Respiration-Dependent Proton Translocation and the Transport of Nitrate and Nitrite in *Paracoccus denitrificans* and Other Denitrifying Bacteria†

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ABSTRACT: Oxidant pulse experiments were performed on *Paracoccus* and *Pseudomonas denitrificans* grown anaerobically on nitrate to determine the stoichiometry of respiration-dependent proton translocation during denitrification. In the presence of valinomycin, the $^{-}H^{+}/2e^{-}$ ratio for oxygen was maximally 7–8 (external acidification), in agreement with the reports of others with aerobically grown *Paracoccus denitrificans*. The ratios observed upon the reduction of nitrate, nitrite, and nitrous oxide to nitrogen gas were maximally 4.3, 3.7, and 4.5, respectively. That for the reduction of nitrite to nitrous oxide in the presence of acetylene was about 3.3. These results are qualitatively consistent with previously reported growth-yield experiments on *Pseudomonas aeruginosa* and *Paracoccus denitrificans*. When valinomycin was omitted, respiration was partially inhibited (controlled), and the $^{-}H^{+}/2e^{-}$ ratio diminished to about 0.2 after several additions of oxygen or nitrous oxide. Under these conditions, the initial event observed upon the addition of nitrite or nitrate was a transient alkalinization with $^{-}H^{+}$/anion = −1 maximally. These alkaline transients were prevented by certain inhibitors of respiration and by dicyclohexylcarbodiimide but not weak acid-type uncouplers of oxidative phosphorylation. Under appropriate conditions the transient exhibited by nitrate showed a substructure in which an acid transient appeared between two alkaline transients. These results show that the uptake of nitrite and nitrate by denitrifiers is coupled with the uptake of one proton (or exit of one hydroxide ion) per anion, and that the site of their reduction is inside the cell or on the inner aspect of the membrane. The proton (or hydroxide) transporter involved is probably the membrane ATPase (Maloney, P. C. (1977) *J. Bacteriol. 132,* 564), inasmuch as it was blocked by dicyclohexylcarbodiimide and exhibited a threshold for function which seems to be related to the value of the membrane potential. The entry of nitrate and nitrite would appear to be by facilitated diffusion down concentration gradients created by their reductive destruction within the cell. Concerted uptake of a proton with each nitrate (nitrite) anion renders the uptake electrically neutral and also almost exactly compensates for the net alkalinization associated with denitrification of nitrate and nitrite.

Aerobically grown *Paracoccus denitrificans* has been reported to exhibit a respiration-dependent transport ratio for protons, $^{-}H^{+}/O$, of 7–8.5 during oxidation of endogenous substrates, L-malate, and succinate (Scholes & Mitchell, 1970b; Lawford et al., 1976; Meijer et al., 1977; Lawford, 1978). The ratio of 7–8.5 has been explained variously in terms of four sites of respiratory proton translocation with $^{-}H^{+}/2e^{-}$ = 2 at each site (Scholes & Mitchell, 1970b), two sites with ratio of about 4 per site (Van Verseveld et al., 1977) and three sites with ratio of about 3 per site (Lawford, 1978). $^{-}H^{+}/O$ ratios of 3.4–6.2, depending on growth conditions, were reported by Van Verseveld et al. (1977) for *Pa. denitrificans* grown on nitrate or nitrite anaerobically.

There are no reports of $^{-}H^{+}/2e^{-}$ ratios for nitrogen oxide respirations in denitrification-adapted denitrifiers. There is reason to believe, however, that proton translocation may be less efficient in the case of the nitrogen oxide respirations of denitrifiers. Koike & Hattori (1975a,b) have reported that oxygen is about twice as efficient per electron as nitrate, nitrite, and nitrous oxide in supporting growth of *Pseudomonas aeruginosa* and that there is little difference per electron among these nitrogen oxides. Similar results with *Pa. denitrificans* have been reported by Van Verseveld et al. (1977). John & Whatley (1970) reported a P/O ratio of about 1.5 and a P/NO_3 ratio of about 1 for phosphorylating subcellular particles derived from *Pa. denitrificans*. Assuming two-electron reduction to nitrite, these phosphorylation ratios suggest a difference of 1.5-fold in phosphorylation efficiency between oxygen and nitrate. We note, however, that spheroplasts of *Escherichia coli* exhibited $^{-}H^{+}/2e^{-}$ ratios of about 4 for both oxygen and nitrate ($NO_3^- \rightarrow NO_2^-$) respirations (Garland et al., 1975).

