The Mechanism of Proton Translocation Driven by the Respiratory Nitrate Reductase Complex of *Escherichia coli*

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Low concentrations (1–50 μM) of ubiquinol₁ were rapidly oxidized by spheroplasts of *Escherichia coli* derepressed for synthesis of nitrate reductase using either nitrate or oxygen as electron acceptor. Oxidation of ubiquinol₁ drove an outward translocation of protons with a corrected $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometry [Scholes & Mitchell (1970) *J. Bioenerg.* 1, 309–323] of 1.49 when nitrate was the acceptor and 2.28 when oxygen was the acceptor. Proton translocation driven by the oxidation of added ubiquinol₁ was also observed in spheroplasts from a double quinone-deficient mutant strain AN384 (*ubiA*[–] *menA*[–]), whereas a haem-deficient mutant, strain A1004a, did not oxidize ubiquinol₁. Proton translocation was not observed if either the protonophore carbonyl cyanide *m*-chlorophenylhydrazone or the respiratory inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide was present. When spheroplasts oxidized Diquat radical (DQ⁺) to the oxidized species (DQ⁺⁺) with nitrate as acceptor, nitrate was reduced to nitrite according to the reaction:

and nitrite was further reduced in the reaction:

Nitrite reductase activity (2) was inhibited by CO, leaving nitrate reductase activity (1) unaffected. Benzyl Viologen radical (BV⁺) is able to cross the cytoplasmic membrane and is oxidized directly by nitrate reductase to the divalent cation, BV⁺⁺. In the presence of CO, this reaction consumes two protons:

The consumption of these protons could not be detected by a pH electrode in the extra-cellular bulk phase of a suspension of spheroplasts unless the cytoplasmic membrane was made permeable to protons by the addition of nigericin or tetrachlorosalicylanilide. It is concluded that the protons of eqn. (3) are consumed at the cytoplasmic aspect of the cytoplasmic membrane. Diquat radical, reduced *N*-methylphenazonium methosulphate and its sulphonated analogue *N*-methylphenazonium-3-sulphonate (PMSH) and ubiquinol₁ are all oxidized by nitrate reductase via a haem-dependent, endogenous quinone-independent, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide-sensitive pathway. Approximate $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries were zero with Diquat radical, an electron donor, 1.0 with reduced *N*-methylphenazonium methosulphate or its sulphonated analogue, both hydride donors, and 2.0 with ubiquinol₁ (QH₂), a hydrogen donor. It is concluded that the protons appearing in the medium are derived from the reductant and the observed $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries are accounted for by the following reactions occurring at the periplasmic aspect of the cytoplasmic membrane.:

Abbreviations used: $\rightarrow\text{H}^+ / 2\text{e}^-$, number of protons translocated/electron pair transferred; $-\Delta\text{H}^+ / 2\text{e}^-$, number of protons consumed/electron pair transferred.

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The observed proton translocation in this segment of the respiratory chain arises not from the translocation of protons across the membrane, but from the translocation of electrons associated with consumption of protons at the cytoplasmic aspect of the membrane and proton liberation at the periplasmic aspect.

It is generally agreed that under appropriate experimental conditions respiration in mitochondria, and also in bacteria, causes a rapid movement of protons across the inner mitochondrial membrane or the cytoplasmic membrane of bacteria. This process of respiration-driven proton translocation has been the subject of several reviews (Papa, 1976; Brand, 1977, 1979; Mitchell, 1979; Mitchell & Moyle, 1979; Wikström & Krab, 1979). There is not yet agreement on the mechanism of respiration-driven proton translocation, nor its relationship to the synthesis of ATP (Mitchell, 1966; Boyer *et al.*, 1977*a,b*; Williams, 1978). These two problems can be considered independently of each other, and it is the former with which this paper is concerned. The respiratory nitrate reductase of *Escherichia coli* was chosen to study this problem for several reasons: it has a relatively simple polypeptide subunit composition (Clegg, 1976) compared to mitochondrial respiratory complexes; the genetics of *E. coli* are well characterized (Bachmann *et al.*, 1976) and mutants are available that are affected in specific components of the respiratory chain (Cox & Gibson, 1974; Haddock, 1977); it functions anaerobically and can be explored by the use of artificial electron donors which are otherwise autoxidizable (Kemp *et al.*, 1975; Jones & Garland, 1977). These experimental advantages have enabled discrimination between the various hypotheses for the mechanism of respiratory-driven proton translocation, and led to the conclusion that in at least this system the mechanism is chemiosmotic in the sense used by Mitchell (1972) i.e. the translocation of protons across the membrane is a direct and inevitable consequence of the chemistry and vectorial organization of the oxidation-reduction reactions within the membrane. Alternative hypotheses, e.g., a proton-motive 'Q-cycle' (Garland *et al.*, 1975*a*; Mitchell, 1976; Downie & Cox, 1978) or a conformational proton pump (Papa, 1976; Artzabanov *et al.*, 1978; Wikström & Krab, 1979) can probably be excluded for this system. Several preliminary reports of parts of this work have been published (Garland & Jones, 1977, 1978; Lamont *et al.*, 1977; Ingledew *et al.*, 1978; Jones & Garland, 1978; Jones *et al.*, 1978*a*).

