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The Protonmotive Force in Phosphorylating Membrane Vesicles from *Paracoccus denitrificans*

MAGNITUDE, SITES OF GENERATION AND COMPARISON WITH THE PHOSPHORYLATION POTENTIAL

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1. The magnitude of the protonmotive force in phosphorylating membrane vesicles from *Paracoccus denitrificans* was estimated. The membrane potential component was determined from the uptake of $S^{14}CN^-$, and the transmembrane pH gradient component from the uptake of [^{14}C]methylamine. In each case a flow-dialysis technique was used to monitor uptake. 2. With NADH as substrate, the membrane potential was about 145mV and the pH gradient was below 0.5 pH unit. The membrane potential was decreased by approx. 15mV during ATP synthesis, and was abolished on addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. In the presence of KCl plus valinomycin the membrane potential was replaced by a pH gradient of 1.5 units. 3. Succinate oxidation generated a membrane potential of approx. 125mV and the pH gradient was below 0.5 pH unit. Oxidation of ascorbate (in the presence of antimycin) with either 2,3,5,6-tetramethyl-*p*-phenylenediamine or *NNN'N'*-tetramethyl-*p*-phenylenediamine as electron mediator usually generated a membrane potential of approx. 90mV. On occasion, ascorbate oxidation did not generate a membrane potential, suggesting that the presence of a third energy-coupling site in *P. denitrificans* vesicles is variable. 4. With NADH or succinate as substrate, the phosphorylation potential ($\Delta G_p = \Delta G^{0'} + RT \ln [ATP]/[ADP][P_i]$) was approx. 53.6kJ/mol (12.8kcal/mol). Comparison of this value with the protonmotive force indicates that more than 3 protons need to be translocated via the adenosine triphosphatase of *P. denitrificans* for each molecule of ATP synthesized by a chemiosmotic mechanism. In the presence of 10mM-KNO_3 the protonmotive force was not detectable (<60mV) but ΔG_p was not altered. This result may indicate either that there is no relationship between the protonmotive force and ΔG_p, or that for an unidentified reason the equilibration of SCN^- or methylamine with the membrane potential and the pH gradient is prevented by NO_3^- in this system.

The respiratory chain of *Paracoccus denitrificans* shows many similarities to the mitochondrial respiratory chain, both in the types of components present and in its sensitivity to respiratory inhibitors (for review see John & Whatley, 1977). Phosphorylating membrane vesicles prepared from the plasma membrane of *P. denitrificans* resemble submitochondrial particles in that the vesicle membrane has an orientation opposite to that of the intact cell, so that the ATPase is directly accessible to ATP and ADP present in the reaction medium. The *P. denitrificans* phosphorylating membrane vesicles differ from submitochondrial particles in that they exhibit the respiratory control characteristic of intact mitochondria (John & Hamilton, 1971). We have determined (Sorgato *et al.*, 1978) the magnitude of the protonmotive force (Δp) (Mitchell, 1966) generated by submitochondrial particles, and proceeded to compare this value with the phosphorylation potential (ΔG_p) attained. The principal aim of the present work was to make similar determinations with the phosphorylating vesicles from *P. denitrificans* and to ascertain whether the tight respiratory control of these vesicles is associated with a significantly higher Δp.

Abbreviation used: ATPase, adenosine triphosphatase (EC 3.6.1.3).

Further, comparison of Δp with ΔG_p has allowed us to determine a minimal H^+/ATP ratio for *P. denitrificans*, since according to the chemiosmotic hypothesis (Mitchell, 1966) ΔG_p is related to Δp by the following relationship:

$$\Delta G_p = -zF\Delta P \quad (1)$$

where F is the Faraday constant and z is the number of protons translocated via the ATPase for each molecule of ATP synthesized, and is thus numerically equal to the H^+/ATP ratio.

In the absence of previous determinations of the H^+/ATP ratio for respiratory bacteria, an H^+/ATP ratio of 2 has been widely adopted (see, e.g., Haddock & Jones, 1977) to convert experimentally determined H^+/O ratios into P/O ratios. Although in chromatophores from *Rhodopseudomonas capsulata* the relatively high Δp values (250–400mV), by comparison with ΔG_p, have been shown to be compatible with an H^+/ATP ratio of 2 (Casadio *et al.*, 1974), from similar experiment with chloroplasts (Hauska & Trebst, 1977; Junge, 1977), submitochondrial particles (Sorgato *et al.*, 1978) and chromatophores from *Rhodospirillum rubrum* (Leiser & Gromet-Elhanan, 1977; Kell *et al.*, 1978) there is evidence that the H^+/ATP ratio is at least 3.

When *P. denitrificans* is grown heterotrophically in the absence of O_2, with nitrate as the added terminal electron acceptor, electron flow through the terminal oxidase region is not coupled to ATP synthesis (John & Whatley, 1970). There is conflicting evidence as to whether the terminal oxidase reaction in aerobically heterotrophically grown *P. denitrificans* is a site of free-energy conservation (van Verseveld & Stouthamer, 1976; Edwards *et al.*, 1977; Lawford, 1978; John & Whatley, 1977; Meijer *et al.*, 1977; Stouthamer, 1977). A further purpose of the present work was to investigate whether electron flow through the terminal oxidase in membrane vesicles derived from cells grown with nitrate as added terminal electron acceptor was linked to the generation of a protonmotive force.

Materials and Methods

Paracoccus denitrificans (*Micrococcus denitrificans* N.C.I.B. 8944) was maintained and grown anaerobically with succinate as the carbon source and nitrate as the added terminal electron acceptor as described previously (Burnell *et al.*, 1975). Phosphorylating membrane vesicles were prepared by the method of Burnell *et al.* (1975) with the modification of John (1977).