In this paper we determine $^{-}H^{+}/2e^{-}$ ratios for oxygen and nitrogen oxide respirations of *Ps.* and *Pa. denitrificans* grown anaerobically on nitrate. Also considered are the problems of pH change and charge compensation inherent in denitrification of the anions, nitrate and nitrite. If the anions were taken up prior to reduction, counterion movement would be expected to make uptake electrically neutral and proton (or hydroxide) movement would be expected to compensate for the net alkalinization caused by reduction of nitrate and nitrite to nitrogen gas. Our results demonstrate in fact that stoichiometric proton uptake accompanies the reduction of nitrite or nitrate in the near absence of permeant counterions and that the proton transporter, in its ability to be gated and to be inhibited by DCCD,[1] resembles the membrane ATPase involved in energy coupling and oxidative phosphorylation (Maloney, 1977). Our results also confirm the conclusion of John (1977) that the nitrate binding site of nitrate reductase in *Pa. denitrificans* is on the inner aspect of the membrane.

Materials and Methods

Materials. Special products were obtained as follows: CCCP, valinomycin, carbonic anhydrase, and KSCN from

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[1] Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; Tris, tris(hydroxymethyl)-aminomethane.

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Sigma; DCCD from Eastman Kodak. Stock solutions of CCCP, DCCD, and valinomycin were prepared in ethanol.

Microbiology. The denitrifiers used were prototrophs of *Pseudomonas denitrificans,* ATCC 13867, and *Paracoccus* (formerly *Micrococcus*) *denitrificans,* ATCC 19367. Cells were grown anaerobically from 2 to 5% inocula on 20 mM nitrate in a yeast extract medium at 30 °C as previously described by St. John & Hollocher (1977) and were harvested by centrifugation after having reached a cell density of 7–9 × 10^8 cells mL^{-1} in mid-exponential phase. Cells were then washed twice in the suspending medium to be used in a particular experiment. Harvesting and washing were carried out at 0–4 °C. Stock cultures were kept on yeast extract–agar slants at 4 °C and were transferred every 2 months. For routine use, cultures were carried on plates that were transferred every 2 weeks. Inocula for liquid cultures were used immediately after growth overnight. Cell concentrations were determined through use of a Klett colorimeter which was calibrated by cell counts on nutrient agar plates. Cells were grown aerobically by vigorous shaking in the same medium minus nitrate.

Denitrification. The production or uptake of nitrogen and gaseous nitrogen oxides was determined at 30 °C by gas chromatography as described by St. John & Hollocher (1977). To initiate denitrification, 10–20 μmol of nitrate, nitrite, or nitrous oxide was introduced by syringe into stoppered 9-mL serum vials containing 10^9 to 10^{10} cells in 1–2 mL of medium under an atmosphere of helium. Nitrite concentrations were determined spectrophotometrically at 540 nm by a diazotization method (Van't Riet et al., 1968). Reactions were stopped by injecting 50-μL aliquots into 1 mL of ethanol prior to analysis.

Oxygen Uptake. Oxygen uptake was monitored using a Clark oxygen electrode, Yellow Springs Instrument, Model 53. Typically 1–2 × 10^9 cells (30–100 μL of a dense cell suspension) were injected into 3 mL of aqueous solution previously equilibrated at 30 °C with air. Oxygen consumption was calibrated by the method of Chappell (1964).

Oxidant Pulse Experiments. These experiments measured the change in pH of the extracellular medium of a dense anaerobic suspension of bacteria in response to the injection of a small amount of oxidant, i.e., oxygen or a nitrogen oxide. The method used was a modification of that described by Scholes & Mitchell (1970b) and the apparatus, which had an overall response time of about 2 s, was the same as that described for acid pulse experiments (Walter et al., 1978). Cells were suspended in 150 mM KCl containing 80 μg mL^{-1} of carbonic anhydrase to concentrations of 2.5–5.5 × 10^{10} cells mL^{-1}. One milliliter of the suspension was allowed to equilibrate under argon at 25 °C for 30 min at which time the pH of the system was adjusted to a desired starting point of 6.8–7.0 (unless otherwise indicated) by the addition of anaerobic 50 mM NaOH or HCl in 150 mM KCl. Once the pH had stabilized, a solution of oxidant dissolved in 150 mM KCl was injected to provide 5–250 nmol of the oxidant, and the subsequent changes in pH were recorded. The response was calibrated by injecting HCl in anaerobic 150 mM KCl. When valinomycin or DCCD was present, the cells were previously incubated with 50 μg mL^{-1} of valinomycin or 1 mM DCCD for 3 h at 4 °C to allow these compounds to interact with the membrane. Cells were washed after incubation to remove residual DCCD. Oxidant was injected at 2–4-min intervals typically. The response in the case of valinomycin-treated cells was nearly constant and reproducible over at least 15 injections when the amount of oxidant per injection was kept constant. Exhaustion of endogenous substrates was thus not a serious problem. The response in the case of cells without valinomycin was found to be dependent on the number of prior injections, as described under Results.