Experimental

Bacterial strains

E. coli strains A1002 and A1004a (Haddock,

1973) were given by Dr. H. U. Schairer (Gesellschaft für Biotechnologische Forschung mbH, D-3300 Braunschweig-Stöckheim, West Germany). Strain A1004a is unable to synthesize 5-amino-laevulinic acid and is haem-deficient unless the growth medium is suitably supplemented. Strain AN384, a gift from Dr. A. Lagarde (Laboratoire de Microbiologie, Institut National des Sciences Appliquées, F-69621 Villeurbanne, France), is a *ubiA⁻menA⁻* double quinone-deficient mutant (Wallace & Young, 1977) and lacks both menaquinone and ubiquinone unless its growth medium is supplemented with 4-hydroxybenzoic acid. Stock cultures of all strains were kept as 4 ml suspensions in 15% (v/v) glycerol at -22°C .

Growth of bacterial strains

Overnight cultures of strain A1002 and haem-deficient A1004a derepressed for synthesis of nitrate reductase were made as described previously (Jones & Garland, 1977).

An initial culture of strain AN384 was made by inoculating 0.2 ml of stock culture into 100 ml of nutrient broth (Jones & Garland, 1977) supplemented with thiamin (10 µg/ml), uracil (10 µg/ml), glucose (0.5%, w/v) and 1 mM-4-hydroxybenzoate, and then incubating anaerobically at 37°C for 24 h. Several 5 ml samples were taken from this culture and were sedimented in a bench centrifuge in sterile tubes. Each pellet was washed twice in the medium of Cohen & Rickenberg (1956) and resuspended in 5 ml of the same medium. One of these samples was checked for revertants by spreading 0.1 ml onto minimal agar plates supplemented with thiamine (10 µg/ml) and one of the following: (a) glucose (0.5%, w/v); (b) glucose (0.5%, w/v) plus 1 mM-4-hydroxybenzoic acid; (c) potassium acetate, potassium succinate and potassium DL-malate (each 0.2%, w/v); (d) as (c) plus 1 mM-4-hydroxybenzoic acid. The double quinone-deficient mutant grows well on plate (b), less well on plates (a) and (d) and not at all on plate (c). This is because the double quinone-deficient mutant is respiratory-deficient and can grow only fermentatively unless the medium is supplemented with 4-hydroxybenzoic acid. If ten or more colonies appeared on plate (c) after 48 h at 37°C , the level of revertants was considered unacceptable and the starter culture was discarded.

An intermediate culture was made by inoculating the washed and resuspended initial culture into

250 ml of the medium of Cohen & Rickenberg (1956) supplemented with glucose (0.5%, w/v), thiamine (10 µg/ml), uracil (10 µg/ml) and vitamin-free casamino acids (0.1%, w/v) and incubating anaerobically for 24 h at 37°C. The level of revertants was checked as above using the same criteria for acceptance. Once the cells had sedimented after growth the supernatant was carefully decanted and the cell sludge was stored for up to 6 weeks at 0–4°C. An overnight culture was set up by inoculating 2.0 ml of this cell sludge into 500 ml of the medium of Cohen & Rickenberg (1956) supplemented with glucose (0.5%, w/v), vitamin-free casamino acids (0.1%, w/v), uracil (10 µg/ml), thiamine (10 µg/ml), KNO₃ (1%, w/v), 1 µM-(NH₄)₆-Mo₇O₂₄ and 1 µM-K₂SeO₃. This culture was incubated anaerobically at 37°C for 12–16 h.

Preparation of spheroplasts

Cells were harvested, starved and washed as described previously (Jones & Garland, 1977; Jones, 1980). Spheroplasts were prepared as described by Garland *et al.* (1975*b*). The protein content of the spheroplasts was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin (fraction V; BDH Chemicals, Poole, Dorset, U.K.) as standard.