The determination of $KS^{14}CN$ and [^{14}C]methylamine uptake by using a flow-dialysis technique, the determination of oxygen uptake, adenine nucleotides and P_i, and the calculation of Δp, $\Delta\psi$, ΔpH and ΔG_p were all as described in Sorgato *et al.* (1978). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Cohn fraction V) as standard.

The fluorescence of 8-anilinonaphthalene-1-sulphonate was measured as described previously (Ferguson *et al.*, 1976*a*).

Venturicidin was a gift from Dr. D. E. Griffiths, Department of Molecular Sciences, University of Warwick, Coventry, U.K. All other materials were from the sources described previously (Sorgato *et al.*, 1978).

Results

Determination of the internal volume and homogeneity of the vesicles

The internal volume of the vesicles was estimated by the sucrose-impermeable-space method by the procedure described previously (Kell *et al.*, 1978; Sorgato *et al.*, 1978). With three preparations, values of 5.9, 4.1 and 4.0μl/mg of protein were obtained, yielding an average of 4.7μl/mg of protein. This method estimates the total volume enclosed by the vesicles, but the present work is concerned only with those vesicles that have a membrane polarity opposite to that of the intact cell. Burnell *et al.* (1975) showed that vesicle preparations from *P. denitrificans* made in a similar way to those used in the present work contain two types of vesicle: right-side-out vesicles, in which the vesicle membrane has the same orientation as the plasma membrane of the intact cell; and inside-out vesicles, in which the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell. We have estimated, from the degree of latency of the NADH dehydrogenase (EC 1.6.99.3) (Burnell *et al.*, 1975), that on average about 40% of the vesicles in the preparations used in the present work are of an inside-out configuration. We have determined the percentage of the vesicles that are inside-out for each preparation used in the present study, and used this percentage to estimate the volume of the inside-out vesicles in each preparation, for the determination of the $\Delta\psi$ and ΔpH values. Our estimation of the internal volume of the inside-out vesicles will be in error if the right-side-out vesicles are larger (or smaller) than the inside-out vesicles, and hence enclose a larger (or smaller) volume per mg of protein. However, we found no significant correlation between the proportion of inside-out vesicles in a given preparation and the specific internal volume enclosed by that preparation. We note that our value of 4.7μl/mg of protein is higher than the internal volumes of 0.5–4.0μl/mg of protein reported for membrane vesicles isolated from *Escherichia coli* (Kaback & Barnes, 1971) and *Bacillus subtilis* (see Konings, 1977). However, our present determinations are not very sensitive to errors in the estimation of the internal volume; for

example, a 2-fold overestimation of the internal volume would lead to an underestimation of only 18mV in either $\Delta\psi$ or ΔpH (Sorgato *et al.*, 1978).

If some of the vesicles could generate a high Δp and were thus capable of synthesizing ATP, but others could generate only a low Δp that was less than that required to activate the ATPase, the apparent Δp value determined would be an average value, and the ΔG_p would be only due to the more tightly coupled vesicles. Comparison of the Δp and ΔG_p values obtained under these circumstances would be invalid. However, there are two lines of argument to indicate that the inside-out *P. denitrificans* vesicles are not a significantly heterogeneous population with respect to their energy-coupling characteristics. First, the P/O ratio of approx. 1.6 that is observed when NADH is substrate (John & Whatley, 1970, 1975) means that, even if the P/O ratio of fully coupled vesicles is 3, then less than half of the vesicles can be non-phosphorylating. Secondly, the profile of sensitivity of respiration to the inhibitor 2-n-heptyl-4-hydroxyquinoline *N*-oxide (Fig. 1) suggests that only a negligible fraction of the vesicles can be uncoupled. Fig. 1 shows that the rate of NADH oxidation by the vesicles in the presence of an uncoupler was inhibited by increasing concentrations of 2-n-heptyl-4-hydroxyquinoline *N*-oxide, whereas the rate of respiration in the absence of an uncoupler (State 4) (Chance & Williams, 1956) was not affected over the same concentration range. If there had been a significant fraction of vesicles that were naturally uncoupled it would have been expected that the respiration by these vesicles in State 4 would be partially sensitive to 2-n-heptyl-4-hydroxyquinoline *N*-oxide, as the State-4 rate would reflect the controlled rate of respiration by the coupled vesicles as well as the uncontrolled rate of respiration by the uncoupled vesicles.

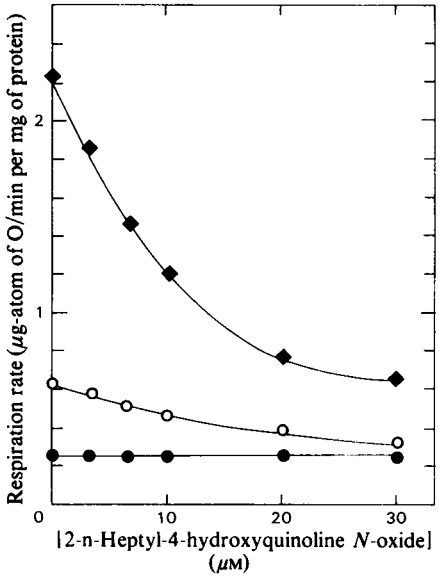

Fig. 1. *Effect of 2-n-heptyl-4-hydroxyquinoline N-oxide on coupled and uncoupled respiration of P. denitrificans vesicles*

Respiration was monitored polarographically in a reaction medium containing 10mM-P_i/Tris, pH7.3, 5mM-magnesium acetate, 1% (v/v) ethanol, 50μg of alcohol dehydrogenase and membrane vesicles (1.0mg of protein) in a final volume of 3ml. The temperature was 30°C. The particles were preincubated with the appropriate concentration of 2-n-heptyl-4-hydroxyquinoline *N*-oxide before initiating respiration by addition of 0.6mM-NAD^+ (●); 0.2mM-ADP (○) or 0.5μg of gramicidin D plus 30mM-ammonium acetate (◆) were present as indicated.

