Inhibition of Denitrification by Uncouplers of Oxidative Phosphorylation†

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ABSTRACT: In the denitrifiers, *Pseudomonas denitrificans* and *Pseudomonas aeruginosa*, nitrogen oxide respirations were reversibly inhibited by several weak acid-type uncouplers of oxidative phosphorylation. Their effectiveness as uncouplers stood in about the same rank as their effectiveness as inhibitors. In contrast, uncouplers increased the rate of oxygen respiration (release of respiratory control) in these two organisms and seemed not to inhibit even at rather high concentrations. The mechanism of inhibition of nitrogen oxide respiration by uncouplers was explored and several lines of evidence ruled out an inhibition which depended on collapse of the proton motive force per se. The concentrations of uncouplers required to inhibit nitrogen oxide respirations in *Ps. denitrificans* were found to be at least ten times higher than those required (a) to inhibit respiration-driven amino acid uptake, (b) to release respiratory control in the case of oxygen respiration, and (c) to increase membrane proton conductivity to such levels that endergonic reactions dependent on proton motive force must, by calculation, be inhibited. Neither valinomycin/K^+ (to collapse membrane potential) nor nigericin (to collapse ΔpH) had an inhibitory effect on nitrogen oxide respirations, whether present separately or together. In addition, nonuncoupling analogues of the nitrophenol and carbonyl cyanide phenylhydrazone uncouplers were effective in inhibiting respiration. The inhibitions are attributed in large part to the hydrophobic properties of these compounds. Spectrophotometric observations suggested that the site of inhibition lay on the reducing side of cytochromes *b* and *c*. In the steady states of nitrogen oxide respirations, cytochromes *b* and *c* remained nearly completely oxidized in the presence of uncouplers, and the transition from steady state to reduced state upon the exhaustion of nitrogen oxide was inhibited. Certain trivial explanations for the inhibitory effect of uncouplers, such as substrate starvation, were ruled out.

A classical uncoupler of oxidative phosphorylation (Loomis and Lipman, 1948), 2,4-dinitrophenol (DNP),[1] has been reported to inhibit denitrification with effects that are apparently diverse and possibly species specific. Pinchinoty and D'Ornano (1961) reported that 0.5 mM DNP strongly inhibited nitrogen production from nitrate, nitrite, and nitrous oxide and partially inhibited oxygen uptake in the case of *Paracoccus* (formerly *Micrococcus*) *denitrificans*. Sacks and Barker (1951) observed with a strain of *Pseudomonas denitrificans* that DNP inhibited nitrogen production from nitrous oxide but not from nitrite. In the case of an organism of the *Alcaligenes/Achromobacter* group (Matsubara, 1975), Matsubara and Mori (1968) reported that 0.1 mM DNP completely inhibited the reduction of nitrous oxide but had no effect on the reduction of nitrite to nitrous oxide. Another uncoupler, FCCP, has been reported to inhibit oxygen uptake partially in aerobically grown *Pa. denitrificans* (Scholes and Mitchell, 1970a).

It is unknown whether uncouplers as a class can inhibit denitrification and, if so, what the basis for inhibition may be. There are at least two possibilities. An uncoupler may inhibit respiration at an endergonic step by collapsing the proton motive force upon which that step depends, i.e., by acting as an uncoupler per se. At least one example of this situation has been provided by Cobley (1976a) who showed that CCP inhibited the aerobic oxidation of nitrite in membrane particles derived from *Nitrobacter winogradskyi* by collapsing the electrical component of the proton motive force. The transfer of electrons from cytochrome a_1 to cytochrome *c* was the step specifically inhibited (Cobley, 1976b). Alternatively, uncouplers may inhibit by complexing with electron-transfer components or simply by changing membrane structure through their ability to dissolve in lipid phases.

In this paper we rule out the idea that collapse of the proton motive force may account for the inhibition of denitrification by uncouplers, and we provide data to suggest that the cause involves hydrophobic interaction of uncouplers with one or another membrane component.

Materials and Methods

Microbiology. The denitrifiers used were prototrophs of *Pseudomonas denitrificans*, ATCC 13867, and *Pseudomonas aeruginosa*, strain 1, CHL-2, FP^- obtained from B. W. Holloway, Monash University, Clayton, Australia. In some experiments a strain of *Ps. denitrificans* was used which seemed to be identical with ATCC 13867 and was obtained from C. C. Delwiche, University of California, Davis. Cells were grown in a yeast extract medium at 30 °C as previously described (St. John and Hollocher, 1977) and were harvested at about 10^9 cells mL^{-1} in late exponential or early stationary phase. The oxidant for anaerobic growth was 20 mM nitrate. After harvest, cells were washed twice at 0 °C in the particular suspending medium to be used in an experiment. All assays and reactions involving cells were carried out at 30 °C unless otherwise indicated.

Materials. Chemicals were obtained as follows: L-[^{14}C]-glutamate (262 Ci mol^{-1}) and L-[^{14}C]proline (130 Ci mol^{-1}) from New England Nuclear; CCCP, valinomycin, and car-

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[1] Abbreviations used: CCP, carbonyl cyanide phenylhydrazone; carboxy-CCP, carbonyl cyanide *p*-carboxyphenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 1799, 2,2′-bis(hexafluoroacetonyl)acetone; DNP, 2,4-dinitrophenol; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; zephiran chloride, a mixture of C-12, -14, and -16 alkylbenzyldimethylammonium chloride.