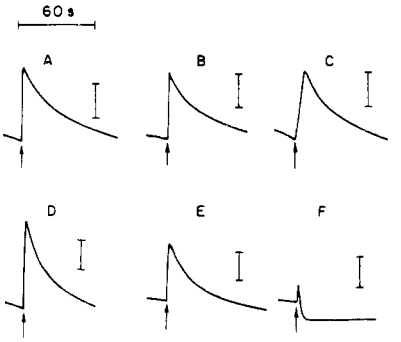

FIGURE 1: Transient acidification following the addition of oxygen or nitrogen oxides to valinomycin-treated *Pa. denitrificans.* Oxidant was injected into 3 × 10^{10} cells at the arrows and the bars correspond to 50 nmol of H^+. Acidification causes an upward deflection. (A) With 23 ng-atom of O_2; (B) 27 nmol of N_2O; (C) 20 nmol of KNO_3; (D) 37 nmol of KNO_2; (E) like D but under 1 atm of acetylene instead of argon; (F) like D but in the presence of 5 μM CCCP.

The initial rapid changes in pH induced by the injection of an oxidant were followed by a slow passive diffusion of protons across the cell membrane (Scholes & Mitchell, 1970a,b). This latter process tended to diminish the amplitude of the former rapid pH transient by perhaps 5–20% depending on conditions. To correct for this effect, the decay curve for passive proton diffusion was extrapolated back to a time at which the initial transient had reached half its final amplitude, as suggested by Scholes & Mitchell (1970b).

The solubility of oxygen in 150 mM KCl was taken to be 0.235 mM under air at 25 °C (Chappell, 1964); that of nitrous oxide was taken to be 25 mM under 1 atm of nitrous oxide (*Merck Index,* 1968). Solutions of 150 mM KCl equilibrated under air, 1.2 atm of oxygen, or 1.2 atm of nitrous oxide were used.

Intracellular pH. The intracellular pH of dense cell suspensions, such as those used in oxidant pulse experiments, was estimated by a method described by Scholes & Mitchell (1970a) involving cell lysis. The method requires determination of the external buffering capacity of the cells, the total buffering capacity after cell lysis caused by 4% butanol, and the ΔpH realized as the result of lysis.

Results

Stoichiometry of Respiration-Driven Proton Translocation. As shown in Figure 1, *Paracoccus denitrificans,* which was grown anaerobically on nitrate and previously incubated with valinomycin, acidified the exterior phase rapidly in response to small additions of oxygen, nitrate, nitrite, and nitrous oxide. Oxygen is reduced to water and the nitrogen oxides to nitrous oxide in the presence of acetylene and to nitrogen otherwise (Balderston et al., 1976; Yoshinari & Knowles, 1976). The addition of 5 μM CCCP greatly diminished the observed amplitudes of the acid transients (Figure 1). The stoichiometry of proton translocation, $^{-}H^+/2e^-$, depended on the amount of oxidant added (Figure 2) with the true ratio being the limit when the amount of oxidant approaches zero (Scholes & Mitchell, 1970b). Larger amounts of valinomycin did not increase the ratios further. The $^{-}H^+/2e^-$ ratios obtained with oxygen, nitrate, nitrite, and nitrous oxide approached about 7.5, 4.3, 3.7, and 4.5, respectively, when extrapolated to zero amount of oxidant. That for the reduction of nitrite to nitrous oxide in the presence of acetylene was about 3.3 (data not shown).

The results with *Pseudomonas denitrificans* (data not

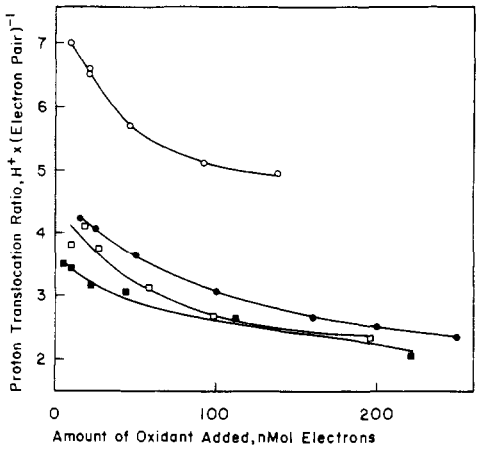

FIGURE 2: Correlations between the observed $^{-}H^{+}/2e^{-}$ ratio and the amount of oxidant added in oxidant pulse experiments with valinomycin-treated *Pa. denitrificans*. The number of cells used was 3 to 3.7 × 10^{10}. (○) O_2; (●) N_2O; (□) KNO_3; (■) KNO_2. Amplitudes for the transients were estimated by extrapolation as explained in Materials and Methods.

