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Substrate Binding Site for Nitrate Reductase of *Escherichia coli* Is on the Inner Aspect of the Membrane

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Escherichia coli grown anaerobically on nitrate exhibited the same transport barrier to reduction of chlorate, relative to nitrate, as that exhibited by *Paracoccus denitrificans*. This establishes that the nitrate binding site of nitrate reductase (EC 1.7.99.4) in *E. coli* must also lie on the cell side of the nitrate transporter which is associated with the plasma membrane. Because nitrate reductase is membrane bound, the nitrate binding site is thus located on the inner aspect of the membrane. Nitrate pulse studies on *E. coli* in the absence of valinomycin showed a small transient alkalinization ($^{-}H^{+}/NO_3^{-} \simeq -0.07$) which did not occur with oxygen pulses. By analogy with *P. denitrificans*, the alkaline transient is interpreted to arise from proton-linked nitrate uptake which is closely followed by nitrite efflux. The result is consistent with internal reduction of nitrate, whereas external reduction would be expected to give $^{-}H^{+}/NO_3^{-}$ ratios approaching -2.

The nitrate binding site of the respiratory nitrate reductase (EC 1.7.99.4) of *Paracoccus denitrificans* and other denitrifiers appears to lie on the inner aspect of the plasma membrane (8, 10). John (8) established that reduction of chlorate, a good substrate for nitrate reductase, is impeded by a permeability barrier in the case of intact cells. The barrier is abolished by Triton X-100 and is not observed to exist with inside-out membrane vesicles. Kristjansson et al. (10) concluded that the substrate site must lie on the inner aspect of the membrane on the basis of the stoichiometry of proton and nitrate uptake in oxidant pulse experiments with *Paracoccus denitrificans* and *Pseudomonas denitrificans*.

On the other hand, Garland et al. (5) concluded that the substrate site in *Escherichia coli* lies on the outer aspect of the membrane, largely because the nitrate flux calculated from osmotic swelling experiments seemed to be about 1,000 times lower than the maximum flux of nitrate respiration. This result is difficult to reconcile with the work of MacGregor and Christopher (12), who showed that the catalytic subunit A (11) of *E. coli* nitrate reductase can be labeled efficiently by dansyl cadaverine only when the catalyst for the transamidation, transglutaminase, has access to the inner aspect of the cytoplasmic membrane.

We demonstrate in this paper that the permeability barrier to reduction of chlorate exists in *E. coli* exactly as it does in denitrifiers and that apparent nitrate fluxes in osmotic swelling experiments bear little or no relationship to fluxes observed during nitrate respiration. In addition, the direction and magnitude of proton flux in response to nitrate pulses are consistent with the intracellular reduction of nitrate.

MATERIALS AND METHODS

Materials. Special products were obtained as follows: carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), valinomycin, lysozyme, and KSCN from Sigma Chemical Co.; nigericin from Eli Lilly & Co.; NADH from Boehringer Mannheim; and nutrient media from Difco. Stock solutions of CCCP, valinomycin, and nigericin were prepared in ethanol.

Microbiology. The organisms used were prototrophs of *Paracoccus* (formally *Micrococcus*) *denitrificans* ATCC 19367 and *Escherichia coli* K-12, wild type (CGSC 5073). A genetically uncharacterized *nar* mutant of *E. coli* K-12, strain C600, lacking nitrate reductase activity, was obtained from David Freifelder of this department.

Cells were normally grown anaerobically from 2 to 5% inocula on 20 mM nitrate in yeast extract medium at 30°C as previously described by St. John and Hollocher (18). For most oxidant pulse experiments *E. coli* was grown anaerobically on 20 mM nitrate and 100 mM ethanol in a medium consisting of 0.8 g of yeast extract, 0.5 g of peptone, 1 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4$, and 3 mg of $FeSO_4$ in 1 liter of 10 mM potassium phosphate buffer, pH 7.1. Cultures were harvested by centrifugation after having reached a cell density of 7×10^8 to 9×10^8 cells/ml in mid-exponential phase, and cells were washed two or more times at 0 to 4°C in the suspending medium to be used in a particular experiment. Stock cultures were kept on yeast extract-agar slants at 4°C, and inocula for liquid cultures were used immediately after growth overnight. Cell concentrations were determined on a Klett colorimeter. Cells were grown aerobically with shaking in the yeast extract medium minus nitrate.