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TABLE I: Respiration Rates of *Ps. denitrificans* toward Different Oxidants.[a]

Reaction	rates (μg atom of N or O min^{-1} by 10^{10} cells) with yeast extract	10 mM L-glutamate	endogenous substrates
N_2 production from N_2O	2.40	0.14	<0.02
N_2 production from NO_2^-	0.94	0.10	<0.02
NO_2^- uptake	0.85		
N_2 production from NO_3^-	0.98		
NO_3^- uptake	1.30		
O_2 uptake (anaerobically grown cells)	1.18	0.48	0.12
O_2 uptake (aerobically grown cells)	1.35	0.40	

[a] Nitrogen and nitrous oxide were measured by gas chromatography, nitrite by colorimetric assay, nitrate uptake by the production of nitrite plus nitrogen plus nitrous oxide (if any), and oxygen by Clark electrode. For nitrogen oxide reductions, 3.5–4 × 10^{10} cells were used, except when yeast extract provided the substrates in which case 1–2 × 10^9 cells were used. For oxygen reduction, 1–2 × 10^9 cells were used. Where yeast extract medium was not used, cells were suspended in 90 mM NaCl, 20 mM potassium phosphate buffer, pH 7.1.

bonic anhydrase from Sigma; DNP from Eastman Kodak; FCCP and carboxy-CCP from P. G. Heytler, E. I. duPont de Nemours; nigericin from Lilly; 1799 from C. Miller, Brandeis University; various nitrophenols, nitrobenzoates, and nitroanisoles from W. P. Jencks, Brandeis University; zephiran chloride from Winthrop Laboratories. CCCP, FCCP, 1799, valinomycin, nigericin, carboxy-CCP, and nitroanisoles were dissolved in ethanol for use.

Denitrification. The production or uptake of nitrogen and gaseous nitrogen oxides was determined by gas chromatography as previously described (St. John and Hollocher, 1977). To initiate denitrification, nitrate (15–25 μmol), nitrite (10–20 μmol), or N_2O (13–20 μmol) were introduced into 9-mL vials stoppered with serum caps containing 1 × 10^9 to 3 × 10^{10} cells in 1–2 mL 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.

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) 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).

Spectrophotometry. Difference spectra of cell suspensions in 1-cm path-length cuvettes were obtained using a Varian Techtron spectrophotometer, model 635. Dual wavelength difference extinctions were obtained using a Perkin-Elmer two-wavelength spectrophotometer, Model 356.

The cuvettes were fitted with serum caps to permit anaerobic conditions to be established. Nitrous oxide was added as a solution in equilibrium with nitrous oxide at 1 atm (about 25 mM N_2O at 25 °C).

Active Transport. The respiration-dependent active transport of L-[^{14}C]proline or L-[^{14}C]glutamate into cells was measured as described by Lieberman and Hong (1974). Oxygen respiration utilized endogenous substrates; nitrogen oxide respirations occurred anaerobically and utilized added lactate.

Acid Pulse Equilibration. The method, which measures the equilibration of protons across the cell membrane of bacteria, was a modification of that described by Scholes and Mitchell (1970a). Cells were suspended in 150 mM KCl containing 80 μg mL^{-1} carbonic anhydrase and were introduced into a specially designed 1-mL chamber containing a magnetic stirrer and a collar which allowed the insertion of an argon lance and a Markson 445 micro combination electrode. The system was allowed to equilibrate under argon at 25 °C for 30 min, at which time the pH of the system was adjusted to 6.88–6.90 with anaerobic 50 mM NaOH or 50 mM HCl in 150 mM KCl. Once the pH had stabilized in the desired range, 30 μL of anaerobic 5 mM HCl in 150 mM KCl was injected and pH was monitored with an Orion Ionalyzer Model 801 digital pH meter connected to an Omniscribe Recorder. The overall response time of the instrument was about 2 s. Any subsequent additions were made 1.5 min after the HCl. In this and other methods where valinomycin or nigericin was used, cells were incubated with these compounds for 3 h at 5 °C prior to the experiment in order to allow time for them to penetrate to the bacterial membrane.

Calculation of C_M. The effective proton conductance (C_M) was calculated as described by Scholes and Mitchell (1970a):

$$C_M = \frac{B_O B_I}{B_T t_{1/2} A} \ln 2 \quad (1)$$

where B_O, B_I, B_T, $t_{1/2}$, and A represent, respectively, the buffering capacity outside the membrane, buffering capacity inside the membrane, total buffering capacity, half-time for the relaxation of protons following an acid pulse (ΔH^+), and total cell-membrane area:

$$B_O = -\frac{\Delta H^+}{\Delta pH_\alpha} \quad (2)$$

where ΔpH_α represents the instantaneous change in pH after the addition of a quantity, ΔH^+, of protons

$$B_T = -\frac{\Delta H^+}{\Delta pH_\omega} \quad (3)$$

where ΔpH_ω is the final pH after equilibration of ΔH^+ across the membrane and

$$B_I = B_T - B_O \quad (4)$$

Cells treated with 4% 1-butanol no longer show a permeability barrier toward protons and so reach equilibrium ΔpH very rapidly following an acid pulse. In our experience, $\Delta pH_{butanol} \simeq \Delta pH_\omega$ and

$$B_T \simeq -\left(\frac{\Delta H^+}{\Delta pH_{butanol}}\right) \quad (5)$$

Values of $t_{1/2}$ were obtained from semilog plots of $\Delta pH_\omega - \Delta pH_t$, where ΔpH_t is any ΔpH value during the exponential decay.

The surface area of one *Ps. denitrificans* cell is calculated to be 6.3 × 10^{-8} cm^2 from its apparent shape and dimensions of about 1 × 1.5 μm. Proton flux is obtained by multiplying C_M by the proton motive force across the membrane in terms of equivalent pH units. At 30 °C, the conversion factor is about 60 mV (pH unit)$^{-1}$.