Determination of $\Delta\psi$ with NADH as substrate

Uptake of $S^{14}CN^-$ into vesicles respiring with NADH is shown in Fig. 2, in which is plotted the radioactivity in sequentially collected fractions of the outflow from the lower chamber of the flow-dialysis cell. The experiment was started by adding $KS^{14}CN$ to the upper chamber of the dialysis cell, at which time collection of the outflow from the lower chamber

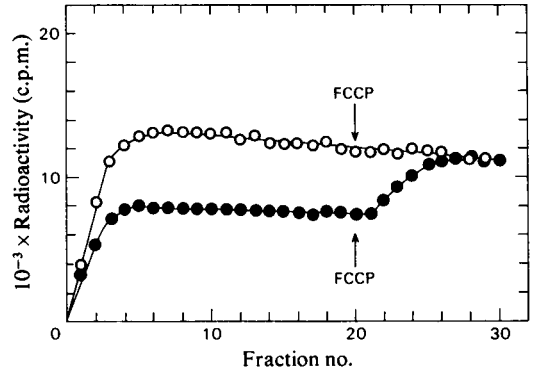

Fig. 2. *Uptake of $S^{14}CN^-$ by membrane vesicles from P. denitrificans*

The radioactivity measured in the outflow from the flow-dialysis cell is plotted against the fraction number. Reaction mixtures contained in a final volume of 1ml: 10mM-P_i/Tris, pH7.3, 5mM-magnesium acetate, 50μg of yeast alcohol dehydrogenase, 1% (v/v) ethanol and 0.6mM-NAD^+. Membrane vesicles (0.94mg of protein) were included in (●) or omitted from (○) the reaction mixture as indicated. The temperature was 23°C. At time zero, 10μl of 2.08mM-$KS^{14}CN$ (60mCi/mmol) was added to the upper chamber and the flow started. After fraction 20 had been collected 2μl of 2.5mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to the upper chamber as indicated by the arrow in the Figure.

was begun. The presence of respiring vesicles in the upper chamber resulted in decreased steady-state amounts of radioactivity in the outflow. This is attributed to a decreased steady-state concentration of $S^{14}CN^-$ in the upper chamber due to the accumulation of $S^{14}CN^-$ by the vesicles. An addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone resulted in an efflux of $S^{14}CN^-$ from the vesicles, which was reflected in the increased amounts of radioactivity in the outflow (Fig. 2). $\Delta\psi$ was calculated from the extent of $S^{14}CN^-$ uptake, which was, for the reasons described previously (Sorgato *et al.*, 1978), obtained from the difference between the radioactivity in a given fraction after carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone addition and the amount that would have been in that fraction had carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone not been added. This latter value is obtained by extrapolation. From a series of experiments performed under the conditions of Fig. 2 with 22 different vesicle preparations (0.6–2.7mg of protein/ml of reaction medium) an average value for $\Delta\psi$ of 145mV (S.E.M. $\pm$3mV) was obtained.

When the initial concentration of $KS^{14}CN$ added to the reaction mixture in the upper chamber was increased there was a slight decrease in $\Delta\psi$ (Fig. 3). When the initial concentration of $KS^{14}CN$ was maintained at 20μM and the vesicle concentration increased there was a slight increase in $\Delta\psi$ (Fig. 4). This apparent variation of $\Delta\psi$ with various concentrations of $KS^{14}CN$ and of vesicles may be due to some binding of $S^{14}CN^-$ to the vesicles, or it may be due to a slight uncoupling effect of $S^{14}CN^-$ at the higher $S^{14}CN^-$/vesicle ratios. These effects can be seen to be of little significance, however, since extrapolation of the measured $\Delta\psi$ values back to zero KSCN concentration (Fig. 3*b*) or to an infinite vesicle concentration (Fig. 4*b*) increases $\Delta\psi$ by only 5–10mV (Figs. 3 and 4) compared with the $\Delta\psi$ values measured with the 20μM-$KS^{14}CN$ and approx. 2mg of protein/ml present as a routine.

When ADP, glucose and hexokinase were included in the reaction medium, the extent of $S^{14}CN^-$ uptake was decreased by an amount that was equivalent to a decrease in $\Delta\psi$ of about 15mV (Fig. 5). A subsequent addition of venturicidin, which inhibits the ATPase of *P. denitrificans* (Ferguson & John, 1977), caused $\Delta\psi$ to return to the value observed in the absence of ADP (Fig. 5).