Oxidant pulse studies. The experiments were carried out on whole cells as described by Kristjansson et al. (10).

Preparation of spheroplasts. Spheroplasts used in osmotic swelling experiments and in nitrate uptake assays were prepared by the method of Garland et al. (5), except that the digestion time with lysozyme was 25 min, 0.5 M sucrose–10 mM glycylglycine, pH 7, was used as the final suspending medium, and the spheroplasts were concentrated to about 25 mg of protein/ml in the final suspension.

Spheroplasts which were sonically disrupted before use were also prepared by the method of Garland et al. (5), except that the suspending medium during and after digestion was 10 mM potassium phosphate buffer, pH 7.2, and the spheroplasts were concentrated to about 8 mg of protein/ml.

EDTA was omitted from these procedures when *P. denitrificans* was used.

Osmotic swelling of spheroplasts. A 2- to 3-μl amount of spheroplast suspension at 25°C was injected into a 1-cm optical cuvette containing 1.4 ml of a salt solution in which the sum of the concentrations of the ions was 0.5 M, e.g., 0.25 M KCl, KNO_3, etc. Swelling and lysis of the spheroplasts resulted in a decrease in optical density, which was followed at 500 nm. The volume of spheroplast suspension used gave an initial optical density of 0.07 to 0.09 depending on the particular preparation. Swelling was facilitated by one or another ionophore as described in the text.

Nitrate uptake. Nitrate and chlorate uptake by whole cells or spheroplasts was monitored as described by John (8) with the use of an Orion nitrate electrode model 93-07 with an Orion Ionalyzer model 801 digital pH meter connected to an Omniscribe recorder. The reference electrode was an Orion double-junction electrode model 92-02. Cells were incubated in 10 ml of yeast extract in a narrowly vented container under constant flow of argon gas, and the reaction was started by injecting anaerobic nitrate or chlorate. Spheroplasts were incubated in anaerobic yeast extract containing 0.4 M sucrose.

Calculation of ion fluxes across bacterial membranes. During nitrate respiration in the case of whole cells or spheroplasts, nitrate flux was calculated from

$$\text{respiratory flux} = \frac{R}{NA} \quad (1)$$

where R is the rate of nitrate uptake, N is the number of cells from cell counts, and A is the membrane area per cell, which we take to be about 6×10^{-8} cm^2.

During osmotic swelling of spheroplasts, salt flux was calculated under the assumptions that the optical end point (lysis in most cases) occurred at an internal salt concentration of 0.25 M and that the initial rate of change of optical density applied to the situation where $C_0 - C_i = \Delta C = C_{0\,\text{initial}} = 0.25$ M. The following equation was used

$$\text{swelling flux} = \frac{2.5 \times 10^{-4} D}{6t} \text{ at } \Delta C \text{ of } 0.25 \text{ M} \quad (2)$$

where 2.5×10^{-4} is the end point of swelling in moles per cubic centimeter, t is the time required to reach the end point at the initial rate of change of optical density, and $D/6$ is the ratio of spheroplast volume to surface area for which we take D, spheroplast diameter, to be about 10^{-4} cm.

In comparing respiratory and osmotic swelling fluxes, we assumed that Fick's law applies in osmotic swelling (5) so that flux is proportional to $C_{0\,\text{initial}}$. Thus, the two fluxes can be compared at the same value of $C_{0\,\text{initial}}$.

Sonic treatment. Spheroplasts prepared in phosphate buffer were disrupted by exposing them for 3 min at 23°C under anaerobic conditions at the focus of a water bath-type sonic oscillator (Laboratory Supply Co.). Disruption was indicated by a dramatic clarification of the suspension and was confirmed by microscopic examination.