Results

Characteristics of the Denitrifiers. Certain characteristics of *Ps. aeruginosa* as a denitrifier were described by St. John and Hollocher (1977). An analogous characterization of *Ps. denitrificans* when grown on nitrate is given in Table I. In yeast

TABLE II: Uncoupler Concentrations Required to Inhibit Denitrification by 50% under Standard Assay Conditions.[a]

organism	uncoupler	concn (μM) for 50% inhibition of N_2O uptake	NO_2^- uptake	NO_3^- uptake
Ps. denitrificans ($1-3 \times 10^9$ cells)	DNP	1000	500	
	1799	400	300	
	FCCP	25	15	
	CCCP	10	10	10
Ps. aeruginosa ($5-15 \times 10^9$ cells)	DNP	5000	6000	
	1799	330	100	
	FCCP	45	25	45
	CCCP	70	25	50

[a] Cells were suspended in yeast extract medium. Nitrous oxide and nitrate were measured as described in Table I; nitrite uptake was determined through the production of nitrogen and nitrous oxide (if any).

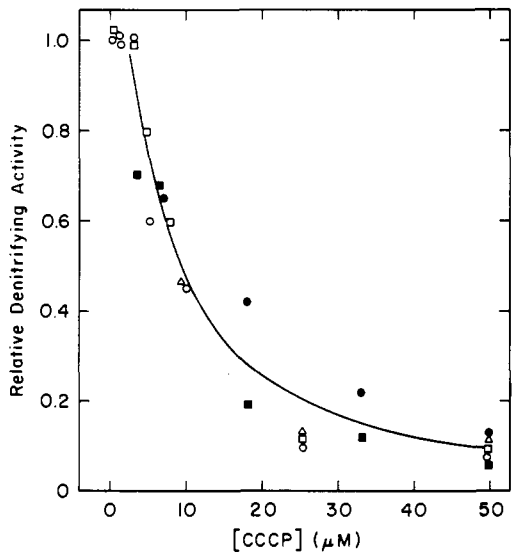

FIGURE 1: Inhibition of denitrification by CCCP in *Ps. denitrificans*. Open symbols, 2×10^9 cells suspended in 1 mL of yeast extract medium; closed symbols, 2.7×10^{10} cells in 1 mL of the NaCl-glutamate-KPO_4 buffer of Table I. Uptake of 12.5 μmol of nitrate, triangles; of 11 μmol of nitrite, squares; of 13 μmol N_2O, circles. Nitrate uptake was measured by the appearance of nitrite, N_2, and N_2O (if any); nitrite uptake was measured colorimetrically.

extract the reduction of nitrous oxide was faster than that of nitrite, as was the case with *Ps. aeruginosa*. In consequence, little or no nitrous oxide was evolved during the denitrification of nitrite (St. John and Hollocher, 1977). In the denitrification of nitrate, the reduction of nitrite was largely rate determining, so that nitrite appeared transiently but nitrous oxide did not. Oxygen respiration was found to be constitutive and similar in rate to the nitrogen oxide respirations (Table I). Inhibition of protein synthesis with 100 μg mL^{-1} chloramphenicol confirmed that the apparatus for oxygen respiration was not synthesized in anaerobically grown cells during harvest, at which time they were exposed to air. The presence of nitrate, nitrite, or nitrous oxide at 10–20 mM had little effect on oxygen uptake, and oxygen seemed not to inhibit reduction of these nitrogen oxides. *Pa. denitrificans* is reported, however, to utilize oxygen in preference to nitrate (Chance, 1955).

As prepared, cells contained sufficient endogenous substrate to drive steady-state oxygen respiration at about 0.1 of the rate observed with yeast extract, but they seemed not to contain

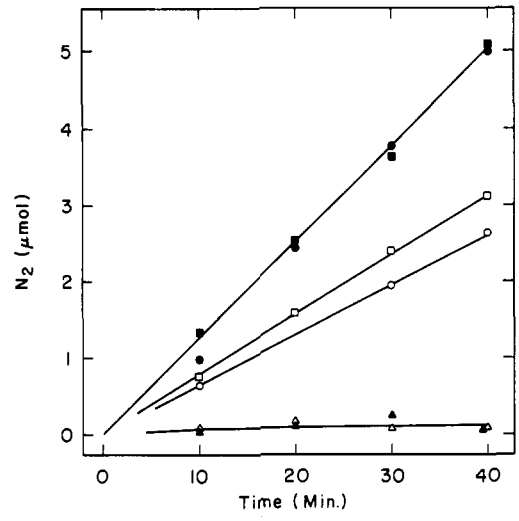

FIGURE 2: Reversibility of the inhibition of denitrification by CCCP in *Ps. denitrificans*. Cells were suspended in 2 mL of yeast extract medium. Closed symbols, 7.5×10^8 cells with 13.4 μmol of N_2O as oxidant; open symbols, 1.5×10^9 cells with 20 μmol of nitrite as oxidant. Circles, cells alone; triangles, cells + 75 μM CCCP; squares, cells washed four times with 2 mL of medium to remove the CCCP.

sufficient endogenous substrates to support steady-state denitrification at easily detectable rates. This difference is consistent with the rather stringent substrate requirements that apply in denitrification (Koike and Hattori, 1975). Cells harvested in midexponential phase growth apparently contain greater stores of substrates able to support denitrification (Kristjansson et al., 1978).