Measurement of respiratory-driven proton translocation

Measurement of small changes of pH in suspensions of spheroplasts were made with the apparatus described by Lawford & Garland (1972). The incubation medium was at 25°C and consisted of 1 ml of 0.3 M-sucrose/0.15 M-KCl/3 mM-MgCl₂/1.5 mM-glycylglycine containing valinomycin (5 µg/ml) and spheroplasts (approx. 1.5 mg of protein). The pH was maintained at 6.8–7.0 by small additions, with a microsyringe, of N₂-saturated 50 mM-KOH/100 mM-KCl. Other additions were as indicated in the legends to the Figures. Calibration was made by addition of 5 µl of N₂-saturated 5 mM-HCl/150 mM-KCl.

Chemical reduction of ubiquinone₁ and the assay of ubiquinol₁

Ubiquinone₁, 2–3 mg in 1 ml of methanol, was reduced by adding a few grains of solid KBH₄. The pH of the solution was adjusted to approx. 7 by additions of 10 mM-HCl. The solution was stored under N₂ at 0–4°C. The preparation was made immediately before use with a batch of spheroplasts and discarded immediately afterwards. Ubiquinol₁ was assayed polarographically as described by Lawford & Garland (1973) except that submitochondrial particles were replaced by spheroplasts from *E. coli*. The results obtained by the two methods were identical, showing that in *E. coli*, as in mitochondria, each molecule of ubiquinol₁ is oxi-

dized by one atom of oxygen to ubiquinone₁. Oxidation of ubiquinol₁ by spheroplasts was assayed spectrophotometrically (Lawford & Garland, 1973) with nitrate as terminal electron acceptor.

Chemical reduction of N-methylphenazonium methosulphate

N-methylphenazonium (PMS⁺) (as its methosulphate salt) can be reduced in two steps (Zaugg, 1964), first to the semiquinone (PMS[•]):

and then to the fully reduced species (PMSH):

The oxidized species is yellow, the semiquinone is green and the fully reduced species gives a white precipitate in water and a colourless solution in ethanol. To obtain a sample of the fully reduced species, approx. 2 mg of the oxidized species was dissolved in 1 ml of ethanol and then reduced by adding a few grains of KBH₄ under a stream of N₂ gas at 0–4°C in a test tube protected against the light by aluminium foil.

The pH was adjusted to approx. 7 by adding 10 mM-HCl. The preparation was made immediately before use and was discarded if a green tinge appeared due to autoxidation to the semiquinone. The concentration of the reduced species was measured polarographically as for ubiquinol₁ except that 30 µg of catalase (EC 1.11.1.6) was added to the reaction medium to decompose H₂O₂ formed by autoxidation. One molecule of the reduced species reacts with one atom of oxygen to give the oxidized species. The sulphonated analogue, *N*-methylphenazonium-3-sulphonate, was reduced and assayed in the same way.

Calcium estimation

The calcium content of suspensions of spheroplasts was measured using an atomic absorption spectrophotometer (model 303, Perkin-Elmer Corp. Beaconsfield, Bucks. HP9 1QA, U.K.).

Reagents

Valinomycin, uracil, thiamin hydrochloride and carbonyl cyanide *m*-chlorophenylhydrazon were purchased from Sigma. Vitamin-free casamino acids were purchased from Difco Laboratories, Detroit, Michigan, U.S.A. and nigericin from Eli Lilly & Co., Indianapolis, IN, U.S.A. Catalase (EC 1.11.1.6) was obtained from Boehringer. Tetrachlorosalicylanilide was a gift from Dr. W. A. Hamilton (Department of Microbiology, University of Aberdeen, Scotland, U.K.). Ubiquinone₁ was kindly donated by Eisai Co., Bunkyo-ko, Tokyo, Japan, and also by Hoffman-LaRoche & Co., CH-4002 Basel, Switzerland. Diquat dibromide was a generous gift from

the Plant Protection Division of ICI, Bracknell, Berks. RG12 6EY, U.K. *N*-methylphenazonium-3-sulphonate was kindly given by Dr. W. Draber, Bayer, A. G., Wuppertal, West Germany and also by Dr. G. Hauska, Institut für Botanik, University of Regensburg, West Germany.