Nitrate reduction followed by NADH oxidation. A 50-μl amount of sonically disrupted spheroplast preparation was injected into a sealed 1-cm optical cuvette containing 2.5 ml of anaerobic 10 mM phosphate buffer, pH 7.2, and 80 μM of NADH. The reaction was started by injecting anaerobic nitrate or chlorate and was followed at 340 nm. The V_{max} in this assay is lower than that obtained with reduced benzyl viologen as reductant (9) but has an advantage in reducing nitrate via the electron transport system including, presumably, the C (cytochrome *b*) component of the nitrate reductase complex.

RESULTS AND DISCUSSION

Chlorate reduction. Table 1 summarizes the comparative rates of nitrate and chlorate respiration in *P. denitrificans* and *E. coli* (K-12 wild type) grown anaerobically on nitrate. Because V_{max} and K_m values differ little between nitrate and chlorate in the case of respiratory nitrate reductases (3, 4, 15, 16), a barrier to chlorate reduction must exist in whole cells and in spher-

TABLE 1. *Comparison of the rates of nitrate and chlorate respiration in E. coli and P. denitrificans*[a]

Prepn	Rate of respiration		Rate ratio
	Nitrate	Chlorate	
E. coli K-12			
Whole cells	205	≤5	≥41
Spheroplasts	165	10	17
Sonically disrupted spheroplasts	55	50	1.1
P. denitrificans			
Whole cells	720	10	72
Sonically disrupted spheroplasts	210	205	1.02

[a] In the case of whole cells and spheroplasts, nitrate and chlorate uptake were measured by a nitrate-specific electrode, and the units are nanomoles of anion per minute per milligram of protein. For sonically disrupted spheroplasts, respiration was measured by following the oxidation of NADH, and the units are nanomoles of NADH per minute per milligram of protein. For comparisons among tables, 10^{10} cells ≃ 2 mg of protein ≃ 600 cm^2 of membrane area. The initial concentration of nitrate or chlorate was 0.5 mM.

oplasts, but not in spheroplasts which have been sonically disrupted. The rate ratio, nitrate to chlorate, was generally somewhat lower in spheroplasts than in whole cells as a consequence of inadvertent damage in preparation. The barrier can be abolished by Triton X-100 (8) and certain other detergents and solvents. In the case of *P. denitrificans*, John (8) interpreted the barrier as a nitrate transporter which strongly discriminates against chlorate with the result that permeation of chlorate is rate limiting in respiration. The rate ratio of about 50 in whole cells (Table 1) would correspond to the permeability ratio of nitrate to chlorate if permeation of nitrate were also rate limiting in respiration. But because the rates of nitrate respiration supported by benzyl viologen in *E. coli* spheroplasts can be as high as 2 μmol/min per mg of protein (9), or about 10 times the rates supported by yeast extract (Table 1), nitrate reduction must be largely rate limiting. Thus, the true permeability ratio of nitrate to chlorate could be as great as $50 \times 10 = 500$. Because the outer membrane of gram-negative bacteria is highly permeable to salts and solutes with molecular weights below 700 to 900 (1, 2, 14), it cannot be the permeability barrier for chlorate. The plasma membrane must be the barrier. Thus, the nitrate binding site of nitrate reductase must lie on the inner aspect of the plasma membrane in order to lie on the cell side of the permeability barrier.

Oxidant pulse alkalinization. Denitrifiers take up one proton per nitrate ($^{-}H^{+}/NO_3^{-} = -1$) in the absence of K^+/valinomycin or other permeant counterions (10). The transient efflux of some nitrite (an intermediate in denitrification of nitrate) following addition of nitrate may temporarily reverse the uptake of protons. Proton uptake may resume when the extracellular nitrite is itself reassimilated and converted to nitrogen gas shortly thereafter. In *E. coli*, however, the reduction of nitrate ends with nitrite. We should thus predict that the proton to nitrate ratio would be some small negative number greater than −1 (or be zero) to reflect an incomplete kinetic separation between influx and efflux. The scheme shown in Fig. 1 represents expected ion movements in *E. coli* if nitrate were reduced internally. In contrast, external nitrate reduction (5) would be expected to yield a ratio approaching −2:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \qquad (3)$$

