$) is not affected by [NO$_3^-$]. The first step in the reaction sequence for which the relative success of the forward versus the backward reaction is influenced by [NO$_3^-$] is E-NO$^*$. The rate of the forward reaction of E-NO$^*$ (step 5) depends on the concentration of the immediate electron donor, R, (probably heme d$_1$ - Fe$^{3+}$), as well as [E-NO$^*$] and the rate constant $k_b$, whereas the rate of the reverse reaction (step 4) depends only on [E-NO$^*$] and $k_a$. The concentration of R, is affected by [NO$_3^-$]. For example, increasing the concentration of NO$_3^-$ causes an increase in the ratio of oxidized to reduced species in the electron transport chain. Therefore, the ratio of the forward to the reverse rate of reaction of E-NO$^*$ decreases with [NO$_3^-$]. However, this also cannot account for the observed variation of $\beta_{\text{obs}}$ with [NO$_3^-$] because, in the case that the rate of hydration/protonation limits the rate of entry of oxygen atoms from H$_2$O into NO$_3^-$, the isotopic preference of the subsequent step 5 (the reduction of E-NO$^*$) would be only slightly expressed (19). The conclusion from the above argument is that, whereas $\beta_{\text{obs}}$ might be significantly greater than 1.00, there is no way that $\beta_{\text{obs}}$ could have differed substantially at the two NO$_3^-$ concentrations used in the experiments reported in this paper. Whereas we believe most readers will find the above discussion convincing, some have suggested that the small observed incorporation of oxygen into NO$_3^-$ from H$_2$O is large enough to allow sufficient variation in the expression intrinsic isotope effects associated with steps beyond step 3 to account for the observed variation of $\beta_{\text{obs}}$ with NO$_3^-$ concentration. A more quantitative discussion is required to meet that objection to our conclusion; namely, that the data of this paper are inconsistent with the operation of Mechanism I in P. stutzeri under the conditions of our experiment.

Such a discussion is based on the equations derived in the Miniprint, where expressions for $\beta_{\text{obs}}$ for the two mechanisms are derived. The derivation for Mechanism I is based on the assumption that step 5 is unidirectional. (If step 5 is unidirectional, it is irrelevant to the derivation of an expression for $\beta_{\text{obs}}$ whether subsequent steps are or are not unidirectional since isotopic fractionation associated with steps beyond the first unidirectional step following entry of substrate is not expressed in $\beta_{\text{obs}}$.) The assumption of unidirectionality of step 5 is based on the strongly exergonic ($\Delta$G = -320 mV) nature of the half-reaction for the one-electron reduction of NO$_2^+$ to NO (20) as well as the exergonic nature of the half-reaction for the oxidation of the stoichiometric electron donor used in the experiments here, succinate ($\Delta$G = -320 mV for its two-electron oxidation to fumarate). If the assumption that step 5 is partially or wholly incorrect, then the effect of the error would be that isotopic fractionation associated with later steps, up to and including the first unidirectional step (but not beyond), would be folded into the isotope effect for step 5 ($\beta_5$).

Equation 36 of the Miniprint (shown below) gives the expression for $\beta_{\text{obs}}$ for Mechanism I in terms of intrinsic isotope effects for the individual steps (1 - $\beta_5$ for steps 1 - 5) and the two partition factors,

$$P = \frac{k_5}{k_3 + k_5}$$

and

$$N = \frac{k_1[R_1]}{k_2 + k_3[R_2]}$$

where $k_2$ - $k_3$ are rate constants for steps 2 - 5, respectively, and $R_1$ is the immediate reductant (the reduced form of heme d$_1$ on cytochrome c,d$_1$ at the end of a chain of electron carriers).

$$\frac{(1 - P) (1 - N) \beta_5 \beta_3}{(1 - P) (1 - N) + N (1 - P) + NP} \beta_{\text{obs}} = \frac{\beta_5 \beta_3 + N P \beta_5}{(1 - P) (1 - N) + N (1 - P) + NP}$$

At first glance, this expression does not appear to permit variation of $\beta_{\text{obs}}$ with [NO$_3^-$] since it does not explicitly include [NO$_3^-$], and the rate constants of $P$ and $N$ and the intrinsic isotope effects are constant. However, the concentration of $R_1$ is affected not only by the concentration of the ultimate reductant (succinate, in our experiments), but also by [NO$_3^-$]. NO$_3^-$ was the principal oxidant in the anaerobic system used in the experiments. As such, [NO$_3^-$] will influence the steady-state ratio of oxidized to reduced species in the electron transport chain.

### Table IV

| Initial NO$_3^-$ | Quantity of O in NO$_3^-$ pool, $\mu$g atoms | Duration of incubation, $t$ | O in NO$_3^-$ pool derived from H$_2$O, $q_0 = t$ | $\nu_{\text{obs}}^a$ | $\nu_{\text{obs}}^b$ | $\nu_{\text{obs}}/\nu_{\text{exp}}^a$ |
|-----------------|-----------------------------|-----------------|-----------------------------|-----------------|-----------------|-----------------|
| mM             | $\mu$g atoms | min | $\mu$g atoms | $\mu$g atoms/min | $\mu$g atoms/min |
| 0.107           | 214            | 61   | 5.8 ± 1.2     | 1.70 ± 0.02    | 0.135 ± 0.006   |
|                 | 2.290          | 70   | 16.8 ± 1.9    | 3.65 ± 0.04    | 0.327 ± 0.08    |