Inhibition of Denitrification by Uncouplers of Oxidative Phosphorylation. The novel ability of uncouplers to inhibit denitrification is shown in Figure 1 and Table II. Figure 1 shows that inhibition increased with uncoupler concentration above some threshold concentration, that the reductions of nitrate, nitrite, and nitrous oxide were all inhibited, and that inhibition seemed not to depend strongly on the reducing substrate used, e.g., L-glutamate as opposed to the variety of substrates in yeast extract. Table II shows that the various reactions of denitrification were not all identically inhibited by an uncoupler, that species differences can occur, and that the rank order of uncouplers as inhibitors of denitrification was similar to or the same as that for inhibition of oxidative phosphorylation, release of respiratory control, increase in proton permeability of membranes, and osmotic swelling, i.e., CCCP $\simeq$ FCCP > 1799 > DNP (Bakker et al., 1975, 1973; Finkelstein, 1970; Hopfer et al., 1968; Reed and Lardy, 1975; Ting et al., 1970).[2] In the range of cell concentrations used, 1–30 $\times 10^9$ cells mL^{-1}, the effect of an uncoupler seemed not to depend much on cell concentration. Inhibition by uncouplers was fully reversible (Figure 2). Uncouplers seemed not to inhibit oxygen respiration in *Ps. denitrificans* (Figures 4 and 5) or *Ps. aeruginosa* (data not shown).

Energy Coupling and Proton Permeability. In this section we offer evidence that collapse of the proton motive force per se does not account for the inhibition of denitrification.

Figure 3 shows the time course for the respiration-dependent active transport of L-glutamate (endogenous substrates; O_2)

[2] The order reported by Cunarro and Weiner (1975) in studies of the release of respiratory control and swelling in rat liver mitochondria in 150 mm naCl was CCCP > 1799 $\simeq$ FCCP > DNP. The ranking is based on the minimum concentrations of uncoupler required to elicit an observable effect. This criterion differs from that in most other studies where half or maximum effects are considered.

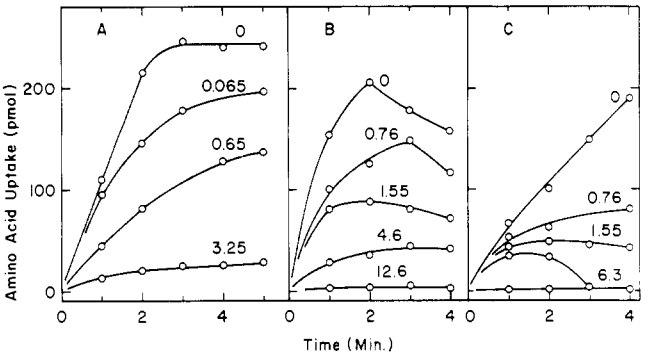

FIGURE 3: Inhibition of active transport of labeled amino acids by CCCP in *Ps. denitrificans*. 10^8 cells were suspended in a final volume of 0.05 mL and respiration was driven by endogenous substrates when dissolved O_2 was oxidant (A) and by lactate when nitrate (B) or N_2O (C) was oxidant. (A) 90 mM NaCl, 20 mM KPO_4 buffer (pH 7), containing 380 pmol of L-[^{14}C]glutamate; (B) anaerobic 90 mM NaCl, 10 mM Tris-Cl buffer (pH 6.9), containing 10 mM lactate, 390 pmol of L-[^{14}C]proline, and 20 mM KNO_3; (C) same as B except the system contained 1 atm of N_2O rather than nitrate. Lactate was added to the cells about 1 h before the experiments of B and C. The numbers indicate the concentrations of CCCP in μM.

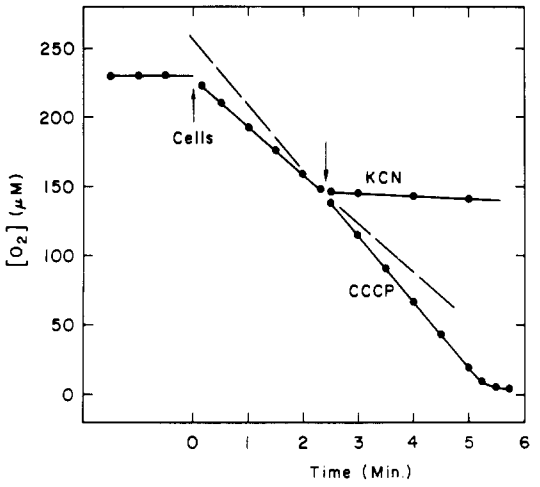

FIGURE 4: Oxygen electrode trace showing the enhancement of respiration in *Ps. denitrificans* by CCCP and its inhibition by KCN. At time 0, 2×10^9 cells were injected into 3 mL of aerated yeast extract medium. At the second arrow, 10 μM CCCP or 1 mM KCN was added. The equilibrium O_2 concentration is taken to be 0.23 mM at 30 °C.

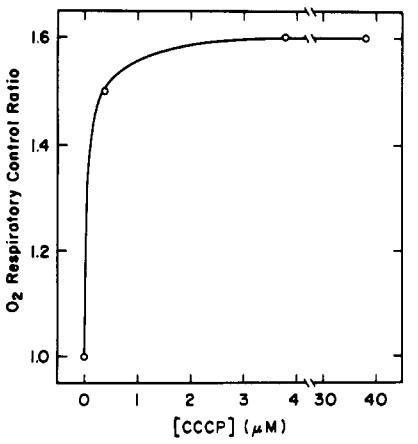

FIGURE 5: Release of respiratory control in *Ps. denitrificans* by CCCP. The oxygen electrode chamber contained 2×10^9 cells suspended in aerated yeast extract medium at a final volume of 3 mL. The respiratory control ratio is the rate of oxygen uptake in the presence of CCCP divided by the rate in its absence.