Determination of ΔpH with NADH as substrate

Fig. 6 shows that vesicles respiring with NADH as substrate did not take up a detectable amount of [^{14}C]methylamine; thus the addition of 5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which caused efflux of $S^{14}CN^-$ under the same conditions (Figs. 2–5), had no effect on the [^{14}C]-

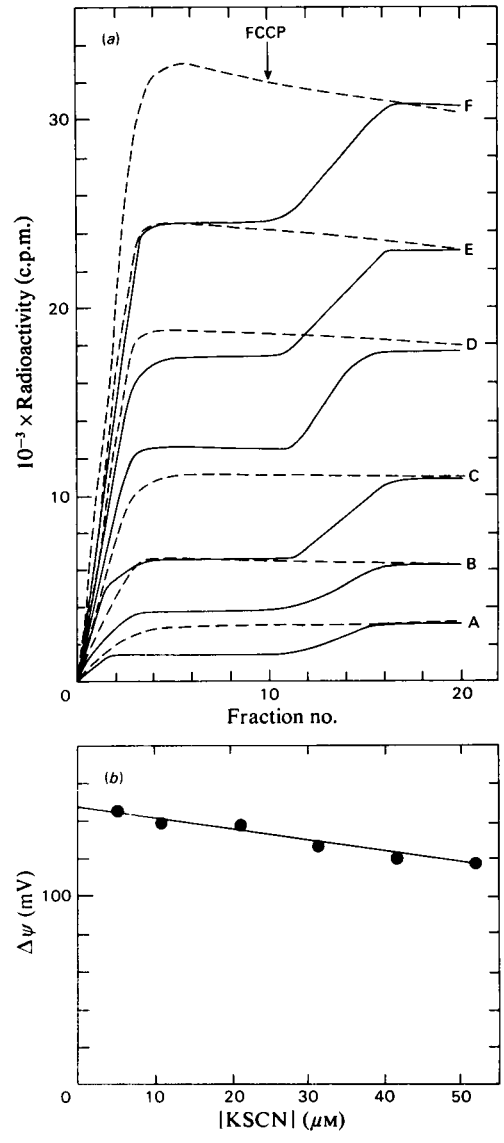

Fig. 3. *Effect of varying the KSCN concentration on the uptake of $S^{14}CN^-$ by P. denitrificans vesicles*

(*a*) Reaction conditions were as described in the legend to Fig. 2, except that the concentration of radioactive $S^{14}CN^-$ used to start the reaction was varied at constant specific radioactivity. Membrane vesicles (0.94mg of protein) were either present (——) or absent (– – – –). The initial $KS^{14}CN$ concentrations (μM) in the upper chamber of the flow-dialysis cell were: (A) 5.2; (B) 10.4; (C) 20.8; (D) 31; (E) 42; (F) 52. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added to the upper chamber after ten fractions had been collected as indicated by the arrow. (*b*) Data of (*a*) replotted to show the effect of KSCN concentration on the value of $\Delta\psi$.

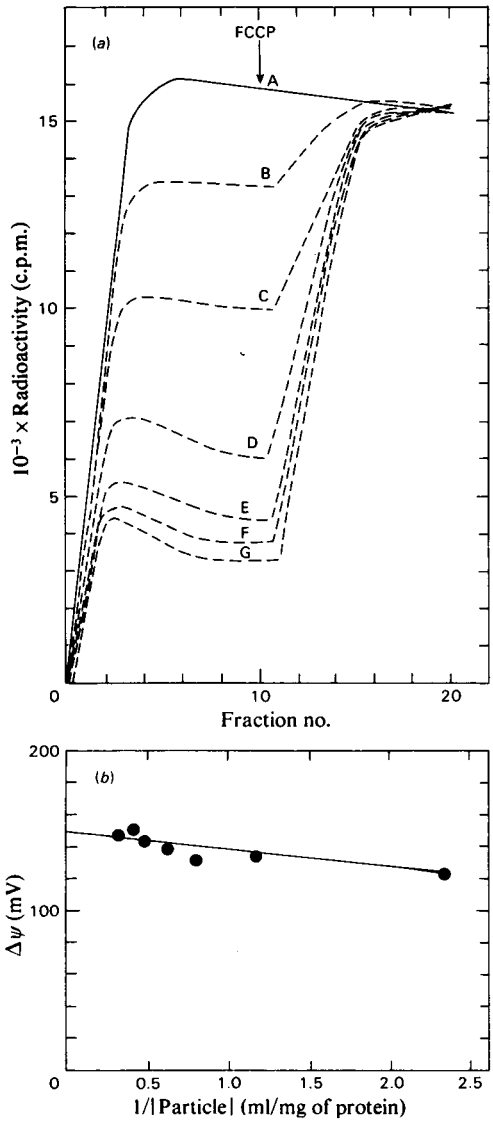

Fig. 4. *Effect of vesicle concentration on the uptake of $S^{14}CN^-$ by P. denitrificans vesicles*

(*a*) Flow dialysis was performed as described in the legend to Fig. 2 except that the $KS^{14}CN$ concentration was 20.8 μM, and the vesicle concentrations were (mg of protein/ml): (A) 0; (B) 0.43; (C) 0.85; (D) 1.70; (E) 2.13; (F) 2.55; (G) 2.98. (*b*) Effect of particle concentration on Δψ, calculated from the data plotted in (*a*). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5 μM) was added as indicated.

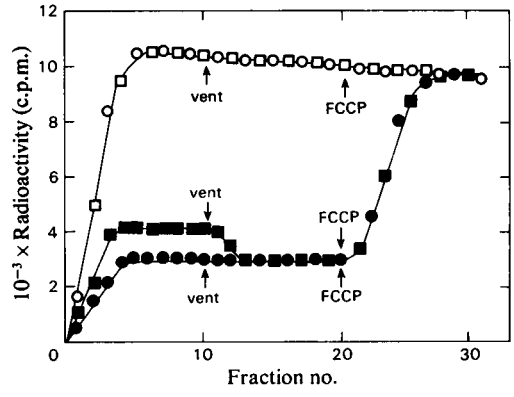

Fig. 5. *Effect of ATP synthesis on $S^{14}CN^-$ uptake by P. denitrificans vesicles*