Results

Reconstitution of respiratory-driven proton translocation in a double quinone-deficient mutant by exogenous ubiquinone₁

When respiration is initiated in spheroplasts derepressed for the synthesis of nitrate reductase and incubated anaerobically in medium containing formate by the addition of a small amount of air-saturated KCl or N₂-saturated KNO₃, a rapid acidification of the medium is observed (Garland *et al.*, 1975b). Observed $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries were 3.63 and 1.6 with oxygen and nitrate respectively as electron acceptors (Garland *et al.*, 1975b). When such an experiment was performed using spheroplasts from the double quinone-deficient mutant AN384, no such pH change was observed (Fig. 1, broken lines). However, when 25 μg of exogenous ubiquinone₁ was included in the incubation medium, addition of air-saturated KCl (Fig. 1a, solid line) or N₂-saturated KNO₃ (Fig. 1b, solid line) caused a rapid acidification, complete within 4 s, followed by an exponential relaxation to the initial pH with a half-time of approx. 30 s. The pH changes were not observed if the respiratory inhibitor 2-n-heptyl-4-hydroxyquinoline-*N*-oxide or the protonophore carbonyl cyanide *m*-chlorophenylhydrazine were included in the incubation medium. Observed $\rightarrow\text{H}^+ /$

2e^- stoichiometries were 1.40 ± 0.12 (mean $\pm$ s.d., nine determinations) with nitrate as electron acceptor and 2.26 ± 0.30 (mean $\pm$ s.d., six determinations) with oxygen.

Suitability of ubiquinol₁ as a pulsed reductant

Fig. 2 shows the oxidation of ubiquinol₁ by spheroplasts with air (Fig. 2a) and nitrate (Fig. 2b). From these experiments an apparent V_{max} of 160 nmol of ubiquinol₁ oxidized/min per mg of protein was calculated with either acceptor. The K_m for ubiquinol₁ was approx. 5 μM . We concluded from these studies that low concentrations of ubiquinol₁ would be almost completely oxidized within 2–3 s by suspensions of spheroplasts at protein concentrations of 1 mg/ml or greater in the presence of an excess of either oxygen or nitrate.

Proton translocation associated with oxidation of ubiquinol₁ by nitrate or oxygen

When small amounts of ubiquinol₁ were added to spheroplasts incubated in either air-saturated medium or N₂-saturated medium containing 10 mM-KNO₃, a rapid acidification of the medium was observed (Fig. 3). This phase was complete within 5 s of the addition of ubiquinol₁ and relaxed back towards the initial pH with a half-time of approx. 10–30 s. Whether or not the pH relaxed completely to its initial value depended on the pH of the added ubiquinol₁ solution; if too acidic it caused a permanent offset in the pH trace and this is shown for a particularly bad case in Fig. 3 (traces *e–f*). Generally the pH of the ubiquinol₁ solution can be adjusted to close to 7.0. The rapid acidification resulting from the addition of ubiquinol₁, allowing

Fig. 1. *Reconstitution by ubiquinone₁ of respiratory-driven proton translocation in a double quinone-deficient mutant Spheroplasts*. prepared from double quinone-deficient mutant strain AN384 derepressed for nitrate reductase synthesis, were incubated at a concn. of 4 mg of protein/ml in 150 mM-KCl/300 mM-sucrose/3 mM-MgCl₂/1.5 mM-glycylglycine/5 mM-potassium formate containing 5 μg of valinomycin. In the upper curve of each trace (solid line), 25 μg of ubiquinone₁ was added to the medium. Additions, indicated by arrows, were (a) 25 μl of air-saturated 150 mM-KCl and (b) 5 μl of N₂-saturated 10 mM-KNO₃/150 mM-KCl.

Fig. 2. Oxidation of ubiquinol₁ by oxygen or nitrate

In (a) a Clark electrode was used to measure oxygen consumption in 3.0 ml of air-saturated 150 mM-KCl/300 mM-sucrose/20 mM-Tris/HCl buffer, pH 7.0 at 25°C. Spheroplast suspension (0.1 ml containing 0.7 mg of protein, prepared from strain A1002) was added at the point shown in the Figure. Further additions were 10 µl of 30 mM-ubiquinol₁ in methanol, 20 µl of 1 mM-KCN, and a further addition of 10 µl of 30 mM-ubiquinol₁. The vertical bar gives the oxygen electrode sensitivity and corresponds to 50 nmol of oxygen/ml. The horizontal bar corresponds to 1 min. In (b), the formation of ubiquinone₁ from ubiquinol₁ was measured spectrophotometrically at 275 nm in a stoppered cuvette of 1 cm light path containing 3.6 ml of N₂-saturated 150 mM-KCl/300 mM-sucrose/3 mM-glycylglycine, pH 7.0. The contents of the cuvette were stirred magnetically, and further additions were made by microsyringe. There was no gas phase in the cuvette. Anaerobic spheroplast suspension was added (0.1 ml containing 0.5 mg of protein from strain A1002) and left for 1–2 min to enable respiration of endogenous substrates to remove any remaining traces of oxygen. At this point the reading of absorbance was adjusted to zero. Further additions were: 20 µl of 30 mM-ubiquinol₁ followed after approx. 1 min by 25 µl of N₂-saturated 1 M-KNO₃.

cyanide *m*-chlorophenylhydrazine (Fig. 3c,f); it was dependent on the presence of oxygen or nitrate; and it was greatly diminished in rate and extent when valinomycin was omitted from the incubation medium (data not shown). These features are exactly those expected of respiratory-driven proton translocation (Lawford & Haddock, 1973).