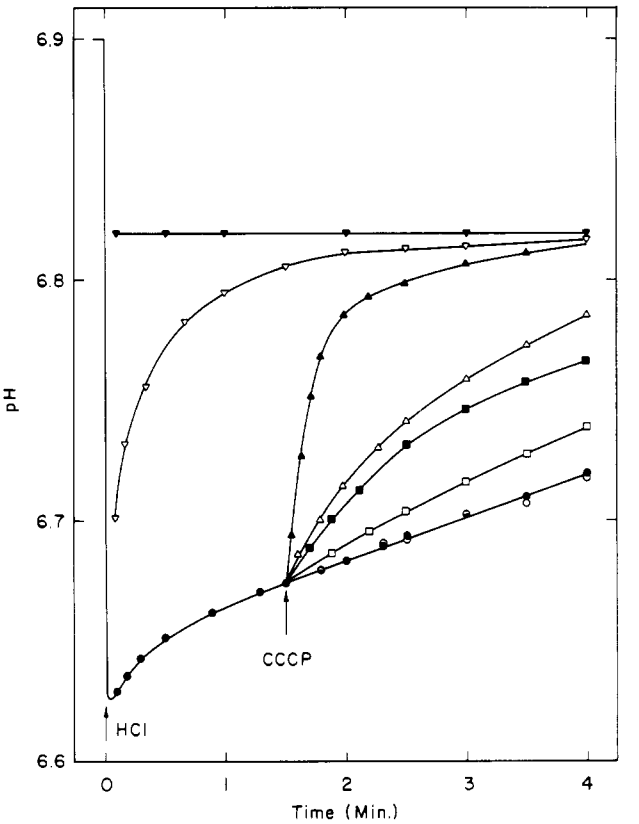

FIGURE 6: Acid pulse relaxations by *Ps. denitrificans* in the presence and absence of CCCP and ionophores. At the first arrow, 150 nmol of HCl was added to anaerobic suspensions of 4.5×10^{10} cells in 1 mL of 150 mM KCl: (●) cells alone; (▼) +4% 1-butanol; (○) +30 μg mL^{-1} valinomycin; (▽) +30 μg mL^{-1} nigericin. At the second arrow 1 μM CCCP was added after the HCl to: (□) cells alone; (■) +15 mM KSCN; (△) +30 mM KSCN. For ▲, 0.5 μM CCCP was added to a system containing 30 μg mL^{-1} valinomycin. Valinomycin and nigericin were incubated with cells before the experiment as described in Materials and Methods; butanol and KSCN were added just before the experiment. Each curve represents the result of a separate experiment for which a separate but similar butanol curve exists. The single butanol curve shown (▼), which approximates an immediate titration of both the internal and external buffering capacities of the cells, is representative in showing an amplitude about one-third of the pulse amplitude.

and L-proline (lactate; nitrate or nitrous oxide). The behavior of these systems was similar and involved a concentration of the labeled amino acid intracellularly by a factor of 200–300 maximally. The concentrations of CCCP required to decrease the internal steady-state levels of amino acid to half maximum were about 1 μM as compared with 10 μM required for the half-inhibition of denitrification (Table II). Thus, concentrations of CCCP that virtually abolished active transport had little effect on the rates of nitrogen oxide reductions. A possibility considered was that inhibition of active transport by an uncoupler might lead to substrate starvation and to a shutdown of denitrification. But inhibition was observed (Figure 1) even with high external concentrations of an oxidizable substrate (10 mM L-glutamate). The inhibition of denitrification by uncouplers seemed not to depend on substrate concentrations or on the nature of the substrate, as long as it could support nitrogen oxide reduction.

Figures 4 and 5 show that the effect of an uncoupler on oxygen respiration in *Ps. denitrificans* was of the normal kind; i.e., the rate increased presumably due to a release of respiratory control following the collapse of the proton motive force (Mitchell, 1966; Scholes and Mitchell, 1970a; Mitchell and Moyle, 1967). Release of respiratory control was virtually

TABLE III: The Failure of Valinomycin/K^+ and Nigericin to Inhibit Denitrification in *Ps. denitrificans*.[a]

additions	rel act. N_2O uptake	rel act. NO_2^- uptake
none	1.00	1.00
nigericin	0.90	0.96
valinomycin	0.91	0.87
valinomycin + nigericin	0.94	0.94
valinomycin + potassium acetate (100 mM)	1.25	0.94

[a] Cells were suspended at 2.8×10^{10} cells mL^{-1} in the glutamate–NaCl–KPO_4 solution of Table I and incubated with 30 $\mu g\ mL^{-1}$ of valinomycin and/or nigericin for 3 h at 5 °C. One-milliliter aliquots were used in denitrification assays. Potassium acetate was added immediately before assay.

complete at 1 μM CCCP (Figure 5). Again, this was well below the concentrations that strongly inhibited denitrification.