* Calculated as $\mu$g atoms of oxygen in NO$_3^-$ = 2 x $\mu$mol of NO$_3^-$.
* Calculated as $\mu$g atoms of oxygen in the NO$_3^-$ pool derived from H$_2$O = $\mu$g atoms of oxygen in NO$_3^-$ x fractional $^{18}$O exchange between H$_2$O and NO$_3^-$ (from Table III) (values are mean ± S.E.).
* Calculated as ng atoms of oxygen (total) leaving the NO$_3^-$ pool via denitrification/min = 2 x rate of NO$_3^-$ loss in nmol/min (values are mean ± S.E.).
* Calculated as described in text (values are mean ± S.E.).
* Values are mean ± S.E.
* Error terms include only the error in $^{18}$O exchange measurements.
transport chain of denitrification for exactly the same reason that the ratio of reduced to oxidized species in the electron transport system of oxidative metabolism is influenced by pO2. A decrease in [R] with increasing [NO3-] has been demonstrated in both whole cells (21) and crude extracts (22) of Paracoccus denitrificans. Thus, we expect [R] to decline with increasing [NO3-]. Measurement of \( \nu_{\text{SW}}/V_{\text{OBS}} \) permits determination of the relationship between \( P \) and \( N \) (Equation 35 of the Miniprint):

\[
N = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}
\]

where

\[
a = 1,
\]

\[
b = \frac{1}{P} \left( 1 - P \left( \frac{V_{\text{OBS}}}{\nu_{\text{SW}}} + 1 \right) + 1 \right),
\]

and

\[
c = - \frac{V_{\text{OBS}} (1 - P)}{\nu_{\text{SW}} P}.
\]

Since \( P \) and \( N \) necessarily lie between 0 and 1 (see definition above) and since \( b > 0 \), only one root of this equation is physically possible. Substituting this expression into the equation for \( \beta_{\text{obs}} \) gives an expression which contains only intrinsic isotope effects, the constant \( P \), and measured parameters. The above equation for \( N \) shows that, for Mechanism I, at any given value of \( P \), \( N \) is inversely related to \( \nu_{\text{SW}}/V_{\text{OBS}} \). Because \( N \) is necessarily inversely related to [NO3-] for Mechanism I (since [R] decreases as [NO3-] increases), \( \nu_{\text{SW}}/V_{\text{OBS}} \) must necessarily increase with [NO3-]. Contrary to the prediction of Mechanism I, the mean measured value for \( \nu_{\text{SW}}/V_{\text{OBS}} \) at high [NO3-] was smaller than that at low [NO3-].

The remaining question is whether the error in the measured value of \( \nu_{\text{SW}}/V_{\text{OBS}} \) is large enough to encompass values of \( N \) which could lead to plausible values of the intrinsic isotope effects consistent with the observed variation in \( \beta_{\text{obs}} \). Examination of this question is described below.

Because isotopic fractionation is not expected to be associated with binding of NO3- to the enzyme or with enzyme-NO3 dissociation, \( \beta_1 \) and \( \beta_2 \) may be taken to be approximately equal to 1,000. Therefore, the equation for \( \beta_{\text{obs}} \) for Mechanism I may be rewritten as the following.

\[
\beta_{\text{obs}} = \frac{(1 - P)(1 - N)}{(1 - P)(1 - N) + N(1 - P) + NP}
\]

If the value of \( P \) and the two values of \( N \) corresponding to low and high [NO3-] were known, values of \( \beta_1 \) and \( \beta_2/\beta_3 \) could be obtained from this equation in order to determine whether these values are consistent with the constraint that intrinsic nitrogen isotopic fractionation rates are within a few percent of 1. Values of \( N \) can be obtained in terms of \( P \) and \( \nu_{\text{SW}}/V_{\text{OBS}} \) from Equation 35 of the Miniprint (shown above), but the data of the present paper give us no information about the value of \( P \). For this reason, we chose to examine the question at hand over the entire range of physically possible values of \( P \) (between 0 and 1) using combinations of values of \( \nu_{\text{SW}}/V_{\text{OBS}} \) which deviate from the experimental mean values for low and high [NO3-] within the constraints that the value of \( \nu_{\text{SW}}/V_{\text{OBS}} \) is greater at high [NO3-] (\( \nu_{\text{SW}}/V_{\text{OBS}} \) high nitrate) than at low [NO3-] (\( \nu_{\text{SW}}/V_{\text{OBS}} \) low nitrate) and that \( 0 < N < 1 \). To meet these constraints, it was necessary to assign values to \( \nu_{\text{SW}}/V_{\text{OBS}} \) low nitrate that were lower than the experimental mean and to assign values to \( \nu_{\text{SW}}/V_{\text{OBS}} \) high nitrate that were higher than the experimental mean. The combinations used encompassed 2 standard errors below the mean for \( \nu_{\text{SW}}/V_{\text{OBS}} \) low nitrate and 2 standard errors above the mean for \( \nu_{\text{SW}}/V_{\text{OBS}} \) high nitrate. For Mechanism I to account for the experimentally observed variation of \( \beta_{\text{obs}} \) with [NO3-], it is necessary that the parameter \( N \) also vary with [NO3-]. Fig. 4 shows the difference in values of \( N \) at low and high [NO3-] over the range of possible values of \( P \) calculated from these assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \) using Equation 35 of the Miniprint. As might be expected, the difference in values of \( N \) for the two NO3- concentrations increases with deviation of \( \nu_{\text{SW}}/V_{\text{OBS}} \) from the measured mean. Values of \( \beta_1 \) and \( \beta_2/\beta_3 \) were calculated from the measured values of \( \beta_{\text{obs}} \) at the two NO3- concentrations and the equation for \( \beta_{\text{obs}} \) given above using the same assigned combinations of values for \( \nu_{\text{SW}}/V_{\text{OBS}} \) at the low and high NO3- concentrations used for Fig. 4. The most plausible values for intrinsic isotopic fractionation factors of the \( \beta_{\text{obs}} \) equation were (predictably) obtained when the assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \) resulted in the largest difference in values of \( N \) for the two NO3- concentrations; namely, \( \nu_{\text{SW}}/V_{\text{OBS}} \) low nitrate --2 standard errors from the measured mean, and \( \nu_{\text{SW}}/V_{\text{OBS}} \) high nitrate =+2 standard errors from the measured mean. Fig. 6 shows the values of \( N \) as a function of \( P \) for the two calculated NO3- concentrations using these assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \), and Fig. 6 shows values of \( \beta_1 \) and \( \beta_2/\beta_3 \) for the same assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \). Fig. 6 shows that the most plausible values for \( \beta_1 \) and \( \beta_2/\beta_3 \) occur in the limit as \( P \to 0 \). (In the other limit, \( P \to 1 \), \( \beta_1 \) and \( \beta_2/\beta_3 \) drop out and