The $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometry of respiratory-driven proton translocation associated with the oxidation of ubiquinol₁ by oxygen or nitrate was measured with several independent cultures and over a limited range of concentrations of ubiquinol₁ of 1–50 µM. Higher concentrations of ubiquinol₁ accelerated the rate of relaxation of the acidification phase and were therefore not used in calculation of the mean stoichiometry. Corrected stoichiometries were obtained by an adaptation of the method of Scholes & Mitchell (1970). A plot of $\log'(\rightarrow\text{H}^+/2\text{e}^-)$ versus time after addition of ubiquinol₁ was drawn for each trace (e.g., Fig. 4c,f) and extrapolation was made to the time at which half of the added ubiquinol₁ was oxidized. The $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometry at this time is considered to be an estimate of the true stoichiometry; the observed stoichiometry is considered an underestimate due to the leakage of translocated protons back into the cytoplasm, and this extrapolation is believed to correct for this. With either oxygen or nitrate as electron acceptor the corrected stoichiometry was close to 2.0 (Table 1). This stoichiometry was independent of the pH of the incubation medium over the range 5.5–7.5.

The haem-deficient strain A1004a was unable to oxidize ubiquinol₁ with either oxygen or nitrate. Addition of ubiquinol₁ to spheroplasts from the double quinone-deficient mutant AN384 incubated in air-saturated medium or N₂-saturated medium containing 10 mM-KNO₃ caused proton translocation as in the wild-type strain (Table 1).

Possible errors in the measurement of $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometries

Possible sources of error in the measurement of $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometries were examined, particularly in view of reports that $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometries in mammalian mitochondria have been seriously underestimated due to rapid electroneutral movement of phosphate in response to the pH difference generated across the membrane (Brand *et al.*, 1976a,b; Wikström & Krab, 1979). Phosphate transport in *E. coli* is effected by two systems (Rosenberg *et al.*, 1977). One system, designated PST, is repressed by phosphate concentrations above 1 mM (Rosenberg *et al.*, 1977) and involves a periplasmic binding protein that is lost during the preparation of spheroplasts (Gerdes *et al.*, 1977) and would therefore not be operative during these experiments (Figs. 3 and 4). The other system, PIT, is constitutive and phosphate transport is driven by

for any pH mismatch, showed the following features: it was not observed in the presence of the respiratory inhibitor 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (Fig. 3b,e) and the protonophore carbonyl

Fig. 3. Proton translocation by spheroplasts with ubiquinol₁ as electron donor

The apparatus, experimental conditions and procedure were as described in the Experimental section. The spheroplasts were at a protein concentration of 2 mg/ml. The electron acceptor was oxygen in traces (a–c) and 10 mM-KNO₃ in traces (d–f). In (b) and (e) 58 μ M-2-n-heptyl-4-hydroxyquinoline-*N*-oxide was present and in (c) and (f) 3 μ M-carbonyl cyanide *m*-chlorophenylhydrazine was present. Ubiquinol₁ (8.6 nmol) was added where indicated by arrows.

Table 1. $\rightarrow H^+/2e^-$ ratios for the oxidation of ubiquinol₁ by oxygen and nitrate

Corrected $\rightarrow H^+/2e^-$ stoichiometries were measured from experiments such as those of Figs. 3 and 4. Results are the mean $\pm$ s.d. for several different spheroplast preparations, each from a separate overnight culture, the number of which is given in parentheses. For any one preparation between five and ten individual measurements of $\rightarrow H^+/2e^-$ were made over a range of ubiquinol additions of 2–50 nmol. The $\rightarrow H^+/2e^-$ ratios within one preparation did not vary beyond an s.d. of $\pm 10\%$.