Figure 2 shows the occurrence of small alkaline transients upon nitrate addition with ratios of about −0.07 in the case of *E. coli* oxidizing ethanol. Alkaline transients were not observed upon oxygen addition. The results are consistent

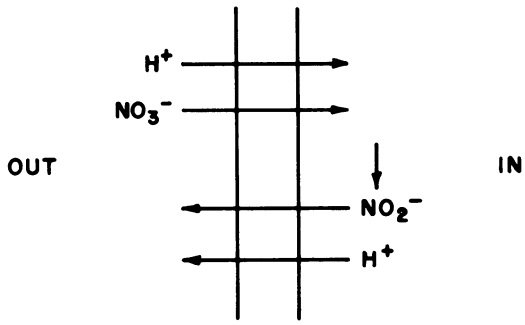

FIG. 1. *Scheme representing possible ion movements in E. coli.*

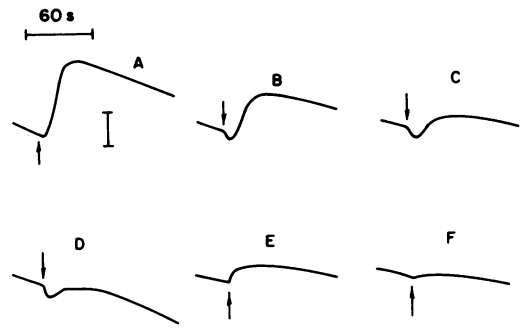

FIG. 2. *Oxidant pulse response of E. coli K-12 grown on nitrate and ethanol. At the mark 200 nmol of KNO_3 or 30 ng atom O_2 was added. Acidification causes an upward deflection, and the bar corresponds to 30 nmol of H^+. (A) First addition of KNO_3; (B) one of several subsequent additions of KNO_3, each separated from the next by about 3 min; (C) the fifth or subsequent addition of nitrate; (D) same as C but in the presence of 10 μM CCCP; (E) first addition of O_2; (F) sixth addition of O_2 in a series at 3-min intervals. The reaction system contained 150 mM KCl, 20 mM ethanol, and 3×10^{10} cells in 1 ml. Gas phase was argon. Ethanol was used to assure that substrate exhaustion did not occur, as determined by repeatedly pulsing cells previously incubated with valinomycin (9).*

with nitrate reduction at the inner aspect of the membrane.

Analysis of an opposing view. The conclusion of Garland et al. (5) that the substrate binding site is on the outer aspect of the membrane was based chiefly on two observations:

(i) The ability of azide, a potent competitive inhibitor of nitrate reductase, to inhibit nitrate respiration in *E. coli* was not influenced by external pH from 5.5 to 8, contrary to what might be expected of the conjugate base of a permeant weak acid ($pK_{HN_3} = 4.7$) if the base acted within the cell. Thus, azide did not inhibit at a site within the cell.

(ii) The nitrate flux calculated during the val-

inomycin-induced swelling of spheroplasts by KNO_3 was about 1,000 times lower than the maximum respiratory flux during nitrate respiration. Thus, nitrate need not enter the cell to be reduced.

In the first observation above, the oxidation-reduction state of cytochrome *b*, considered to be the direct reductant of nitrate reductase, served as an index of the duration of steady-state respiration upon addition of nitrate. It is noteworthy that the *reduction* of cytochrome *b* seemed to be inhibited by azide rather than its *oxidation* and that cytochrome *b* remained largely oxidized in the steady state. If azide had blocked nitrate reductase, then the oxidation of cytochrome *b* by nitrate reductase ought to have been inhibited and cytochrome *b* ought to have been largely reduced in the steady state. This is the situation observed when azide inhibits nitrous oxide reduction in denitrifiers (13, 20) and when azide inhibits nitrate reductase in *E. coli* extracts (7). The results suggest that azide may have been inhibiting chiefly the reduction of cytochrome *b* by L-malate. Thus, a pH dependence of azide inhibition may not have been seen because nitrate reductase may not have been the primary target.