\[
P \to 1; \quad \beta_{\text{obs}} = \beta_1.
\]

That is, in the limit \( P \to 1 \), \( \beta_{\text{obs}} \) is invariant and equal to about 1,000.)

In the limit \( P \to 0 \), \( N \) may be calculated from Equation 34 of the Miniprint.

\[
P \to 0 \quad N = \frac{1}{\frac{2\nu_{\text{SW}}}{V_{\text{OBS}}} + 1}
\]

Thus, in the limit \( P \to 0 \), the values of \( N_{\text{low nitrate}} \) and \( N_{\text{high nitrate}} \) for assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \) low nitrate =--2 standard errors from the measured mean and \( \nu_{\text{SW}}/V_{\text{OBS}} \) high nitrate =+2 standard errors from the measured mean.

![Fig. 4. Difference in calculated values of \( N \) \( \left( N \approx \frac{k_2[R]}{k_3 + k_2[R]} \right) \) for Mechanism I for low and high [NO3-] as a function of \( P \) \( (P \approx \frac{k_3}{k_3 + k_2}) \) using assigned values of \( \nu_{\text{SW}}/V_{\text{OBS}} \) which are consistent with the constraints that \( \nu_{\text{SW}}/V_{\text{OBS}} \) for low [NO3-] be greater than \( \nu_{\text{SW}}/V_{\text{OBS}} \) for high [NO3-] and that \( 0 < N < 1 \). Assigned values for low and high [NO3-] were: --1 and +1 standard error from measured mean (curve a) --1 and +2 standard errors from measured mean (curve b), --2 standard errors from measured mean for low [NO3-] and the measured mean for high [NO3-] (curve c), --2 and +1 standard error from measured mean (curve d), --2 and +2 standard errors from measured mean (curve e).](image)
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}$O Exchange

FIG. 5. Calculated values of $N$ (\(N = \frac{k_5[R_i]}{k_3 + k_5[R_i]}\)) for Mechanism I for low and high [NO$_3$] as a function of $P (P = \frac{k_3}{k_3 + k_5})$ using assigned values of $v_{sw}/v_{om}$ of -2 (for low [NO$_3$]) and +2 (for high [NO$_3$]) standard errors from measured mean.

Errors from the measured mean are 0.9208 and 0.8741, respectively. Substituting these values into the equation for $\beta_{obs}$ yields values of 0.9930 and 1.2159 for $\beta_3$ and $\beta_4/\beta_3$, respectively. Thus, even in the unlikely event that the true mean of $v_{sw}/v_{om}$ was 2 standard errors below the measured mean at low [NO$_3$] and 2 standard errors above the measured mean at high [NO$_3$], $\beta_3$ and $\beta_4$ would need to be at least 200% different from each other to satisfy the equation. Such a difference between two nitrogen isotope effects is implausible.

This analysis is based on derivations which assume, for reasons previously stated, that step 5 is unidirectional. If this step and all subsequent steps up to release of NO$^-$ from the enzyme were freely reversible, which seems quite unlikely, $\beta_3$ and $\beta_4/\beta_3$, where $\beta_3$ and $\beta_4$ are isotope effects associated with steps 7 and 9 (Fig. 1) and $\beta_3$ and $\beta_4$, the isotope effects associated with the reverse reaction of steps 5 and 7. That is, $\beta_3/\beta_4$ would be equal to $\beta_3/\beta_4$. Even if this were the case, the 200% difference between $\beta_3$ and $\beta_4/\beta_3$ is implausible. On this basis, we consider Mechanism I to be inconsistent with experimental results.

Mechanism II—Unlike Mechanism I, Mechanism II provides a means by which $\beta_{obs}$ can vary with [NO$_3$] even though $v_{sw}/v_{om}$ is very small. Since NO$_3$ enters the reaction sequence twice (once at step 1, enzyme-substrate association, and again at step 5, the attack of NO$_3^-$ on $E\cdot$NO$^+$), isotope effects associated with steps beyond step 3 can be expressed even if step 1 or (and) step 3 is unidirectional. This can be seen formally in Equation 24 of the Miniprint (shown below), [NO$_3$] enters the expression for $\beta_{obs}$ explicitly in term $B$ (defined below). Moreover, $[R_i]$, which varies with [NO$_3$], is also explicitly included in the expression for $\beta_{obs}$ in the partition factor, $Q$ (defined below):

$$\beta_{obs} = \frac{2\Pi A(A + B)}{A(1 + \frac{[R_i]}{k_4}Q(1 + \frac{k_1}{k_3})) + \frac{\beta_1\beta_3}{\beta_3\beta_4}(A + B)}$$

where

- $[S] = [NO_3]$,
- $Q = \frac{\beta_4}{\beta_3}k_3$,
- $\Pi\beta = \frac{\beta_1\beta_3\beta_7}{\beta_3\beta_4\beta_5}$,
- $A = 1 - Q\left(1 - \frac{\beta_8}{\beta_7}\right)$,

and

$$B = \frac{\beta_6\beta_7k_4[NO_3]}{\beta_3\beta_4}Q\left(1 + \frac{\beta_4}{\beta_3}\right).$$