Figure 6 illustrates the ability of CCCP to enhance proton permeability and thus to accelerate the migration of protons from the medium into the cells following the addition of a small amount of HCl to the medium (acid pulse). CCCP alone had little apparent effect because counterion transport strongly limited proton flux (Scholes and Mitchell, 1970a). Counterion transport was facilitated to some extent by thiocyanate, a membrane-permeant anion (Mitchell and Moyle, 1956, 1969), and very strongly by valinomycin, a potassium-specific ionophore (Pressman, 1970). Figure 6 also shows that 4% (0.54 M) butanol destroyed the permeability barrier to protons and that nigericin, an ionophore which can exchange H^+ for K^+ across membranes (Pressman et al., 1967; Chance and Montal, 1972), was similar in effect to valinomycin plus CCCP. The changes in membrane permeability, expressed as effective membrane conductance, C_M, are shown in Figure 7 as a function of CCCP concentration. The value of C_M in Figure 7 at 1 μM CCCP in the presence of valinomycin, 40–50 pmol of H^+ (pH unit)$^{-1}$ s^{-1} cm^{-2}, was based on the observed $t_{1/2}$ and was uncorrected for instrument response time. The true value must be about 100 pmol of H^+ (pH unit)$^{-1}$ s^{-1} cm^{-2}.[3] This compares well with the value of 38 pmol of H^+ (pH unit)$^{-1}$ s^{-1} cm^{-2} obtained by Scholes and Mitchell (1970a) with aerobically grown *Pa. denitrificans* at 2 μM FCCP. The inward proton flux implied by the conductance value can be compared with an outward proton flux driven by respiration. Taking 60 mV (pH unit)$^{-1}$ as the electrical equivalent of pH in the proton motive force and assuming 150 mV as a threshold below which respiration-dependent endergonic processes should be inhibited,[4] we obtain a nominal proton flux of about 0.25 nmol of H^+ s^{-1} cm^{-2} at 1 μM CCCP. *Ps. denitrificans* can sustain respiration rates of about 4 μg of electron min^{-1} for 10^{10} cells under optimum conditions (Table I). Using a cell area of 6.3×10^{-8} cm^2 $cell^{-1}$ and a ratio of protons translocated outward to electrons passed through the electron-transport system ($\rightarrow H^+/e^-$) of 2 in the case of nitrogen oxides (Kristjansson et al., 1978), the outward proton flux becomes 0.2 nmol of H^+ s^{-1} cm^{-2}. This rate approximates the possible inward proton flux

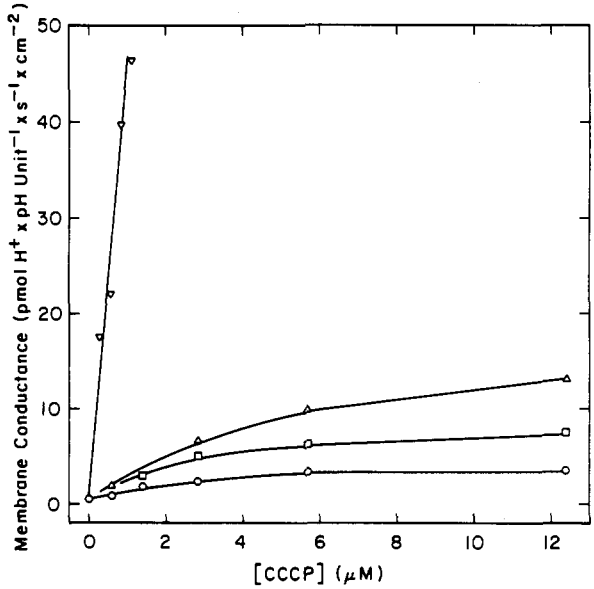

FIGURE 7: Effective proton conductance (C_M) of the membrane of *Ps. denitrificans* as a function of CCCP concentration. C_M was calculated from the results of acid pulse relaxation experiments (Figure 6) by the method of Scholes and Mitchell (1970a), uncorrected for instrument-response time: (○) cells alone; (□) +15 mM KSCN; (△) +30 mM KSCN; (▽) +30 $\mu g\ mL^{-1}$ valinomycin. The C_M values for the last two points on the valinomycin curve are minimum values due to the fact that the observed acid-pulse relaxation half-times (3.5–4 s) approach the instrument-response time of about 2 s.

at 150 mV and implies that 1 μM CCCP should create a leak of protons across the membrane so massive that respiration-dependent processes should be largely inhibited. The result of this calculation is consistent with the results of Figures 3 and 5.

Studies of proton permeability (Figure 6) established that nigericin and valinomycin can penetrate to the bacterial membrane and serve effectively as ionophores. Nigericin can collapse ΔpH but not the membrane potential, whereas valinomycin can collapse membrane potential but not ΔpH (Pressman, 1976). Together they behave much as does CCCP. Table III shows that nigericin and valinomycin, alone or together, did not much inhibit denitrification. Similarly, valinomycin plus a permeant weak acid (acetic acid in equilibrium with acetate) used to collapse ΔpH, acid external, failed to inhibit.

Nonuncoupling Analogues of Uncouplers. In the nitrous oxide reduction assay with *Ps. denitrificans*, 3-nitrophenol, 3,5-dinitrobenzoate, phenol, $NaDodSO_4$ and zephiran chloride caused 50% inhibition at 2, 2.5, 20, 15, and 0.1 mM, respectively. The concentrations of these compounds at which inhibition was observed were well below those at which cell lysis occurred. A nondissociable analogue, 3,5-dinitroanisole caused 60 to 80% inhibition when the aqueous phase was saturated. Butanol had no effect in the 20 mM range nor did ethanol at 700 mM. Carboxy-CCP, a nonuncoupling analogue of CCCP and FCCP (P. G. Heytler, personal communication) caused 50% inhibition at 12 μM. These results can be compared with those of Table II.

Spectrophotometric Studies. Suspensions of *Ps. denitrificans* grown on nitrate showed cytochromes *b* and *c* but not cytochromes of the *a* (600, 445 nm) or *d*(a_2) (625–655, 460 nm) type. Figure 8 shows difference spectra between oxidized and reduced states of cytochromes *b* and *c* and also steady-state difference spectra during turnover in the oxidation of glutamate by nitrite or nitrous oxide. The reduced cytochromes *c*

[3] For two consecutive first-order processes, $k_{obsd} = k'k''/k' + k''$ and $t_{1/2,obsd} = \ln 2/k_{obsd}$. Thus, if the instrument half-time, $t_{1/2}''$, were 2 s and $t_{1/2,obsd}$ were 4 s, then the half-time for pH relaxation, $t_{1/2}'$, would be 2 s and k' twice k_{obsd}.

[4] At one proton per amino acid transported, a sequestration ratio of 300 in active transport is equivalent to a proton motive force of 150 mV. In a similar comparison Scholes and Mitchell (1970a) used an assumed potential of 220 mV.