We confirm the second observation above. Table 2 shows that the nitrate respiratory flux supported in *E. coli* by yeast extract is about 500 times greater than the valinomycin-promoted nitrate flux calculated to apply in a hypothetically equivalent osmotic swelling experiment. The method of calculation assumes, as did Garland et al. (5), that the flux in osmotic swelling is proportional to the difference in nitrate concentration across the membrane. The concentration of valinomycin (a potassium transporter [17]) was such that the apparent rate of swelling was independent of its concentration. Figure 3 illustrates the time course of osmotic swelling experiments and shows essential controls for Fig. 3 and 4. Note in Table 2 that a similar rate disparity applies to *P. denitrificans*, for which the criteria of John (8) and Kristjansson et al. (10) locate the nitrate binding site on the inner aspect of the membrane. A comparison of flux data apparently provides no information regarding location of the nitrate binding site.

Table 3 compares osmotic swelling fluxes induced by valinomycin for the potassium salts of four rather permeant anions, nitrate, nitrite, chlorate, and thiocyanate, into spheroplasts of *E. coli* and *P. denitrificans*. Note that the fluxes are similar and little influenced by the anion. This is remarkable, inasmuch as the permeability ratio, nitrate to chlorate, must lie between 50

TABLE 2. *Comparison of nitrate flux during nitrate respiration in whole cells with its apparent flux during the osmotic swelling of spheroplasts*[a]

Organism	Respiratory flux at C_0 = 0.5 mM A	Flux in osmotic swelling at		A/B
		C_0 = 250 mM	C_0 = 0.5 mM B	
E. coli K-12	0.68	0.72	0.0014[b]	480
P. denitrificans	2.40	1.85	0.0037[b]	650

[a] The term C_0 refers to the initial external concentration of nitrate. The methods used to calculate fluxes are described in Materials and Methods, and the results are given in nanomoles per minute per square centimeter. Valinomycin (1.5 μg/ml) was used in the osmotic swelling experiments.

[b] Calculated assuming the initial flux is proportional to C_0.

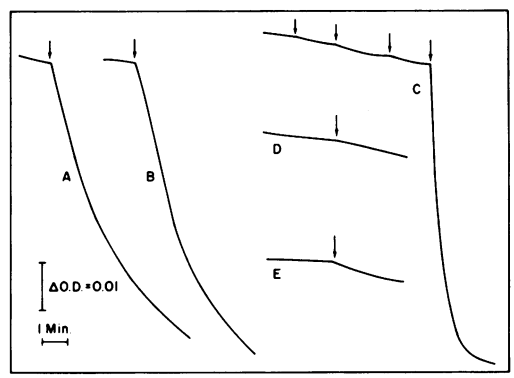

FIG. 3. *Osmotic swelling of spheroplasts promoted by valinomycin and control experiments. The agent added at the mark is indicated in parenthesis: (A) E. coli in KNO_3 (valinomycin); (B) P. denitrificans in KNO_3 (valinomycin); (C) either organism in KCl (first nigericin, then CCCP, then valinomycin, then butanol for lysis); (D) either organism in KCl (valinomycin or CCCP); (E) either organism in KNO_3 (CCCP). Cells were grown anaerobically on nitrate. The concentrations of valinomycin and nigericin were 1.5 μg/ml; those of CCCP, butanol, and the salts were 5 μM, 0.43 M, and 0.25 M, respectively. A decrease in optical density (downward deflection) indicates swelling. Curves C, D, and E represent controls for curves A and B and for Fig. 4.*

TABLE 3. *Fluxes of four salts during the osmotic swelling of spheroplasts*[a]

Organism	Flux in osmotic swelling at C_0 = 250 mM for			
	KNO_3	KNO_2	$KClO_3$	KSCN
E. coli K-12	0.7	0.6	0.6	0.7
P. denitrificans	1.8	1.2	0.9	1.2