This equation for $\beta_{obs}$ contains the elements of three partition factors:

$$P = \frac{k_3}{k_3 + k_5},$$

$$N' = \frac{k_5[R_i]}{k_4 + k_5[R_i]},$$

and

$$Q = \frac{\beta_4}{k_3 + k_5[R_i]}.$$

B may be rewritten as follows.

$$B = \frac{\beta_6\beta_7N'Q}{\beta_3\beta_4}(1 - N')\left(1 + \frac{\beta_8}{\beta_7}\right).$$

Equation 52 of the Miniprint (shown below) relates $N'Q/(1 - N')$, $P$, and $v_{sw}/v_{om}$:

$$\frac{N'Q}{1 - N'} = \frac{-b' + \sqrt{b'^2 - 4a'c'}}{2a'}$$

where

- $a' = 1$,
- $b' = 1 - \frac{P - \frac{v_{om}}{v_{sw}}}{P - \frac{v_{om}}{v_{sw}}}$,

and

$$c' = \frac{v_{om}}{v_{sw}}(1 - P).$$

Using this expression together with the expression for $\beta_{obs}$, we can examine the consistency of experimental results with predictions from Mechanism II in the two limits $P \to 1$ and $P \to 0$. Given the assumption that $\beta_3$ and $\beta_2$ are 1.000, in the limit $P \to 1,$

$$\beta_{obs} = \frac{2 - 2Q\left(1 - \frac{\beta_8}{\beta_7}\right)}{1 - Q\left(1 - \frac{\beta_8}{\beta_7}\right)} + \frac{\beta_6}{\beta_3\beta_4}.$$
In the limit just discussed ($P \to 1$), the term $N'/Q/(1 - N')$.

Bryan et al. (7) found that in the limit as [NO$_3^-$] approaches 0, $\beta_{obs}$ approached 0.9955 $\pm$ 0.0055 and in the limit as [NO$_3^-$] approaches $\infty$, $\beta_{obs}$ approached 1.0245 $\pm$ 0.0032. Since $Q$ is constrained to lie between 0 and 1, $\beta_0 < 0.991$ and $\beta_0/\beta_b > 1.051$ in the limit $P \to 1$ if Mechanism II is correct, that is, $\beta_0$ is required to be in the limit. In the case $P \to 1$ if Mechanism II is correct. Step 5 is a bond-making step, a nitrogen–nitrogen bond being formed as NO$_2^-$ reacts with E$\cdot$NO$_2^-$ to form E$\cdot$N$_2$O$_4$. An inverse isotope effect for a bond-making step is entirely consistent with theory (see, for example, Ref. 23). For Palding bond order $>0.5$ of the forming bond in the transition state, theoretical calculations for carbonoyl addition reactions predict an inverse isotope effect (see Fig. 4 in Ref. 24). Inverse nitrogen atom isotope effects for bond-making reactions are, in fact, observed, for example, in the alalnine dehydrogenase-catalyzed reductive amination of pyruvate to alanine and in the reductive amination of $\alpha$-ketoglutarate to glutamate catalyzed by glutamate dehydrogenase.7

By using the values of $\beta_{obs}$ high and low [NO$_3^-$] (2.290 and 0.107 mM) reported here and assigning values of 0.99 to $\beta_0$ and 1.053 to $\beta_b/\beta_0$ (i.e. assigning the value of 1.064 to $\beta_b/\beta_0$), $Q_{low}$ (the value of Q at low [NO$_3^-$]), calculated from the above equation, is 0.517, and $Q_{high}$ (the value of Q at high [NO$_3^-$]) is 0.192. Assigning the same values to $\beta_0$ and $\beta_0/\beta_b$ and using the extrapolated values of $\beta_{obs}$ as [NO$_3^-$] $\rightarrow 0$ and [NO$_3^-$] $\rightarrow \infty$ reported by Bryan et al. (7), the values for $\beta_0$ as [NO$_3^-$] approaches 0 and infinity are 0.978 and 0.659, respectively. Thus, all of the calculated values of $Q$ lie between 0 and 1 and decrease with [NO$_3^-$], as required by the decline of [R$_0$] as [NO$_3^-$] increases. These results are summarized in the upper portion of Table V.

The values are examples only.

| NO$_3^-$ | $\beta_{obs}$ measured | Assigned | Q calculated |
|----------|------------------------|----------|--------------|
| mM       | $\beta_b$ | $\beta_0$ | $\beta_0/\beta_b$ |
| 0        | 0.9955$^5$ | 0.9900 | 1.0640 | 0.978 |
| 0.107    | 1.0100$^7$ | 0.9900 | 1.0610 | 0.517 |
| 2.290    | 1.0208$^7$ | 0.9900 | 1.0620 | 0.192 |
| $\infty$| 1.0245$^5$ | 0.9900 | 1.0650 | 0.0059 |

*Step 2 limits the entry of oxygen from H$_2$O into NO$_3^-$; 1. From Ref. 6. The calculation for the limit [NO$_3^-$] $\rightarrow \infty$ involves the assumption that [R$_0$] does not approach 0 as [NO$_3^-$] approaches infinity.