Flow dialysis was carried out as described in the legend to Fig. 2. The reaction mixtures contained in a final volume of 1 ml: 10 mM-P_i/Tris, pH 7.3, 5 mM-magnesium acetate, 50 μg of alcohol dehydrogenase, 10 μl of ethanol and 0.6 mM-NAD^+. At zero time 20.8 μM-$KS^{14}CN$ (60 mCi/mmol) was added. Additional components were: ○, none; □, 10 mM-glucose, 5 units of hexokinase and 0.5 mM-ADP; ●, membrane vesicles (2.67 mg of protein); ■, 10 mM-glucose, 5 units of hexokinase, 0.5 mM-ADP and membrane vesicles (2.67 mg of protein). The arrows indicate the successive additions of 2 μg of venturicidin (vent) (after collection of fraction 10), and 5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (after collection of fraction 20). One unit of hexokinase catalyses the phosphorylation of 1.0 μmol of glucose/min at 25°C, pH 8.5.

methylamine concentrations in the reaction mixture, even in the presence of 5 μM-rotenone to restrict electron transport and thus enhance the effectiveness of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone as an uncoupler. Similarly, an addition of 0.1% Triton X-100 instead of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone under the conditions of Fig. 6 did not result in any efflux of [^{14}C]methylamine from the vesicles. An uptake of 3% of the methylamine present would have been detectable. This would have been equivalent to a ΔpH of 0.5 unit. Thus we conclude that ΔpH under our conditions is below 0.5 unit.

Further evidence that a significant ΔpH was absent during NADH oxidation by the *P. denitrificans* vesicles was that the addition of potassium acetate (10 mM) and nigericin (1 μg/mg of protein) under the conditions of Fig. 2 did not result in a measurable increase in the uptake of $S^{14}CN^-$. An analogous addition of K^+ and nigericin to chromatophores replaces the ΔpH with an energetically equivalent Δψ as a result of the electroneutral exchange of internally accumulated H^+ for external K^+ (Gromet-Elhanan, 1977).

A ΔpH was detected when KCl and valinomycin were included in the reaction mixture (Table 1). The

appearance of a ΔpH under these conditions was accompanied by the disappearance of $\Delta\psi$. When nigericin was present in addition to KCl and valinomycin both $\Delta\psi$ and ΔpH were no longer detected (Table 1). Presumably the replacement of

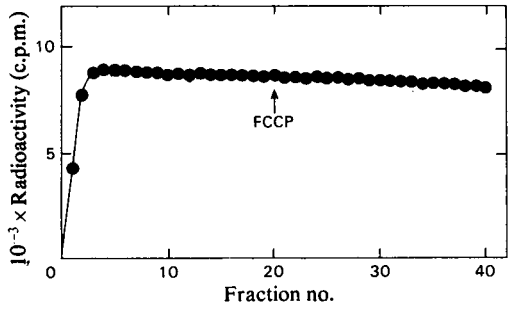

Fig. 6. *Absence of detectable [^{14}C]methylamine uptake by respiring P. denitrificans membrane vesicles*
Flow dialysis was performed with the reaction mixture and by the method described in the legends to Fig. 2, except that the $S^{14}CN^-$ was replaced by 10 μl of 2.25 mM-[^{14}C]methylamine hydrochloride (55.5 mCi/mmol), and 1.82 mg of vesicle protein was present. After 20 fractions had been collected (as arrowed), 2 μl of 2.5 mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to the upper chamber. The addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone did not affect the rate of flow of methylamine through the dialysis membrane, as was shown in a control experiment in which vesicles were omitted from the upper chamber (not shown for reasons of clarity).

Table 1. *Effect of salts and ionophores on the components of the protonmotive force in P. denitrificans vesicles*
$\Delta\psi$ and ΔpH were measured from the extent of $S^{14}CN^-$ or [^{14}C]methylamine uptake by the flow-dialysis procedure. The upper chamber of the flow-dialysis cell contained in a volume of 1 ml: 10 mM-P_i/Tris, pH 7.3, 5 mM-magnesium acetate and membrane vesicles (Expt. 1, 1.8 mg of protein; Expt. 2, 2.9 mg of protein; Expt. 3, 2.0 mg of protein), plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. For measurements of $\Delta\psi$ 20 μM-$KS^{14}CN$ (60 mCi/mmol) was added to the upper chamber, and for measurements of ΔpH 20 μM-[^{14}C]methylamine hydrochloride (55.5 mCi/mmol) was added. NADH was the substrate and 0.6 mM-NAD^+, 1% (v/v) ethanol and 50 μg of alcohol dehydrogenase were added. For experiments in which either $\Delta\psi$ or ΔpH was not observed the lower limit of detection is signified by <. The values of Δp given in parentheses represent upper limits on Δp, obtained by adding the lower limit of detection for ΔpH and $\Delta\psi$ to the observed ΔpH or $\Delta\psi$.