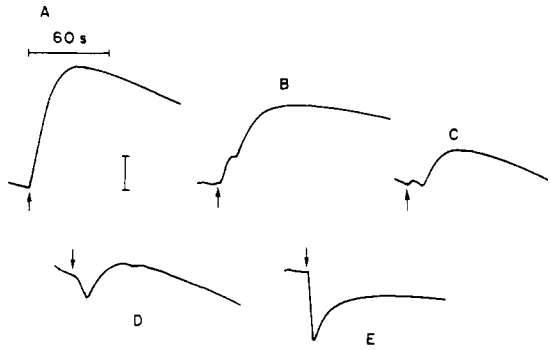

FIGURE 3: The response of *Pa. denitrificans* not treated with valinomycin to the first few pulses of KNO_2. At the arrows 74 nmol of KNO_2 was added to 5.25 × 10^{10} cells. The bar corresponds to 25 nmol of H^+ and acidification causes an upward deflection. Tracings A-E represent the responses at 3-min intervals beginning with the first pulse.

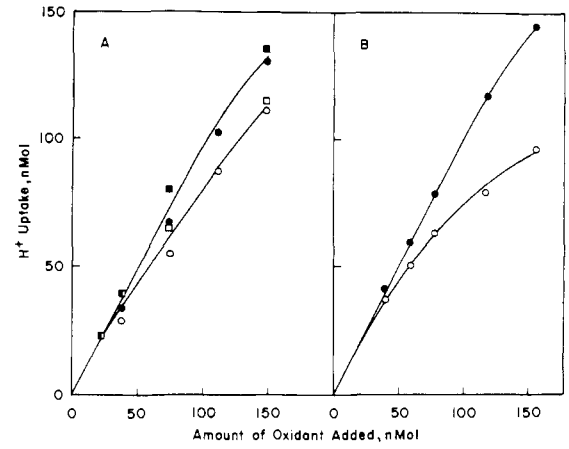

FIGURE 4: Alkaline transients following the addition of nitrite or nitrate to denitrifiers not treated with valinomycin as a function of the amount of the oxidant. (A) KNO_2; (B) KNO_3. Extrapolated response as described in the text (closed symbols) and observed response (open symbols) with 4.25 × 10^{10} *Ps. denitrificans* (circles) or 3 × 10^{10} *Pa. denitrificans* (squares). The cells had received 5 or 6 pulses over 10-15 min prior to the one used for the figure in order to maximize the alkaline transients. The slopes of the curves approach 1 when the amount of oxidant added is small, thus indicating a stoichiometry of one proton taken up for each nitrite or nitrate anion added.

shown) were virtually identical with those of *Pa. denitrificans* under comparable conditions. Valinomycin penetrates to the cell membrane more slowly in *Ps.* then in *Pa. denitrificans* and this necessitated a longer prior incubation with or a higher concentration of valinomycin in order to avoid the effects of membrane polarization.

Kinetics. We have shown previously that 10^{10} denitrifiers in nutrient broth can reduce nitrite, for example, at a maximum steady-state rate of 1-2 μmol min^{-1} (St. John & Hollocher, 1977; Walter et al., 1978). If the same rate were to apply in the oxidant pulse experiments, where endogenous substrates serve as reductants, then 50 nmol of nitrite added to 3 × 10^{10} cells would be exhausted in 0.5-1 s. Typically pulses in the 50-nmol range gave acidification rise times of 2-3 s using an instrument with a response time of about 2 s (Figure 1). The observations are therefore consistent with the idea that respiration proceeds at nearly maximal rates following oxidant pulses (Scholes & Mitchell, 1970b; Lawford et al., 1976).

Proton-Linked Uptake of Nitrate and Nitrite. It is well established that electron transport in bacteria in the absence of valinomycin (a potassium transporter (Pressman, 1976)) develops a membrane potential which both inhibits (controls) respiration and limits the extent of proton translocation (Scholes & Mitchell, 1970b; Harold, 1972). For example, oxygen or nitrous oxide produced $^{-}H^{+}/2e^{-}$ = ca. 0.5-0.8 for pulses in the range 10-100 nmol in both *Pa.* and *Ps. denitrificans*. Because the proton efflux required for purely capacitive charging of the bacterial membrane is too small to be easily observed in these oxidant pulse experiments,[2] most of the proton efflux in the absence of valinomycin must represent electrically neutral movement supported by trace amounts of endogenous permeant ions. To a large extent these permeant ions can be exhausted (temporarily relocated or sequestered) and proton efflux minimized ($^{-}H^{+}/2e^{-}$ = ca. 0.2) by administering several pulses of oxygen or nitrous oxide at 2-3 min intervals. Recovery to $^{-}H^{+}/2e^{-}$ = ca. 0.5-0.8 typically took 10 min with *Pa. denitrificans*. That suppression of proton efflux was not due to substrate exhaustion was determined by control experiments containing valinomycin, in which case proton efflux did not diminish upon repeated pulsing.