7From this study.

8Step 4 limits the entry of oxygen from H$_2$O into NO$_3^-$.

9This analysis is based partially on measurements of incorporation of NO from H$_2$O into substrate NO$_3$ provided that oxygen atoms from H$_2$O are not incorporated into intermediates in protonation-dehydration steps later than step 3, additional information concerning the relative rates of the forward and reverse steps of reduction of NO$_2$ to NO could be obtained by measuring both $v_{low}$ and $v_{high}$ and the ratio of the rate of entry of oxygen atoms from H$_2$O into the product of the reaction ($v_{low}$) to the rate of entry of oxygen atoms from all sources into the product (v$_{high}$), as discussed in the third section of the Miniprint. Specifically, for Mechanism I, values for both N and P could be obtained. For Mechanism II, values for both P and $N'/Q/(1 - N')$ could be obtained.

P *** $\rightarrow 0$ $\cdot N'/Q/(1 - N')$ ***
Denitrification Mechanism: Nitrogen Isotope Effect and 18O Exchange

nitrogen isotopic fractionation data in combination with 18O exchange data are consistent with Mechanism I and are consistent with Mechanism II.

Two issues need to be addressed concerning the viability of Mechanism II. First, it is well known that NO can serve as sole substrate in P. stutzeri and other denitrifying microorganisms for the enzymatic reduction to N2O (see Refs. 2, 5, 26, and 27), albeit at a reduced rate. Aerssens et al. (5) pointed out that this need not be taken as evidence against Mechanism II. They proposed that NO may be oxidized to NO+ and then converted to NO2- by way of steps 4 and 2 (Fig. 2). This NO2- would then be available for entry into the pathway at step 5. Data reported in this paper show that the rate of back-reaction of $E$.NO+ to free NO2- is very slow in P. stutzeri compared to the overall rate of reduction (<10%). However, it may be fast enough to support the suggestion of Aerssens et al. (5). Garber and Hollocher (27) reported that the rate of NO reduction was less than 25% of the rate of NO2- reduction in P. stutzeri. If Mechanism II is correct, only half of the nitrogen in the product (N2O) would need to be derived from NO2- in the presence of NO as the sole substrate, the other half being derived from the oxidation of NO to E. NO+. Thus, the rate of reduction of NO relative to the rate reduction of NO2- (<25%) observed by Garber and Hollocher is reasonably consistent with a 10% return of enzyme-bound nitrogen to the free NO2- pool. Moreover, oxidation of E.NO to E.NO+ might cause the concentration of E.NO+ to be increased relative to its concentration in the absence of NO. Mass action might well increase the rate of the reverse reaction, yielding more NO2- for entry into the reaction at step 5 than would be expected on the basis of the rate of formation of NO2- from E.NO+ in the absence of NO. (On the other hand, the net oxidation of NO to E.NO2- would have to take place in the reducing environment responsible for the production of N2O.) It is also possible that NO is reduced in a separate enzymatic process. An enzyme apparently distinct from nitrite reductase which reduces NO has been reported in at least one denitrifying microorganism (28).

A second issue arises from the observation that the major product of reduction of NO2- by cell-free extracts and partially purified nitrite reductase of most denitrifying organisms is N2 (29). Moreover, in whole cell experiments, Goretzki and Hollocher (3) succeeded in trapping NO with hemoglobin, indicating that at least some fraction of NO2- is reduced by a pathway in which NO is a free intermediate, yet NO is not an intermediate in Mechanism II. Averill and Tiedje (4) proposed two competing pathways of reaction in the forward direction of the intermediate, E.NO+ namely, attack on this intermediate by NO2- followed by reduction versus decomposition to NO (i.e. Fe3+ . NO2- → Fe2+ . NO → NO + Fe3+). They considered the first pathway to predominate in vivo, but pointed out that disruption of the cell and partial purification of the enzyme could so alter the environment of the enzyme that the ratio of the rates of the two pathways could well be inverted. A second possible competition for an intermediate of Mechanism II which would yield NO is the decomposition of E.N2O3. The chemical precedent for this suggestion is the dissociation of free N2O3 to NO + NO2 (30). In the context of denitrification, the N2O3 would be bound to the enzyme by

In the experiments of Goretzki and Hollocher (3), a large fraction of the nitrogen reduced (about 30%) in the case of P. stutzeri was trapped as HbNO. However, if there are three competing fates of ENO+ (namely the reversal of E.NO2- to ENO+, E.NO+ to E.N2O3, and E.NO- to NO), the presence of Hb might well increase the relative success of the third fate, since NO + Hb → HbNO is irreversible.

M. Doyle, personal communication.
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}\text{O}$ Exchange

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SUPPLEMENTARY MATERIAL TO NITROGEN ISOPTE FRACTIONATION AND INTERCHANGE IN RELATION TO THE MECHANISM OF DENITRIFICATION OF NITRITE BY POND-TIMBERWASHTH

Fig. S1: A schematic representation of the mechanism of denitrification of $^{15}$NO$^{-}$ proposed by Garber and Hollocher (1970). Symbols used in the derivations are shown in this diagram. Fig. 1 of the main article is a flow diagram for the same mechanism. The present paper adds the fact that the ratio of nitrogen oxides and nitrite from hydrogen isotope exchange. One important point shows the ratio of the isotope enrichment of the nitrogen oxides to be highly variable. In the case of the use of $^{15}$NO$^{-}$ in nitrite, results from the paper show that the ratio of the nitrogen from the isotope enrichment of the nitrogen oxides to be highly variable. This is often used in the derivations of the nitrogen oxides to be highly variable. This will allow an examination of the behavior of $\dot{\phi}$, where the ratio of the hydrogen isotope enrichment of the nitrogen oxides at least the rate of uptake of $^{15}$NO$^{-}$ from $^{15}$NO$^{-}$ as well as the case in which the uptake of $^{15}$NO$^{-}$ is limited by the rate of uptake of substrate decomposition.