Strain	Terminal acceptor	Corrected $\rightarrow H^+/2e^-$ ratio
A1002	Oxygen	2.28 ± 0.42 (9)
A1002	Nitrate	1.49 ± 0.36 (8)
AN 384 (<i>ubiA</i> [−] , <i>menA</i> [−])	Oxygen	2.00 ± 0.21 (9)
AN 384 (<i>ubiA</i> [−] , <i>menA</i> [−])	Nitrate	1.86 ± 0.24 (15)

the protonmotive force (Konings & Rosenberg, 1978; Rosenberg *et al.*, 1979; Russell & Rosenberg, 1979). However, several lines of evidence demonstrate that $\rightarrow H^+/2e^-$ stoichiometries in *E. coli* are unlikely to be underestimated due to phosphate uptake. Firstly, any extracellular phosphate will have leaked from the spheroplasts. Since the spheroplasts, which despite starving retain some endogenous substrate, were incubated in air-saturated or nitrate-containing medium, a protonmotive force will be maintained prior to the addition of ubiquinol₁ and the leakage of phosphate will be minimal. The concentration of extracellular phosphate would probably be too low to allow rapid uptake by the PIT system, which has a K_m for phosphate of 25 μ M (Rosenberg *et al.*, 1977). Since *N*-ethylmaleimide inhibits phosphate transport by both systems when

present at a concn. of 5 mM (H. Rosenberg, personal communication), we investigated its effect on $\rightarrow H^+/2e^-$ stoichiometries over the range 1–50 mM. Fig. 4 shows that 50 mM-*N*-ethylmaleimide increases the half-time of the relaxation phase, but does not affect the corrected $\rightarrow H^+/2e^-$ stoichiometry. Finally, Cox & Haddock (1978) have shown that $\rightarrow H^+/2e^-$ stoichiometries in a mutant of *E. coli* lacking both phosphate transport systems were the same as in the parent strain.

The possibility that electroneutral exchange of H^+ for Na^+ (West & Mitchell, 1974; Schuldiner & Fishkes, 1978; Beck & Rosen, 1979) or Ca^{2+} (Rosen & McClees, 1974; Brey & Rosen, 1979) could interfere with measurement of $\rightarrow H^+/2e^-$ stoichiometries was investigated. Culturing of cells in the absence of Na^+ and a number of washings in

Fig. 4. The effect of *N*-ethylmaleimide on $\rightarrow H^+/2e^-$ stoichiometry

The apparatus, experimental conditions and procedure are described in the Experimental section. Spheroplasts were at a protein concentration of 4 mg/ml. Traces (a) and (b) were obtained with nitrate as terminal electron acceptor, traces (d) and (e) with oxygen. Addition of 10.6 nmol of ubiquinol, was made where indicated by arrows. In traces (b) and (e), *N*-ethylmaleimide was present at 50 mM. Graphs (c) and (f) show semi-logarithmic plots of $H^+/2e^-$ versus time (zero time is the time at which half of the peak of acidification is obtained), using the data from traces (a, b) and (d, e) respectively. Closed symbols are taken from (b and e) and open symbols from (a and d).

Na⁺-free buffer followed by spheroplast preparation using the K⁺ rather than the Na⁺ salt of EDTA did not increase $\rightarrow H^+/2e^-$ ratios. Analysis of the spheroplasts by atomic absorption spectrophotometry showed that the calcium content was less than 10 nmol/mg of protein, and only 4 nmol/mg of protein when 10 mM-EGTA was added to the washing buffer. It would seem unlikely that all this calcium would be free cytoplasmic Ca²⁺. Unless all this calcium was pumped out during a burst of respiration and immediately re-entered the spheroplast (within 2 min) when respiration was terminated, there would be insufficient Ca²⁺ for a rapid Ca²⁺/H⁺ antiport to cause underestimation of $\rightarrow H^+/2e^-$ in subsequent bursts of respiration. Addition of 1 mM-EGTA or 10 mM-Ca²⁺ to the incu-

bation medium did not affect the $\rightarrow H^+/2e^-$ stoichiometry. It would therefore seem unlikely that Ca²⁺/H⁺ exchange causes underestimation of $\rightarrow H^+/2e^-$ stoichiometries.