Remarkably, nitrite and nitrate pulses elicited responses quite different from that of the neutral oxidents in the absence of valinomycin. Repeated pulses of nitrite (Figure 3) or nitrate (data not shown) brought about an alkaline transient (proton influx) which ultimately dominated the response rather than simply the suppression of proton efflux. Changes in both amplitude and moment of onset (Figure 3) suggest the involvement of a threshold or gating mechanism for the alkaline transients. Once the alkaline transient was fully developed, the ratio $^{-}H^{+}$/anion approached −1 as the limit for both nitrite and nitrate (Figure 4). The limit of −1 was obtained by considering either the observed amplitude or the extrapolated amplitude at the halftime for the transient, according to the method described by Scholes & Mitchell (1970b) for acid transients. The size of the alkaline transients for nitrate tended to be slightly smaller than those for nitrite (Figure 4), because of the greater durations and different kinetics (see below) that occurred in the case of nitrate. To realize the limiting value of −1 for the alkaline transients it was necessary to use small amounts of nitrate.

[2] The electrogenic (capacitive) component of proton ejection for the experiment of Figure 3 can be calculated to be 8-16 nmol of H^+ to achieve 250 mV potential, assuming a membrane capacitance of 1-2 μF cm^{-2} (Bell et al., 1972) and a membrane area of 6 × 10^{-8} cm^2 per cell. This can be compared with an initial ejection of 80 nmol of H^+ (Figure 3A).

TABLE I: Correlation between Inhibition of Respiration and Decrease in Proton Translocation Events in *Pa.* and *Ps. denitrificans* in the Absence of Valinomycin.[a]

inhibitor	concn	organism	reductase activities toward				amplitudes of alkaline transients in response to		amplitudes of acid transients in response to	
			NO_3^-	NO_2^-	N_2O	O_2	NO_3^-	NO_2^-	N_2O	O_2
N_3^-	1 mM	*Ps.*	−	+++	+	+++	−	+++	++	+++
N_3^-	4 mM	*Ps.*	−	++	−	++	−	+++	+	++
CN^-	5 mM	*Ps.*	−	+	−	−	−	+	−	+
CN^-	10 mM	*Ps.*	−	−	−	−	−	−	−	−
CCCP	5 μM	*Ps.*	+++	+++	+++	+++	+++	+++	+++	+++
CCCP	5 μM	*Pa.*	−	+	+	++	−	++	++	++
CCCP	10 μM	*Ps.*	++	++	++	+++	+++	+++	+++	+++
acetylene	1 atm	*Ps.*	++	+++	−	+++	++	+++	−	+++
acetylene	1 atm	*Pa.*	++	+++	−	+++	++	+++	−	+++

[a] The oxidant pulse experiments were carried out as described in Materials and Methods and involved 2 to 4 × 10^{10} cells to which was added 20–40 nmol of KNO_3, 30–50 nmol of KNO_2, 125 nmol of N_2O, or 12–24 nmol of O_2. Data for the table were taken after 5 or 6 previous pulses. Reductase activities toward nitrogen oxides and oxygen were determined as described in Materials and Methods. +++, normal; ++, diminished by 30–50%; +, diminished by 70–90%; −, abolished.

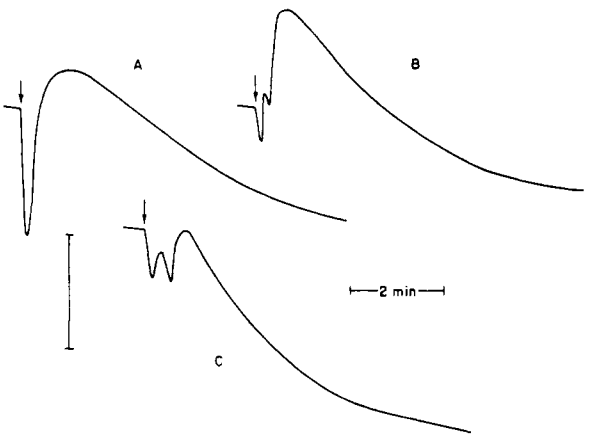

FIGURE 5: Transients following rather large additions of KNO_2 or KNO_3 to *Ps. denitrificans* not treated with valinomycin. Oxidant was injected into 4.5 × 10^{10} cells at the arrows. The bar corresponds to 50 nmol of H^+ and acidification causes an upward deflection. (A) With 125 nmol of KNO_2; (B) 125 nmol of KNO_3; (C) 250 nmol of KNO_3. The cells had received several previous pulses of KNO_2 over 15 min before the event displayed in A. A–C are sequential events.