[a] Conditions as described in footnote *a* of Table 2. The data for KNO_3 from Table 2 are included again for ease of comparison.

and 500 (see above). *P. denitrificans* can reduce nitrite internally at rates about equal to those for nitrate (10), and so nitrite probably also enters this organism via facilitated transport. Although *E. coli* cannot respire on nitrite, it must permit product nitrite to exit in steady-state nitrate respiration as rapidly as nitrate is reduced internally and also must avoid internal nitrite concentrations so high as to be toxic. Thus, we may assume that the *E. coli* membrane can facilitate nitrite transport. Thiocyanate is known to be highly permeant across bacterial membranes and is frequently used as a substitute for K^+/valinomycin in collapsing membrane potential in proton pulse or oxidant pulse experiments (19, 20). We might expect rapid penetration and swelling in the case of KNO_3, KNO_2, and KSCN but a much slower penetration by $KClO_3$. This was not observed. These results imply that anion permeability cannot be rate limiting in osmotic swelling. The involvement of a rate-limiting change in mechanical properties of spheroplasts seems unlikely, inasmuch as the swelling associated with water flux (dilution from an osmotically supporting medium into water) is complete within a few seconds. Although the nature of the rate-limiting step is not entirely clear, we can conclude that ion fluxes calculated from osmotic swelling may bear little or no relationship to ion fluxes measured in other experiments.

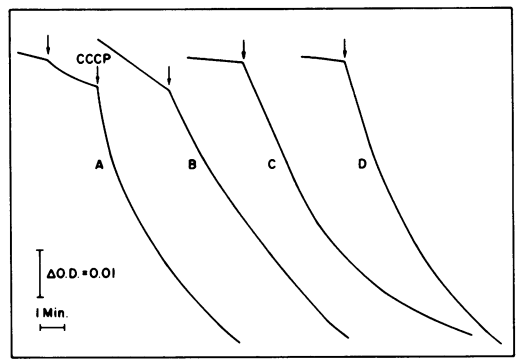

FIG. 4. *Osmotic swelling of E. coli spheroplasts promoted by nigericin. Nigericin was added at the mark except in the one place indicated by CCCP. (A) E. coli K-12 in KNO_3; B, E. coli K-12 in KNO_3 containing 0.1 mM KCN to inhibit respiration; (C) E. coli nar mutant (grown aerobically) in KNO_3; (D) E. coli K-12 in $KClO_3$. Conditions otherwise were as indicated in the legend to Fig. 3. Nigericin-induced swelling of E. coli spheroplasts in KNO_2 and KSCN and of P. denitrificans spheroplasts in KNO_3, KNO_2, $KClO_3$, and KSCN was similar to curves C and D. KCN at the concentrations used seemed to make spheroplasts somewhat leaky. Cells were grown anaerobically on nitrate except in the case of curve C. The kinetics of swelling were independent of nigericin concentration at the concentration used.*

Flux models for osmotic swelling. Figure 4 illustrates the fact that nigericin, an exchange carrier for protons and potassium (17), failed to promote the osmotic swelling by KNO_3 of *E. coli* spheroplasts grown on nitrate but succeeded in conjunction with an uncoupler, as was first noted by Garland et al. (5). In contrast, nigericin promoted swelling of *P. denitrificans* spheroplasts in the case of KNO_3, KNO_2, $KClO_3$, and KSCN (data not shown but similar to curves C and D, Fig. 4). Swelling of *E. coli* spheroplasts in KNO_3 was promoted by nigericin, however, when respiration was inhibited or when a *nar* mutant was used (curves B and C, Fig. 4). Garland et al. (5) interpreted the need for uncoupler (KNO_3, nitrate-grown *E. coli*) to be due to insignificant proton permeability of the membrane. This explanation cannot be correct because the results with the other three anions show that proton permeability must be appreciable in the presence of nigericin in the time frame of osmotic swelling (minutes).