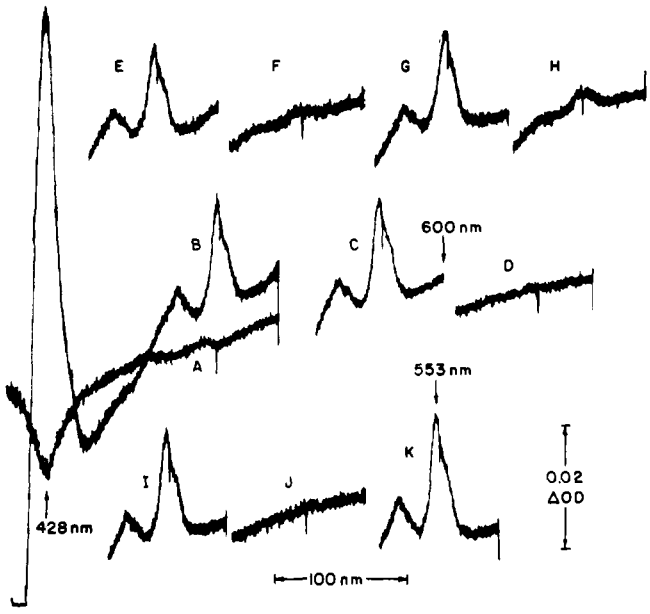

FIGURE 8: Difference spectra of cytochromes *b* and *c* of *Ps. denitrificans* during denitrification. The cuvettes contained 1.75×10^{10} cells in 2.5 mL, 5 mM glutamate or 0.1 mg of dithionite as reductant, 1 mM nitrous oxide or 1 mM nitrite as oxidant, and 40 μM CCCP or 400 μM azide as inhibitor: (A) glutamate minus glutamate; (B) glutamate minus nitrite; (C) dithionite minus nitrite; (D) nitrite minus nitrite; (E) glutamate minus N_2O + glutamate in steady-state oxidation; (F) E after exhaustion of N_2O; (G) glutamate minus N_2O + glutamate + CCCP in steady-state oxidation; (H) glutamate minus N_2O + glutamate + azide in steady-state oxidation; (I) glutamate minus nitrite + glutamate in steady-state oxidation; (J) I after exhaustion of nitrite; (K) glutamate minus nitrite + glutamate + CCCP in steady-state oxidation. All traces begin at 600 nm and sweep from right to left toward shorter wavelengths at 50 nm $\times$ min^{-1}. The mark at about 555 nm is a transient caused by the automatic changing of a filter.

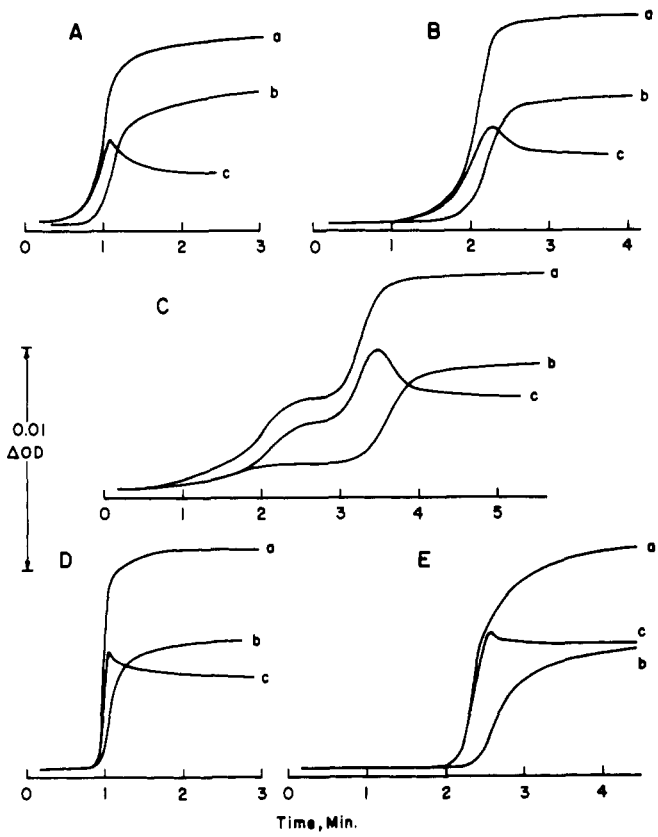

FIGURE 9: Dual wavelength extinction differences in the region of the α bands of cytochromes *b* and *c* of *Ps. denitrificans* during denitrification: (a) 551 minus 600 nm for contribution of cytochromes *c* predominantly; (b) 559 minus 600 nm for contribution of cytochromes *b* predominantly; (c) 551 minus 559 for differential response of cytochromes *b* and *c*. The cuvettes contained 1.75×10^{10} cells in 2.5 mL, 5 mM glutamate as reductant, 0.1 mM nitrite or 0.3 mM nitrous oxide as oxidant, and CCCP as inhibitor: (A) nitrite; (B) nitrite + 6 μM CCCP; (C) nitrite + 10 μM CCCP; (D) N_2O; (E) N_2O + 10 μM CCCP. Oxidant was added at time zero, and the upward deflections represent cytochrome reductions following the exhaustion of oxidant.

component at about 553 nm had an amplitude about twice that of the cytochromes *b* component which appeared as a shoulder at about 559 nm. Cytochromes *b* and *c* were nearly completely oxidized in steady-state turnover both in the absence and presence of CCCP, as illustrated in Figure 8E, G (nitrous oxide as oxidant), I, and K (nitrite as oxidant). In fact, the cytochromes seemed to be more strongly oxidized in the presence of uncoupler than in its absence (Figure 8I, K). As shown in Figure 8H, azide produced a largely reduced steady state. Azide has been shown by Matsubara (1975) to inhibit nitrous oxide reduction at a site on the oxidizing side of cytochromes *b* and *c*. The results of Figure 8 indicate that the site of inhibition by uncouplers lies on the reducing side of cytochromes *b* and *c*.