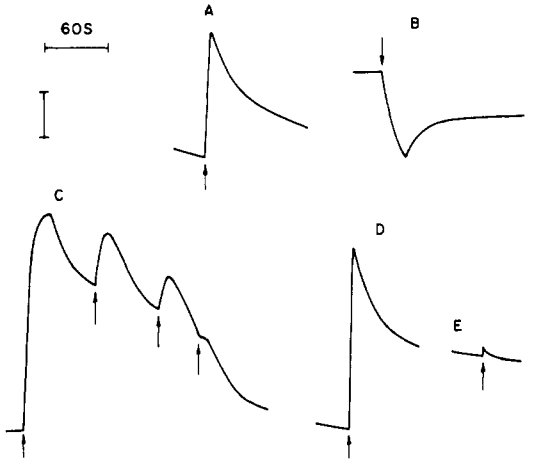

FIGURE 6: The response of *Pa. denitrificans* treated with DCCD to oxidant pulses. Oxidant was injected into 3.25 × 10^{10} cells at the arrows. The bar corresponds to 25 nmol of H^+ and acidification causes an upward deflection. (A) With 25 nmol of KNO_2; (B) 50 nmol of KNO_2 in the presence of 5 μM CCCP; (C) 125 nmol of N_2O at the first arrow (left) followed by three successive additions of 100 nmol of KNO_2; (D) 23 nmol of O_2; (E) 11 nmol of O_2 in the presence of 5 μM CCCP.

Nitrite showed sharp, simple alkaline transients from 10 to 150 nmol. Nitrate also showed sharp alkaline transients for small pulses (10–25 nmol), but with larger amounts (50–100 nmol) the transient became broader and often showed an inflection point or a rather flat broad minimum. Finally, as shown in Figure 5, amounts of nitrate greater than about 120 nmol initiated a complex response in which an acid transient appeared between two alkaline transients.

Table I illustrates the fact that inhibitors of respiration served to diminish or abolish the alkaline transients, but not the uncoupler, CCCP, except insofar as CCCP can also serve as an inhibitor of respiration. CCCP completely uncoupled respiration from endergonic processes in *Ps. denitrificans* at about 1 μM and inhibited nitrogen oxide respirations at concentrations above 10 μM in *Ps. denitrificans* and *Ps. aeruginosa* (Walter et al., 1978) and above about 3 μM in *Pa. denitrificans* (Table I). Chlorate, a slowly reduced analogue of nitrate (John, 1977), elicited neither an acid nor alkaline transient. Furthermore no alkaline transient was elicited by thiocyanate (a nonreducible, permeant anion) even when the solution contained dissolved oxygen which then served as an oxidant.

Treatment of denitrifiers with DCCD did not interfere with the weak acid transient caused by an initial nitrite pulse in the absence of valinomycin (Figure 6A) but completely abolished alkaline transients during a sequence of repeated pulses of nitrite (Figure 6C). The subsequent addition of CCCP, which virtually abolished proton efflux in the case of oxygen or nitrous oxide (Figure 6D vs. 6E), served to restore fully the alkaline transient produced by the addition of nitrite (Figure 6B). Thus DCCD can block the alkaline transients and CCCP can bypass the block. Analogous experiments could not be performed with nitrate because nitrate reduction is almost totally inhibited by DCCD treatment.

Internal pH during Oxidant Pulse Experiments. Under typical conditions (5 × 10^{10} cells mL^{-1} in 150 mM KCl with external pH 6.8–7.0 initially) the internal pH was 7.3–7.4 in resting cells. This increased to a limiting value of 7.6–7.7 following several additions of nitrate or nitrite in the 100-nmol range and at the same time the external pH increased by ca. 0.2 pH unit.

Discussion

An important observation in this study was the stoichio-

TABLE II: Relationship between the Possible Location of the Site of Reduction of Nitrate and Nitrite and the Concomitant Uptake of Protons from the Extracellular Medium.[a]

location of binding site for		proton uptake per anion reduced as the result of				possibility of an acid transient between two alkaline transients during the reduction of nitrate
nitrate reduction	nitrite reduction	oxidant	water formation[b]	transport	total	
outside	outside	nitrate	5	0	5	no
		nitrite	3	0	3	
outside	inside	nitrate	2	1	3	no
		nitrite	0	1	1	
inside	outside	nitrate	3	0	3	yes
		nitrite	3	0	3	
inside	inside	nitrate	0	1	1	yes
		nitrite	0	1	1	