Expt. no.	Additions	$\Delta\psi$ (mV)	ΔpH	Δp (mV)
1	None	150	<0.5	150 (180)
	50 mM-KCl	115	<0.5	115 (145)
	50 mM-KCl + valinomycin (1 μg)	<30	1.5	90 (120)
	50 mM-KCl + valinomycin (1 μg) + nigericin (1 μg)	<30	<0.5	0 (60)
2	None	165	<0.5	165 (195)
	10 mM-KSCN	<30	<0.5	0 (60)
3	None	150	<0.5	150 (180)
	10 mM-KNO_3	<30	<0.5	0 (60)

Table 2. *Protonmotive force measured with vesicles from P. denitrificans oxidizing various substrates*
$\Delta\psi$ and ΔpH were measured from the extent of $S^{14}CN^-$ or [^{14}C]methylamine uptake by the flow-dialysis procedure. The upper chamber of the flow-dialysis cell contained in a volume of 1 ml: 10 mM-P_i/Tris, pH 7.3, 5 mM-magnesium acetate and membrane vesicles (between 1.0 and 2.6 mg of protein), plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. For measurements of $\Delta\psi$ 20 μM-$KS^{14}CN$ (60 mCi/mmol) was added to the upper chamber, and for measurements of ΔpH 20 μM-[^{14}C]methylamine hydrochloride 55.5 mCi/mmol) was added. When NADH was the substrate 0.6 mM-NAD^+, 1% (v/v) ethanol and 50 g of alcohol dehydrogenase were added. When succinate was the substrate 10 mM-sodium succinate was added. When ascorbate was the substrate 10 mM-sodium D-isoascorbate plus either 0.1 mM-2,3,5,6-tetramethyl-*p*-phenylenediamine or 0.1 mM-*NNN'N'*-tetramethyl-*p*-phenylenediamine were added. When present the concentration of antimycin was 1 μg/mg of protein. For experiments in which ΔpH was not observed the lower limit of detection is signified by <. The values of Δp given in parentheses represent upper limits on Δp, obtained by adding the lower limit of detection for ΔpH to the observed $\Delta\psi$. Abbreviation: n.d., not determined.

Substrate	Addition	$\Delta\psi$ (mV)	ΔpH	−59ΔpH	Δp (mV)
NADH		145	<0.5	<30	145 (175)
Succinate		125	<0.5	<30	125 (155)
Ascorbate + 2,3,5,6-tetramethyl-*p*-phenylenediamine		120	<0.8	<50	120 (170)
Ascorbate + 2,3,5,6-tetramethyl-*p*-phenylenediamine	Antimycin	85	n.d.	n.d.	85
Ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine		90	n.d.	n.d.	90
Ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine	Antimycin	70	n.d.	n.d.	70

$\Delta\psi$ by ΔpH is due to the efflux of K^+ via valinomycin neutralizing $\Delta\psi$ and thereby allowing a greatly enhanced H^+ uptake (Jackson *et al.*, 1968). A similar replacement of $\Delta\psi$ by ΔpH should occur when $\Delta\psi$ is neutralized by the electrophoretic uptake of high concentrations of permeant ions. Table 1 shows, however, that the addition of 10mM-KNO_3 or 10mM-KSCN resulted in the loss of a measurable $\Delta\psi$, but not in the appearance of an energetically equivalent ΔpH.

The cells from which the vesicles used in the present work were isolated showed a significant rate of methylamine-dependent oxygen uptake (about 10μmol of O_2/min per g dry wt.) (cf. Cox & Quayle, 1975), which was inhibited by 0.5mM-hydrazine (D. B. Kell, unpublished work). However, the vesicles showed no detectable methylamine-dependent respiration (<3nmol of O_2/min per mg of protein) when incubated with 0.1mM-methylamine hydrochloride, either in the presence or absence of NAD^+ (0.6mM), under reaction conditions similar to those described for Fig. 1. This indicates that oxidation of methylamine by the vesicles could not account for our failure to observe respiration-dependent [^{14}C]methylamine uptake.

Determination of Δp generated by succinate or ascorbate oxidation, or by ATP hydrolysis

The oxidation of succinate, and the oxidation of ascorbate (mediated by either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine), generated a consistently lower $\Delta\psi$ than did the oxidation of NADH (Table 2), but in no case was there a measurable ΔpH. An addition of antimycin, at a concentration just sufficient to inhibit maximally the oxidation of NADH, somewhat decreased the $\Delta\psi$ generated by the oxidation of ascorbate (Table 2). The presence of energy coupling associated with the oxidation of ascorbate, in the presence of 2,3,5,6-tetramethyl-*p*-phenylenediamine, was confirmed by the observation of a carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-sensitive enhanced fluorescence of 8-anilinonapthalene-1-sulphonate, although the magnitude of both the rate and extent of fluorescence enhancement was less than that induced by succinate (Fig. 7). On occasion, ascorbate oxidation mediated by *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine gave no detectable SCN^- uptake nor a significant carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-sensitive enhancement of 8-anilinonaphthalene-1-sulphonate fluorescence, even though the same preparations gave the usual $\Delta\psi$ value and enhancement of 8-anilinonaphthalene-1-sulphonate fluorescence with NADH or succinate as substrate.

When the vesicles were incubated with ATP instead of a respiratory substrate no detectable $\Delta\psi$ or ΔpH was observed. The absence of a detectable Δp associated with the low rate of ATP hydrolysis observed with the *P. denitrificans* vesicles is compatible