A minimum transport scheme for osmotic swelling by a nonmetabolized permeant anion (e.g., $A^- = SCN^-$) can be represented by scheme A in Fig. 5. Nigericin promotes a neutral exchange of potassium and protons, while the driving force for proton return is supplied by the diffusion potential of A^-. If every molecule of A^- were converted internally to another permeant anion, B^- (e.g., $A^- = NO_3^-$; $B^- = NO_2^-$), which can exit down its concentration gradient, then osmotic swelling would not be promoted by nigericin alone, as shown in scheme B in Fig. 5. Both the K^+/H^+ and A^-/B^- exchanges are neutral so that there can be little driving force for the proton return (scheme A, Fig. 5) required for rapid net uptake of salt. The weak driving force can be compensated by greatly increasing the proton permeability through a proton ionophore (uncoupler) such as CCCP. Alternatively, any device that succeeds in abolishing nitrate respiration would prevent $A^- \rightarrow B^-$ and reestablish the situation of scheme A where rapid swelling can occur.

Why does a similar situation not apply with nitrate and nitrite in denitrifiers and with chlorate in both organisms? Reduction of chlorate in both organisms yields chloride (6), which is an impermeant ion. Chloride cannot diffuse away rapidly, and scheme C (Fig. 5) applies. But scheme C is in fact equivalent to scheme A in outcome, so swelling would occur. Similarly, denitrifiers reduce nitrate and nitrite internally to nitrous oxide or nitrogen gas with the concom-

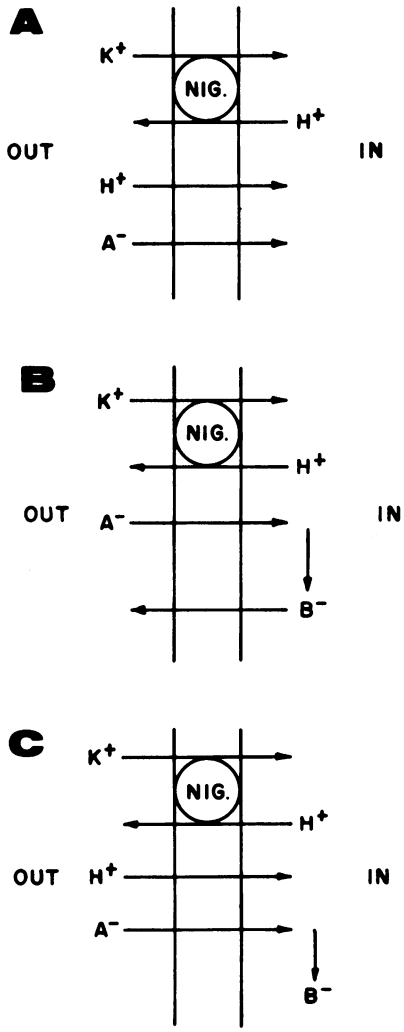

FIG. 5. *Schemes representing ion movement during osmotic swelling in the presence of nigericin (NIG).*

itant stoichiometric production of hydroxide (10). Because hydroxide is also impermeant, scheme C again applies.

The lack of nigericin-induced osmotic swelling of *E. coli* in KNO_3 argues against reduction of nitrate at the outer aspect of the plasma membrane. Nitrite would then simply replace ntirate in the medium, and, because both are permeant anions, scheme A would apply and swelling would occur.

Is nitrate reductase also a proton pump? Garland et al. (5) reported that the $^{-}H^{+}/2e$ ratios for oxygen and nitrate were similar (~4) in oxidant pulse experiments on valinomycin-treated *E. coli* spheroplasts. Because reduction of nitrate to nitrite at an external site must consume two protons per electron pair (equation 3), the gross $^{-}H^{+}/2e$ ratios would necessarily be about 4 for oxygen and 6 for nitrate, with implication that an extra site for proton translocation (extra proton pump) may exist under nitrate respiration. But, because only nitrate reductase lies on the oxidizing side of cytochrome *b*, it appeared that nitrate reductase needed to assume the role of that proton pump. None of this speculation is necessary if nitrate reduction occurs, as our results require, on the inner aspect of the membrane.

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