[a] Consider the model in which nitrate and nitrite are reduced respectively outside and inside the membrane. External reduction of nitrate to nitrite consumes two protons and the transport of nitrite inside consumes a third. In the case where the respective sites of reduction are reversed, nitrate enters with one proton, is reduced internally to nitrite, and then nitrite exists with one proton for a net change of zero protons. Nitrite is now reduced externally to nitrous oxide in a reaction that consumes three protons. For all models, we assume that nitrous oxide is reduced internally and that the release or uptake of protons internally is not monitored by an external pH electrode. An acid transient between two alkaline transients arises by the proton-coupled efflux of nitrite down its concentration gradient following its production internally from nitrate. [b] For the complete reduction of nitrate or nitrite to nitrogen gas.

metric uptake of one proton (or release of one hydroxide ion) for each nitrate or nitrite anion reduced by *Pa.* and *Ps. denitrificans* under conditions favoring the development of appreciable membrane potentials (absence of valinomycin).

Alkaline Transients and the Sites of Reduction of Nitrate and Nitrite. It would appear that nitrate and nitrite are reduced inside denitrifiers or on the inner aspect of their membranes from two lines of evidence.

(1) The stoichiometry of the alkaline transient, $^{-}H^{+}$/anion = −1, for both nitrate and nitrite is consistent with their electrically neutral transport into the cells, but not with their external reductions which require 2 H^{+}/NO_3^{-} ($NO_3^{-} \rightarrow NO_2^{-}$), 3 H^{+}/NO_2^{-} ($NO_2^{-} \rightarrow \frac{1}{2}N_2O$), or 5 H^{+}/NO_3^{-} ($NO_3^{-} \rightarrow \frac{1}{2}N_2O$). In Table II are considered four models which differ depending on the location of the reduction sites for nitrate and nitrite. The model compatible with the data is the last one (inside/inside) where both nitrate and nitrite are reduced internally. The second model (outside/inside) could be compatible with the observed stoichiometry only if the nitrate reductase were also a respiratory proton pump as postulated by Garland et al. (1975) for the *E. coli* nitrate reductase. Then the external reduction of nitrate to nitrite need not consume net protons, whereas the transport of product nitrite would consume one proton. This possibility is ruled out by consideration of 2 below.

(2) Sequential events in the reduction of nitrate can be resolved kinetically into an initial alkaline transient followed by an acid and then by another alkaline transient (Figure 5). Only the third (inside/outside) and last model of Table II could show an acid transient between two alkaline transients. Thus this phenomenon establishes that nitrate reduction occurs inside. Only the last model is consistent with both 1 and 2. In the last model, the complex kinetics are rationalized as follows. Because nitrate is reduced internally to nitrite, nitrate enters down its concentration gradient accompanied by one proton per anion. Nitrite transiently accumulates in the cell and exits down its concentration gradient with one proton per nitrite anion (acid transient). Nitrite is known to accumulate from nitrate in the steady-state denitrification of nitrate (St. John & Hollocher, 1977; Payne, 1973). Internal conversion of nitrite to nitrous oxide and nitrogen reverses the concentration gradient of nitrite shortly thereafter and the remaining external nitrite enters with one proton per anion to cause the second alkaline transient of Figure 5. A similar set of events would occur in the third model, but the second alkaline transient would be expected to be much larger than the first, because it must arise as the result of water formation in the reduction of nitrite to nitrous oxide, a process which would consume 3 protons per nitrite anion rather than the observed value of one.

Because chlorate, a good substrate for respiratory nitrate reductases, lacks access to the enzyme in *Pa. denitrificans*, John (1977) concluded that the nitrate (chlorate) binding site lies on the inner aspect of the membrane and that nitrate enters the cell via a transporter which discriminates against chlorate. Our studies confirm these conclusions (also see below).

Gating the Proton Transporter. Figure 3 suggests that the uptake of protons with nitrite or nitrate is not an obligatory process and that proton uptake depends on some gating mechanism. It is also clear from the literature (Scholes & Mitchell, 1970a,b; Lawford et al., 1976; Walter et al., 1978) and from the slow pH relaxation following an oxidant pulse (Figures 1, 3, 5, and 6) that the passive proton permeability of denitrifiers is small. While the nature of the gating mechanism for proton transport is not established, the evidence suggests a connection with the membrane potential or total proton motive force.

(1) We have been unable to evoke an alkaline transient in systems treated with valinomycin which, because of electrogenic transport of potassium, acts to prevent the generation of a membrane potential. In these systems, potassium rather than protons (or hydroxide) would be expected to move with nitrate or nitrite.

(2) Alkaline transients are observed only when the capacity for electrically neutral proton ejection is repressed.

(3) The increase in intracellular pH (<0.1 unit) following an injection of 80 nmol of protons (Figure 3) is so small that internal pH alone seems to be a poor candidate for a gating signal.