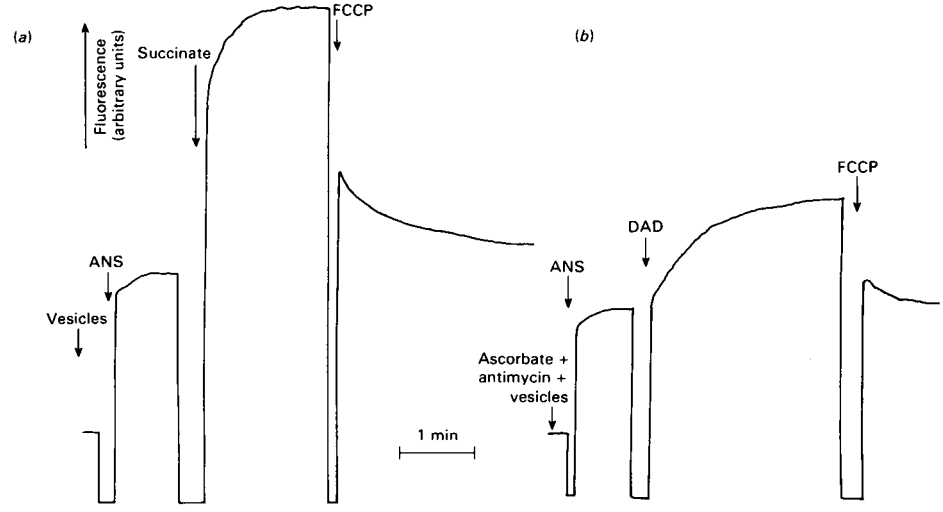

Fig. 7. *Energy-linked enhancement of 8-anilinonaphthalene-1-sulphonate fluorescence*
Anilinonaphthalenesulphonate (ANS) (5μM) was added to 3ml of 10mM-P_i/Tris, pH7.3, containing 5mM-magnesium acetate and *P. denitrificans* vesicles (0.37mg of protein). The temperature was 30°C. (*a*) Sodium succinate (10mM) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (3μM) were subsequently added as indicated. (*b*) Antimycin (0.3μg) and sodium D-isoascorbate (10mM) were present initially; 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) (0.1mM) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (3μM) were added as shown.

Table 3. *Phosphorylation potentials generated by P. denitrificans vesicles*

In Expts. 1 and 2 *P. denitrificans* vesicles (2 mg of protein) from the same preparation were incubated in 1 ml of a reaction mixture that contained 10 mM-P_i/Tris, pH 7.3, 5 mM-magnesium acetate, 20 μM-KSCN and, initially, 1.7 mM-ATP plus 0.2 mM-ADP. When NADH was substrate (Expt. 1) 0.6 mM-NAD^+, 50 μg of yeast alcohol dehydrogenase and 1% (v/v) ethanol were also present. When succinate was the substrate (Expt. 2) 5 mM-sodium succinate was also added. The incubations were carried out at 23°C in the flow-dialysis cell over which water-saturated O_2 was blown. After 8 (Expt. 1) or 10 (Expt. 2) min the reaction mixture was withdrawn from the flow-dialysis cell and added to 0.1 ml of ice-cold 40% $HClO_4$. In Expts. 3 and 4 *P. denitrificans* vesicles (0.05 mg of protein) from the same preparation (which was a different preparation from that used for Expts. 1 and 2) were incubated in 3 ml of a reaction mixture that contained 10 mM-P_i/Tris, pH 7.3, 5 mM-magnesium acetate, 0.6 mM-NAD^+, 50 μg of yeast alcohol dehydrogenase, 1% (v/v) ethanol and initially 0.2 mM-ADP. The incubations were carried out at 30°C in a thermostatically controlled cell for 5 min with the reaction mixture open to atmospheric O_2, and then 2 ml of the reaction mixture was withdrawn and added to 0.2 ml of ice-cold 40% (v/v) $HClO_4$. The acid extracts from Expts. 1–4 were left on ice for 10 min, and then the precipitated protein was removed by centrifugation at 2000*g*. The supernatants were neutralized by addition of the predetermined amount of 0.25 M-Tris/10% (w/v) KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralized extracts was determined with hexokinase and glucose 6-phosphate dehydrogenase, and ADP was assayed with pyruvate kinase and lactate dehydrogenase. P_i was measured by the method of Hurst (1964) in a Technicon Autoanalyzer. In calculating ΔG_p, a value for $\Delta G^{0'}$ of 30.1 kJ/mol (7.2 kcal/mol) was used (Rosing & Slater, 1972).

Expt. no.	Substrate	Addition	Final concentrations (mM) ATP	ADP	P_i	ΔG_p [kJ/mol (kcal/mol)]
1	NADH	—	1.8	0.013	9.8	53.9 (12.9)
2	Succinate	—	1.9	0.017	9.8	53.2 (12.74)
3	NADH	—	0.2	0.003	9.8	52.2 (12.5)
4	NADH	10 mM-KNO_3	0.2	0.003	9.8	52.2 (12.5)

with the effective irreversibility of this ATPase (Ferguson *et al.*, 1976*b*).

Determination of ΔG_p

Table 3 shows the ΔG_p values obtained with the *P. denitrificans* vesicles during the oxidation of NADH or succinate. The ΔG_p obtained with NADH was not significantly altered when 10 mM-KNO_3 was included in the reaction mixture (Table 3).

Discussion

The Δp determined for the respiration of NADH by the phosphorylating vesicles from *P. denitrificans* is similar to the Δp we have determined with submitochondrial particles (Sorgato *et al.*, 1978) and is greater than the Δp we have determined for chromatophores from *Rhodospirillum rubrum* (Kell *et al.*, 1978). However, the Δp values we have determined for all three of these systems are similar to the Δp values that have been claimed for intact cells of a variety of bacteria (see Hamilton, 1977), and for right-side-out vesicles of *Escherichia coli* (Ramos & Kaback, 1977), but are lower than values obtained for chromatophores from *Rhodopseudomonas capsulata* (Casadio *et al.*, 1974) and in some instances for intact mitochondria (Mitchell & Moyle, 1969; Nicholls, 1974). We have discussed the general validity of our methods (Kell *et al.*, 1978; Sorgato *et al.*, 1978). In the present study it appears unlikely that we have failed to detect a significant ΔpH since we have been able to measure a ΔpH when KCl plus valinomycin were added to convert $\Delta\psi$ into a ΔpH; and, further, there was no significant increase in $\Delta\psi$ when nigercin and potassium acetate were added to convert any ΔpH into $\Delta\psi$. Although we realize that other workers using solute-distribution methods have observed a ΔpH in both intact cells (Padan *et al.*, 1976) and right-side-out membrane vesicles (Ramos *et al.*, 1976) or *Escherichia coli* under certain conditions, we note that the magnitude of this ΔpH was insignificant at pH 7.3, the pH used in the present work.