The consumption of H⁺ by nitrate reductase: the problem of nitrite reductase

Setting aside for the moment any consideration of proton translocation, the scalar reaction catalysed by nitrate reductase, i.e.:

must result in the consumption of 2 protons/ion of nitrate reduced to nitrite. Since nitrate reductase is membrane-bound, it is possible that these protons could come from either the cytoplasmic or the periplasmic aspect of the cytoplasmic membrane. Identification of the side from which these protons are derived is essential for understanding how proton translocation is effected during nitrate respiration (Garland *et al.*, 1975b). To investigate this point it is possible to take advantage of the findings of Jones & Garland (1977) that the non-physiological electron donor Benzyl Viologen radical can rapidly cross the cytoplasmic membrane and directly reduce nitrate reductase independently of cytochrome $b_{556}^{NO_3^-}$ and the site inhibited by 2-n-heptyl-4-hydroxyquinoline-*N*-oxide. The appropriate experiment is to measure simultaneously the pH in the bulk phase of a suspension of spheroplasts and the decrease in absorbance as the intensely-coloured Benzyl Viologen radical is oxidized by nitrate to the colourless divalent cation. Any marked lag of the pH change behind the absorbance change would demonstrate that the protons consumed in the nitrate reductase reaction (eqn. 9) do not come from the periplasmic side of the membrane. Such experiments were complicated by the fact that the nitrite formed by nitrate reduction was further reduced by a periplasmic nitrite reductase that uses bipyridylum radicals as reductant (Jones & Garland, 1977). Measurement of bulk-phase pH and absorbance changes during reduction of nitrate by Diquat radical (Fig. 5) showed that the overall reaction was:

Fortunately it was found that this nitrite reductase activity could be inhibited by saturating the suspension of spheroplasts with CO, leaving the nitrate reductase activity unaffected (Fig. 5).

The consumption of H⁺ by nitrate reductase: the protons are derived from the cytoplasm

Fig. 6(a) shows that when a small addition of N₂-saturated KNO₃ was made to spheroplasts suspended in anaerobic CO-saturated medium containing Benzyl Viologen radical, the pH changes due to proton consumption lagged behind the absor-

Fig. 5. *Inhibition by CO of nitrite reductase activity* Spheroplasts were prepared from *E. coli* A1002 derepressed for nitrate reductase activity and incubated at a protein concentration of 4 mg/ml in 3.6 ml of 150 mM-KCl/300 mM-sucrose/3 mM-MgCl₂/1 mM-Diquat/1.5 mM-glycylglycine, pH 7.0 at 25°C. A micro pH electrode mounted in a rubber stopper was inserted into the cuvette such that there was no gas phase and the rubber stopper gave a gas-tight seal. Additions were made by microsyringe via a small (1 mm) hole in the stopper. 25 mM-Na₂S₂O₄/10 mM-KOH was added to give an absorbance of approx. 1.0 at 460 nm. Simultaneous pH (a and b) and absorbance at 460 nm (c and d) recordings were made in response to the addition, where indicated, of 5 μ l of N₂-saturated 10 mM-KNO₃/150 mM-KCl (a and c) or 10 mM-NaNO₂⁻/150 mM-KCl (b and d). The pH traces were calibrated by a 10 μ l addition of N₂-saturated 10 mM-HCl/150 mM-KCl. The upper trace (broken line) of each pair was obtained when the spheroplasts and suspending medium were saturated with CO.

Fig. 6. *pH and absorbance changes due to nitrate-dependent oxidation of Benzyl Viologen radical*

Spheroplasts were prepared from *E. coli* A1002 derepressed for nitrate reductase synthesis and suspended to a protein concentration of 4 mg/ml in 3.6 ml of 150 mM-KCl/300 mM-sucrose/3 mM-MgCl₂/1 mM-Benzyl Viologen/1.5 mM-glycylglycine, pH 7.0. A micro pH electrode was inserted as described in Fig. 5. Na₂S₂O₄ (25 mM) was added to give an absorbance of approx. 1.0 at 600 nm. The upper trace of each pair (broken line) shows the pH change and the lower trace the change in absorbance at 600 nm in response to the addition, shown by arrows, of 5 μ l of N₂-saturated 10 mM-KNO₃/150 mM-KCl. In trace (b), tetrachlorosalicylanilide was present at 8 μ M and in (c) nigericin was present at 6 μ g/ml.

bance changes due to oxidation of the dye. This lag was abolished when the cytoplasmic membrane was made permeable to protons by the addition of the protonophore tetrachlorosalicylanilide (Hamilton, 1968) (Fig. 6b) or nigericin, which effects an electroneutral H^+/K^+ exchange (Harold, 1970; Henderson, 1971) (Fig. 6c). At the point where the absorbance change was complete, $-\Delta H^+/2e^-$ was calculated (Table 2). The value approached 2.0 as predicted by eqn. (9) only in the presence of tetrachlorosalicylanilide or nigericin. It is concluded that the protons of eqn. (9) are obtained from the cytoplasmic space of the spheroplasts where they are not directly detectable by an extracellular pH electrode.