(4) Respiration-dependent proton ejection clearly must be gated off when nitrite-dependent proton uptake is gated on in order to obtain the observed $^{-}H^{+}/NO_2^{-}$ ratio of −1. The simplest mechanism that would inhibit proton ejection momentarily but facilitate proton entry is the development of a

large transient membrane potential (negative inside).

(5) When CCCP is added to the system of Figure 3, an alkaline transient was observed upon the first addition of nitrite, just as one might expect from the action of CCCP as a proton transporter (Mitchell, 1966).

(6) Cells that have been repeatedly pulsed with oxygen or nitrous oxide evoke an alkaline transient upon the first addition of nitrite a few minutes later.

The Membrane ATPase as the Proton Transporter. The gating of proton cotransport by what appears to be the membrane potential and its blockage by DCCD (Figure 6) suggest that the proton transporter observed in denitrifiers is the membrane ATPase (EC 3.6.1.3) involved in oxidative phosphorylation (Beechey et al., 1967; Maloney, 1977).

Maintenance of Intracellular pH during Denitrification. Concerted movement of protons with oxidant anions also assists in maintaining intracellular pH. The internal proton demands for water production during the reduction of nitrate and nitrite anions to nitrogen are 1.2 and 1.33 H^+/e^-, respectively, whereas those for nitrous oxide and oxygen are both 1 H^+/e^-. The compensatory release of protons upon substrate oxidation is about 1 H^+/e^-.[3] Because the reductions of nitrate and nitrite are five- and three-electron processes, respectively, their reductions would create an internal proton deficiency of about 1 H^+/anion. This deficiency would be largely compensated by any mechanism that allows the uptake of one proton per anion.

Facilitation of Nitrate (Nitrite) Uptake. The kinetics of nitrate (nitrite) reduction suggest that transport of these ions across the membrane must be facilitated. Typically 50 nmol of nitrate was exhausted even in the absence of valinomycin within 3 s by 5×10^{10} cells in 1 mL. Assuming an average flux of 17 nmol s^{-1} during uptake and a favorable difference in chemical potential equivalent to 100 mV (favorable activity gradient but opposing electrical potential), the effective ion conductance is 6 μmho cm^{-2}. The average concentration of nitrate is taken to be 25 μM. Because conductance is proportional to concentration, nitrate conductance would be 24 mmho $\times$ cm^{-2} at 100 mM nitrate; but the corresponding conductance across soya bean phospholipid black membrane (unpublished data of C. Miller) is about 10^6 times smaller. The existence of a nitrate transporter in *Pa. denitrificans* is also indicated by the discrimination of the membrane against the nitrate analogue, chlorate (John, 1977).

The $^{-}H^+/2e^-$ Ratio. The ratio for oxygen, about 7.5 in the case of denitrifiers grown on nitrate anaerobically, is in agreement with values of 7–8.5 reported by Scholes & Mitchell (1970b), Lawford et al. (1976), Meijer et al. (1977), and Lawford (1978) for aerobically grown *Pa. denitrificans*, but is in disagreement with values of 3.4–6.2 reported by Van Verseveld et al. (1977) in the case of *Pa. denitrificans* grown anaerobically on nitrate or nitrite in a chemostat.

The $^{-}H^+/2e^-$ ratios for oxygen and nitrogen oxides, 7.5 and about 4, respectively, are in general agreement with the observations of Koike & Hattori (1975a,b) who compared the efficiencies of oxygen and nitrogen oxides in promoting growth of the denitrifier, *Ps. aeruginosa*. They found that oxygen was about twice as efficient as the nitrogen oxides, per electron, but that there was little difference, per electron, among nitrate, nitrite, and nitrous oxide. Similar results were reported for *Pa. denitrificans* by Van Verseveld et al. (1977). It is important in this regard that most denitrifiers, including *Pa.* and *Ps. denitrificans*, cannot grow fermentatively and depend absolutely on respiration for growth. It would appear that nitrogen oxide respiration in denitrifiers cannot utilize one or more of the proton translocation mechanisms available to oxygen respiration. Analogous situations have been reported in other bacteria wherein the proton translocation ratio may depend on reducing agent and the detailed organization of the electron transport system (Jones et al., 1975; Garland et al., 1975).

The ratios for the reductive steps $NO_2^- \rightarrow \frac{1}{2}N_2O$ and $NO_2^- \rightarrow \frac{1}{2}N_2$ are slightly lower than the ratios for the steps $N_2O \rightarrow N_2$ and $NO_3^- \rightarrow \frac{1}{2}N_2$. The numbers suggest that the reduction of nitrite to nitrous oxide may translocate protons with lower efficiency than other steps of denitrification.

[3] The average redox state of cellular material is approximately that of carbohydrate (CH_2O) which releases four protons for the four equivalent oxidation of carbon to carbon dioxide.

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