The ΔG_p value of 53.6 kJ/mol (12.8 kcal/mol) determined for the phosphorylating vesicles of *P. denitrificans* is similar to the ΔG_p values determined for *Azotobacter vinelandii* (Eilermann & Slater, 1970, recalculated by Ferguson & Sorgato, 1977), *Rhodopseudomonas capsulata* (Casadio *et al.*, 1974), *Rhodospirillum rubrum* (Kell *et al.*, 1978), chloroplasts (Kraayenhof, 1969) and mitochondria (Cockrell *et al.*, 1966; Slater *et al.*, 1973), but is higher than the ΔG_p value of about 43.9 kJ/mol (10.5 kcal/mol) determined for submitochondrial particles (Ferguson & Sorgato, 1977). The significance of these similarities and this difference has been discussed (Ferguson & Sorgato, 1977).

If it is assumed that Δp and ΔG_p come into equilibrium in State 4 (as defined by Chance & Williams, 1956) then from eqn. (1), by using values for Δp and ΔG_p given in Tables 2 and 3 for NADH oxidation in the absence of NO_3^-, an H^+/ATP ratio of

more than 3 is derived for the *P. denitrificans* ATPase. Values for the H^+/ATP ratio for *P. denitrificans* may be underestimates since, as noted by Ferguson *et al.* (1976*b*), Δp may not reach equilibrium with ΔG_p. The chemiosmotic hypothesis envisaged an H^+/ATP ratio of 2 (Mitchell, 1966), but the H^+/ATP ratio of 3 or more derived from the present work is in line with findings with mitochondria (Wiechmann *et al.*, 1975; Rottenberg, 1975; Brand, 1977; Nicholls, 1977), submitochondrial particles (Rottenberg & Gutman, 1977; Sorgato *et al.*, 1978) and chloroplasts (McCarty & Portis, 1976; Hauska & Trebst, 1977; Junge, 1977). An H^+/ATP ratio of 3 for *P. denitrificans* would agree with the conclusions of Meijer *et al.* (1977), which were based on the measurements of the H^+/O ratio and growth yields of intact cells of *P. denitrificans*. Lawford (1978) has reported that the respiration-driven proton extrusion from cells of *P. denitrificans* is consistent with an H^+/2e per site ratio of 3. If the P/O ratio for *P. denitrificans* oxidizing NADH is 3 or less (John & Whatley, 1977) then the results of Lawford (1978) suggest that H^+/ATP is 3 or more.

The present observation of an undetectable Δp and an undiminished ΔG_p when 10mM-KNO_3 is present during NADH oxidation, and the previous observation of respiratory control and oxidative phosphorylation in the presence of nitrate (John & Whatley, 1970), could be taken together to suggest that Δp does not represent the sole mode of membrane energization and that there is no relationship between Δp and ΔG_p. However, it would be premature to call into question the essential nature of Δp for ATP synthesis until this curious effect has been more fully characterized.

The widely observed absence of ATP synthesis coupled to ascorbate oxidation by *P. denitrificans* vesicles with *NNN'N'*-tetramethyl-*p*-phenylenediamine as electron mediator has led some authors to conclude that the flow of reducing equivalents along the terminal region of the respiratory chain is not coupled to ATP synthesis (see John & Whatley, 1977). The present work shows that oxidation of ascorbate with either 2,3,5,6-tetramethyl-*p*-phenylenediamine or *NNN'N'*-tetramethyl-*p*-phenylenediamine as electron mediator can be linked to the generation of a protonmotive force. This finding does not necessarily mean that ascorbate oxidation will be able to drive ATP synthesis, as the protonmotive force is lower than when succinate or NADH is a substrate, and may therefore be below a critical threshold that is needed to activate ATP synthesis (cf. Baccarini-Melandri *et al.*, 1977; Junge, 1977). However, when reducing equivalents flow down the whole respiratory chain the contribution of the terminal region to ATP synthesis may prove to be more important then when antimycin is present and ascorbate (plus *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine) is the reductant, since the charge separation at the terminal region of the respiratory chain would be additional to the charge separations occurring at the other regions of the chain. Our finding that on occasion the oxidation of ascorbate plus 2,3,5,6-tetramethyl-*p*-phenylenediamine did not generate a protonmotive force with vesicles in which succinate oxidation did generate a protonmotive force suggests that the presence of an energy-coupling site in the terminal region of the respiratory chain may depend very finely on the exact environmental conditions and the phase of growth, in harmony with the suggestion of Edwards *et al.* (1977). Nevertheless the occasional absence of energy-linked ascorbate oxidation indicates that, when a protonmotive force is generated by ascorbate oxidation, proton translocation does occur as electrons flow through the terminal region of the respiratory chain. This conclusion parallels the arguments made in the accompanying paper (Sorgato *et al.*, 1978) that ascorbate, with either 2,3,5,6-tetramethyl-*p*-phenylenediamine or *NNN'N'*-tetramethyl-*p*-phenylenediamine as mediator, does not donate electrons to a putative proton-pumping redox carrier in the cytochrome bc_1 region of the respiratory chain.

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