B. MECHANISM II

Fig. S2 shows diagrams for the mechanism (Mechanism II) proposed by Amend and Tiedje (1984) modeled to show how $S^+$ (addition of NO$^{-}$ to $^{15}$NO$^{-}$) is converted to NO$^{-}$ and NO$^{-}$ to $^{15}$NO$^{-}$, all of which are not seen in Mechanism I. However, some of the steps of the reaction are more complicated than Mechanism I, because they engage the enzyme nitrate reductase. In the case of Mechanism II, the enzyme nitrate reductase is not involved in the reaction and the ratio of substrate uptake. In the case of Mechanism II, the enzyme nitrate reductase is not involved in the reaction and the ratio of substrate uptake. In the case of Mechanism II, the enzyme nitrate reductase is not involved in the reaction and the ratio of substrate uptake. In the case of Mechanism II, the enzyme nitrate reductase is not involved in the reaction and the ratio of substrate uptake. In the case of Mechanism II, the enzyme nitrate reductase is not involved in the reaction and the ratio of substrate uptake.
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}$O Exchange

Employing the assumption that the concentrations of all the enzyme bound intermediates are constant leads to the result that

$$\text{(1) } \frac{d[N]}{dt} = k_{+} [S] [E]_0 - k_{-} [N] [E]_0$$

$$\text{(2) } \frac{d[S]}{dt} = k_{+} [S] [E]_0 - k_{-} [S] [E]_0$$

$$\text{(3) } \frac{d[E]}{dt} = k_{+} [S] [E]_0 - k_{-} [S] [E]_0$$

In deriving the above equations, $[E]_0$ is the buffer concentration and $[E]_0$ were included in the rate constants $k_{+}$ and $k_{-}$, respectively. The terms $\frac{k_{+}}{k_{-}} [S]_0$ and $\frac{k_{+}}{k_{-}} [E]_0$ were dropped since $k_{+} [S]_0 = k_{-} [S]_0$, and $k_{+} [E]_0 = k_{-} [E]_0$.

Substituting [E]_0 into [E]_0 and [E]_0 into the resulting versions of [E]_0 and [E]_0 into the resulting version of [E]_0 and this final version of [E]_0, the rate equation for the disappearance of NO is

$$\text{(4) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

Unlike the case for the rate of disappearance of NO, the rate of disappearance of NO, represented by $k_{+} [S]_0$, may not be equal to $k_{+} [S]_0$. This is because the different effects on isotope fractionation in the last two reactions are shown in the lower part of Fig. 2. This is the rate of production of two products, $\text{(1)}$ and $\text{(2)}$, which may be different. The net rate of change of $\text{(1)}$ at step $\text{(1)}$ and $\text{(2)}$ at step $\text{(2)}$ is equal to $\frac{k_{+}}{k_{-}} [S]_0$ and the net rate of change of $\text{(1)}$ at step $\text{(1)}$ and $\text{(2)}$ at step $\text{(2)}$ is equal to $\frac{k_{+}}{k_{-}} [S]_0$.

Thus,

$$\text{(5) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

While this is a formidable challenge, the approach to obtaining a useful expression for $\frac{d[N]}{dt}$ is to consider the approach to obtaining the expression for $\frac{d[N]}{dt}$ in the context of the concentration of the enzyme bound intermediates, which constant leads to

$$\text{(6) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

$$\text{(7) } \frac{d[S]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

$$\text{(8) } \frac{d[E]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

Using the relationship $\frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$, the equation for the rate of disappearance of NO can be written as

$$\text{(9) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

Note that $\frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$, due to the mechanism and the fact that $k_{+} [S]_0 = k_{-} [S]_0$, $k_{+} [E]_0 = k_{-} [E]_0$. Using the fact that $k_{+} = k_{-} [S]_0$, we have

$$\text{(10) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

and rearranging leads to

$$\text{(11) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

These equations (9) and (10) are obtained with a stoichiometry term $\text{(11)}$. Using equations (11) and (10), we have

$$\text{(12) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

with $\frac{k_{+}}{k_{-}} [S]_0$ and $\frac{k_{+}}{k_{-}} [E]_0$ one of the denominator and numerator in the equation for the rate of change of $\text{(11)}$.

$$\text{(13) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

Substituting equations (11) and (10) into equations (12) and rearranging gives

$$\text{(14) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

$$\text{(15) } \frac{d[N]}{dt} = \frac{k_{+}}{k_{-}} [S]_0$$

The rate of disappearance of NO from $\text{(11)}$ is equal to $\frac{k_{+}}{k_{-}} [S]_0$, and the rate of disappearance of NO from $\text{(10)}$ is equal to $\frac{k_{+}}{k_{-}} [S]_0$.
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}$O Exchange

Multiplying both sides of this equation by $(k_3 + k_4)k_6$ and adding the common denominator of the left side gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} + \frac{k_6}{k_7} \frac{(k_3 + k_4)k_6}{(k_3 + k_4)k_6 + k_3} \]

(38)

Therefore, the expression for the steady state concentrations of $E^\ast S_2$ and $E^\ast S_3$ differ for Mechanism II because these expressions can be solved for step 5 which is absent in Mechanism I.