This view was reinforced by the results of Figure 9 which shows the kinetics of return from the steady-state to the glutamate-reduced state in a dual-wavelength spectrophotometer. The transition from glutamate-reduced state to steady state was too rapid to measure by the manual technique used and always lay within the 10 s required to inject a nitrogen oxide through the serum cap, mix, and replace the cuvette. The transition from steady state to reduced state was markedly slowed by CCCP, as can be seen from the slopes of the curves in the transition region. Under most conditions, cytochromes *b* and *c* showed similar kinetics in the transition, as evidenced by the fact that the 551 nm minus 559 nm curves showed usually only a small overshoot (Figure 9A,B,D,E). The overshoot implies that cytochromes *c* lead cytochromes *b* by a few seconds. At CCCP concentrations of 10 μM and above, the response of cytochromes *c* during nitrite reduction was biphasic with one component undergoing reduction well before cytochromes *b* and the second preceding cytochromes *b* by 10–15 s (Figure 9C). This complex behavior was not seen when nitrous oxide was the oxidant (Figure 9E). Ignoring this complexity, it is clear overall that uncouplers can inhibit the reduction of cytochromes *b* and *c*.

Figures 8 and 9 illustrate the behavior of cells in the presence of the more effective uncouplers. The intense absorption of DNP and the high concentrations required for an effect defeated efforts to obtain useful spectrophotometric data with this uncoupler.

Discussion

The mechanism by which uncouplers of oxidative phosphorylation reversibly inhibit denitrification is probably related to the disruption of one or another redox reaction of the electron-transport system as the result of the penetration of the uncoupler into the cell membrane: (a) the potency of inhibition of denitrification shows a rank order similar to potency in uncoupling oxidative phosphorylation and in affecting certain membrane properties, (b) the site of inhibition lies specifically on the reducing side of cytochromes *b* and *c*, and (c) certain nonuncoupling analogues of uncouplers are also effective as inhibitors.

Of particular interest among these analogues were 3,5-dinitrobenzoate and carboxy-CCP. These carry carboxylate groups which permit the hydrophobic portions to insert into but not easily traverse the membrane, just as is the case with

ionic detergents. They were, respectively, about as effective as were DNP and CCCP (Table II). Anionic and cationic detergents likewise inhibited, but the lower alcohols were relatively ineffective. Inhibition by 3,5-dinitroanisole proved that an acidic function was not necessary. While 3-nitrophenol and phenol have similar p*K* values (about 10), the former was more effective than the latter, thus suggesting that ring substituents may have at least some importance. The results overall suggest that inhibition minimally requires the interaction of rather bulky organic groups with the membrane or a membrane protein and thus may be thought of as a hydrophobic effect.

Studies concerning the inhibition of active transport, release of respiratory control, and proton permeability, etc., were carried out to test the possibility that some reaction in denitrification may be endergonic and thus may require a proton motive force or ATP to occur. Potential analogies were the energy-dependent transhydrogenases, nitrogenase, or nitrite oxidation in *Nitrobacter* (Cobley, 1976a,b). Our studies show that the idea of endergonic reactions in denitrification is unsupportable. Data of the kind presented in Table II and Figures 3, 5, and 7 show that the concentration of uncoupler needed to inhibit denitrification is at least ten times that required to inhibit endergonic processes or greatly increase proton permeability. Table III shows that a different class of uncouplers, the ionophores valinomycin and nigericin, fails to inhibit denitrification.

Matsubara (1975) reported that the effect of 0.1 mM DNP in a denitrifier of the *Alcaligenes/Achromobacter* group resembled that of azide by inhibiting on the oxidizing side of cytochromes *b* and *c* and in being specific against nitrous oxide reduction. We saw little effect at the 0.1 mM DNP level with our two denitrifiers and have not succeeded with spectrophotometric studies at and above 1 mM DNP. A clear difference between our systems and those of Matsubara (1975), Matsubara and Mori (1968), and Sacks and Barker (1952) is that we observed inhibition of nitrite reduction by DNP. The basis of the differences is unknown. With our strains of *Ps. denitrificans* and *Ps. aeruginosa,* DNP behaved like the other weakly acidic uncouplers in inhibiting the reduction commonly of nitrate, nitrite, and nitrous oxide.

Oxygen respiration in *Ps. denitrificans* seemed to be insensitive to uncouplers and exhibited a degree of respiratory control which we have been unable to observe in nitrogen oxide respirations. It is therefore likely that components of oxygen respiration differ from those associated with nitrogen oxide respirations.

Note Added in Proof

Studies were extended to *Paracoccus denitrificans,* ATCC 19367, with the remarkable result that oxygen respiration in this organism was also inhibited by uncouplers or their analogues at relatively low concentrations. Unlike the situation shown in Figure 5, the initial stimulation in respiration gave way to inhibition in the case of *Pa. denitrificans.* The concentrations of DNP, CCCP, and carboxy-CCP required to inhibit oxygen respiration by 50% were, respectively, about 1000, 7, and 3 μM for both anaerobically (nitrate) and aerobically grown cells. The corresponding values for 50% inhibition of nitrite respiration by anaerobically grown cells were, respectively, about 500, 13, and 35 μM. In *Pa. denitrificans* uncouplers and their analogues seem to be general rather than specific inhibitors of respiration.

Acknowledgment

The authors acknowledge the interest and advice of J.-s. Hong and C. Miller.

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