\[ E^\ast S_2 = \frac{k_6}{k_7} + \frac{k_6}{k_7} \frac{(k_3 + k_4)k_6}{(k_3 + k_4)k_6 + k_3} \]

(39)

Substituting $k_3 = 0$ into (38), we get:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(40)

The steady state concentration of $E^\ast S_2$ is given by:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(41)

The steady state concentration of $E^\ast S_3$ is given by:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(42)

Substituting equations 23, 42 and 43 into 41 gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(43)

Rearranging gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(44)

The branched term on the left side of the equation is equal to:

\[ k_6(k_3 + k_4) + k_7(k_3 + k_4) + k_3 \]

(45)

Multiplying both sides of the equation by $k_3 + k_4$ and adding the common denominator of the left side gives:

\[ \frac{k_6}{k_7} \]

(46)

Substituting this result into the above equations containing $E^\ast S_2$ gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(47)

Equation 35 contains constant intrinsic isotope effects, the measured parameter $P_{sub}$ for the parameter $P$, and $N$, which is expressed in terms of $E^\ast S_2$ because a constant term $E^\ast S_2$ which decreases as $E^\ast S_2$ increases (see discussions in the main text). Equations 35 may be substituted into eqn 36 to give an expression for the mechanism I, which contains only constants and measured parameters, in order to examine the consistency of Mechanism I with experimental data. The result of this examination is given in the main text.

**B. MECHANISM II**

As it is the case of Mechanism I, for the rate at which oxygen atoms leave the NO$_3^-$ pool is equal to $v_{o_{2}} = k_3$.[*E$^\ast S_2$]* = $k_3$.[*E$^\ast S_3$]* = $k_3$.[*E$^\ast S_4$]*. The steady state concentrations of $E^\ast S_2$ and $E^\ast S_3$ are likewise given by the same expressions as for Mechanism I.

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(48)

In finding an expression for the ratio of the rate at which oxygen atoms leave the NO$_3^-$ pool $v_{o_{2}}$ to the rate at which oxygen atoms leave the free NO$_3^-$ pool $v_{o_{2}}$, the latter velocity is twice the rate at which NO$_3^-$ is reductase, or rate $P_{sub}$. In the case of Mechanism II, $P_{sub}$ is given in equation 13 of this supplement. Notice that the denominator of this equation (shown below) is equal to $d$ as defined above:

\[ v_{o_{2}} = \frac{k_6}{k_7} \]

(49)

Dividing eqn 37 by 48 gives:

\[ \frac{k_6}{k_7} = \frac{k_6}{k_7} \]

(50)

Substituting eqn 38 to 37, then eqn 23 and 24 into the result of the first substitution gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(51)

The steady state concentration of $E^\ast S_3$ is given by:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(52)

Substituting eqn 38 to 37, then eqn 23 and 24 into the result of the second substitution gives:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(53)

Rearranging gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(54)

The branched term on the left side of the equation is equal to:

\[ k_6(k_3 + k_4) + k_7(k_3 + k_4) + k_3 \]

(55)

Multiplying both sides of the equation by $k_3 + k_4$ and adding the common denominator of the left side gives:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(56)

Substituting equations 23, 42 and 43 into 22 gives the following expressions for $E^\ast S_2$:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(57)

Notice that the denominator of this equation (shown below) is equal to $d$ as defined above:

\[ v_{o_{2}} = \frac{k_6}{k_7} \]

(58)

Dividing eqn 37 by 48 gives:

\[ \frac{k_6}{k_7} = \frac{k_6}{k_7} \]

(59)

Substituting eqn 38 to 37, then eqn 23 and 24 into the result of the first substitution gives:

\[ E^\ast S_2 = \frac{k_6}{k_7} \]

(60)

The steady state concentration of $E^\ast S_3$ is given by:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(61)

Substituting eqn 38 to 37, then eqn 23 and 24 into the result of the second substitution gives:

\[ E^\ast S_3 = \frac{k_6}{k_7} \]

(62)
Denitrification Mechanism: Nitrogen Isotope Effect and \(^{18}O\) Exchange

\[
\begin{align*}
\text{Denitrification Mechanism: Nitrogen Isotope Effect and } ^{18}O \text{ Exchange} \\
\text{Substituting equations 28 and 29 into the terms of the equation, we find that} \\
\text{This equation implies that} \\
\text{Also from equations 28 and 29,} \\
\text{Substituting these into the expression for } \nu_{\text{NH}_3}\text{ and rearranging gives} \\
\text{This equation may be written in terms of the two parameters,} \\
\text{Additional information could be obtained by measuring, in the same experiment, both } \nu_{\text{NO}}\text{ and the ratio of the} \\
\text{Reading out of } P, N, Q, \text{ and } R \text{ gives} \\
\text{Equation 49 contains elements of the three separate factors of Mechanism I} \\
\text{Also from equation 21 it can be seen that } A \text{ was defined as } \gamma - Q E. \\
\text{It can be rewritten as} \\
\text{If the ratio of } P, N, \text{ and } Q \text{ is defined as} \\
\text{Equation 52 can be rewritten into eq 53 to obtain an expression for } \nu_{\text{NH}_3}, \text{ which contains only constants and} \\
\text{Methane I} \\
\text{It is possible to determine the actual values of } P \text{ and } N \text{ under any given set of conditions (e.g., different substrate concentrations) in the case of Mechanism I. To do this, we start by writing an expression for } \nu_{\text{NO}} \\
\text{where } k_1 \text{ (as calculated in Table 1) is the rate constant for the non-autoionization reaction of NO to form the product } \\
\text{N}_2O, \text{ and } k_{\text{NO}} \text{ is the rate constant for the oxygen exchange reaction from water. From this, we can write an expression for } \\
\text{Equation 54 represents the ratio of } \nu_{\text{NH}_3} \text{ to } \nu_{\text{NO}}. \\
\text{The expression for } \nu_{\text{NH}_3} \text{ and } \nu_{\text{NO}} \text{ in terms of the two parameters,} \\
\text{This equation expresses the ratio of } \nu_{\text{NH}_3} \text{ to } \nu_{\text{NO}} \text{ in terms of the two parameters,} \\
\text{In the absence of } \nu_{\text{NH}_3} \text{ and } \nu_{\text{NO}} \text{ is} \\
\text{Solving this equation simultaneously with eq 53 gives expressions for } P \text{ and } N \text{ for any given set of values of } \\
\text{Methane II} \\
\text{The specific activity of the chemical intermediates beyond the first accidental step following the entry of the} \\
\text{This is not the case for oxygen exchange. The product of the 2 electron reduction of } \text{E}(\text{NO}) \text{ (shown as } E(\text{NO})\text{ in Fig 8b) is} \\
\text{Although } E(\text{NO}) \text{ is shown as } E(\text{NO})\text{ in Fig 8c and 8d, and two oxygen atoms are bound in the second } \\
\text{In the case of nitrous oxide, } E(\text{NO}) \text{ is assumed to be zero in the rate constant for the reduction of } \text{E}(\text{NO})\text{ to } \\
\text{This is not the case for oxygen exchange. The product of the 2 electron reduction of } \text{E}(\text{NO}) \text{ (shown as } E(\text{NO})\text{ in Fig 8b) is} \\
\text{The expression for } \nu_{\text{NH}_3} \text{ and } \nu_{\text{NO}} \text{ in terms of the two parameters,} \\
\text{In this case, } \nu_{\text{NH}_3}/\nu_{\text{NO}} \text{ will be dependent on both time and } \\
\text{The derivation given below is for the first condition (only one oxygen atom from } \text{H}_2\text{O in active } \text{NO}\text{ pool).} \\
\end{align*}
\]
Denitrification Mechanism: Nitrogen Isotope Effect and $^{18}$O Exchange

Using this equation with eqns. 14 and 16 gives

$$\frac{N_{NO}}{N_{HNO}} \cong \frac{k_1(k_2 + k_{13}R_2) + k_{12}R_2(k_1 + k_{13})}{k_{12}R_2(k_1 + k_{13}) + k_2(k_1 + k_{13})} \quad (58)$$

The rate at which oxygen atoms from all sources enter NO (r$_{NO}$) is one fourth the rate given in equation 40 at which oxygen enters from NO$_2$ (r$_{NO}$).

$$r_{NO} = \frac{1}{4} \frac{N_{NO}}{N_{HNO}} \quad (59)$$

Solving equation 58 by the above equation gives the rate of $r_{NO}/r_{NO}$.

$$r_{NO} = \frac{1}{4} \frac{k_1(k_2 + k_{13}R_2) + k_{12}R_2(k_1 + k_{13})}{k_{12}R_2(k_1 + k_{13}) + k_2(k_1 + k_{13})} \quad (60)$$

Dividing numerator and denominator by $k_1(k_2 + k_{13}R_2)$ gives

$$\frac{N_{NO}}{N_{HNO}} = \frac{k_1}{k_2 + k_{13}} \left( \frac{k_1(k_2 + k_{13}R_2) + k_{12}R_2(k_1 + k_{13})}{k_{12}R_2(k_1 + k_{13}) + k_2(k_1 + k_{13})} \right) \quad (61)$$

Dividing numerator and denominator by $k_2(k_1 + k_{13})$ gives

$$\frac{N_{NO}}{N_{HNO}} = \frac{1}{1 + \frac{Q}{P} \left( \frac{k_1}{k_2 + k_{13}} \right)} \quad (62)$$

Substituting $P = \frac{k_1}{k_2 + k_{13}}$, $N = \frac{k_1}{k_2 + k_{13}}$ and $Q = \frac{k_1}{k_2 + k_{13}}$, into this and rearranging gives

$$\frac{N_{NO}}{N_{HNO}} = \frac{1}{1 + \frac{Q}{P} \left( \frac{k_1}{k_2 + k_{13}} \right)} \quad (63)$$

Solving the equation simultaneously with eqn. 51 gives expressions for $P$ and $Q$ for any given pair of values of $N_{NO}$/HNO and $N_{NO}$/HNO at any given [NO$_2$]. Mechanism II, like Mechanism I, requires that the value of $P$ be independent of [NO$_2$]. Mechanism II also requires that the value of $N$ increase with [NO$_2$] and that the value of $Q$ decrease with [NO$_2$], with the result that the ratio, $N_{NO}$/HNO, should increase with [NO$_2$], especially at very high values of [NO$_2$]. Calculated values of $N$ and $P$ can be evaluated into eqn. 53 to give an expression for $R_{NO}$ for mechanism II which contains only measured parameters and which can be evaluated. To solve this equation simultaneously for values of $N_{NO}$/HNO at all values of [NO$_2$], the use of Mechanism II requires measurements of $N_{NO}$/HNO and $N_{NO}$/HNO as [NO$_2$] is varied. $\beta_1$ and $\beta_2$ may be assumed to be equal to approximately 3.00 since, under the conditions of the experiment, the $\delta$-values of the oxygen are not expected to be accompanied by isotopic fractionations. Values of $\beta_1$, $\beta_2$, and $\beta_3$ (acetylene) are unknown.