The ability of the nitrate reductase system to drive proton translocation by using other non-physiological reductants

It has previously been shown (Jones & Garland, 1977, and see below) that in intact spheroplasts two other reductants resemble ubiquinol₁ in their ability to reduce nitrate in a cytochrome *b*-dependent, endogenous ubiquinone-independent, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide-sensitive manner. They are Diquat radical and reduced *N*-methylphenazonium methosulphate.

The ability of Diquat radical to drive proton translocation was investigated in experiments similar to those shown in Fig. 6. Bulk-phase pH changes and absorbance changes due to the nitrate-depen-

dent oxidation of Diquat radical were measured simultaneously. Valinomycin was present to ensure that any electrogenic consequences of the reduction of nitrate by Diquat radical would not set up a membrane potential which might, by driving proton re-entry into the spheroplasts, obscure any appearance of protons into the bulk phase. Fig. 7(a) shows that addition of nitrate to spheroplasts suspended in

Fig. 7. pH and absorbance changes due to nitrate-dependent oxidation of Diquat radical

Apparatus, experimental conditions and procedure are as for Fig. 5. The upper trace of each pair (broken line) shows the pH change and the lower trace the change in absorbance at 460 nm in response to the addition, as shown by the arrows, of 10 μ l of N_2 -saturated 10 mM- KNO_3 /150 mM-KCl. In trace (a) valinomycin was present at 5 μ g/ml and in (c) valinomycin (5 μ g/ml) and tetrachlorosalicylanilide (8 μ M) were present.

CO-saturated medium containing Diquat radical and valinomycin caused an increase in the pH of the extracellular bulk phase. The rate of the pH change lagged behind that of the absorbance change. Omission of valinomycin reduced the lag (Fig. 7b), suggesting that transmembranous electron transfer from Diquat radical to nitrate generates a membrane potential that drives protons into the spheroplasts (see below). At no time was an initial acidification phase observed on addition of nitrate. Addition of tetrachlorosalicylanilide completely abolished the lag (Fig. 7c) and $-\Delta H^+/2e^-$ was very close to 2.0 (Table 2). It is concluded that electron flow from Diquat radical to nitrate via cytochrome $b_{556}^{NO_3}$ does not drive proton translocation, although a protonmotive force may be generated (see the Discussion).

The ability of reduced *N*-methylphenazonium methosulphate to drive proton translocation was investigated (Fig. 8). Addition of reduced *N*-methylphenazonium methosulphate to spheroplasts incubated in medium containing nitrate caused a rapid phase of acidification, complete within 5 s, followed by a slower relaxation past the initial pH to give an overall alkalization (Fig. 8a). In the presence of carbonyl cyanide *m*-chlorophenylhydrazone, only a rapid alkalization phase was observed (Fig. 8b). In some experiments in the presence of carbonyl cyanide *m*-chlorophenylhydrazone, a brief, very rapid acidification was observed which is probably due to a pH mismatch. The adjustment of the pH of the reduced *N*-methylphenazonium methosulphate to 7.0 is difficult because of the need to keep the donor, which is rapidly autoxidizable in air, fully reduced. The presence of the semiquinone will cause underestimation of $\rightarrow H^+/2e^-$ and overestimation of $-\Delta H^+/2e^-$. The $-\Delta H^+/2e^-$ ratio of the alkali-

Fig. 8. pH recording of spheroplasts using nitrate as electron acceptor and reduced *N*-methylphenazonium methosulphate as reductant

Apparatus, experimental conditions and procedure are given in the Experimental section. Spheroplasts were at a concentration of approx. 4 mg/ml; KNO_3 was present at 10 mM. Carbonyl cyanide *m*-chlorophenylhydrazone was present at 3 μ M in trace (b). Reduced-*N*-methylphenazonium methosulphate (5 μ l of 9.6 mM) was added where indicated by arrows.

Table 2. $\rightarrow H^+/2e^-$ stoichiometries and $-\Delta H^+/2e^-$ ratios for nitrate reduction by non-physiological donors $\rightarrow H^+/2e^-$ stoichiometries and $-\Delta H^+/2e^-$ ratios in the presence of an uncoupler, tetrachlorosalicylanilide or carbonyl cyanide *m*-chlorophenylhydrazine, were measured in experiments such as those of Figs. 6, 7 and 8. The values predicted from Scheme 2 are also given. Values are means $\pm$ s.d. for one spheroplast preparation from strain A1002; the number of measurements is given in parentheses. Negative values indicate consumption of protons.

Donor	$\rightarrow H^+/2e^-$		$-\Delta H^+/2e^-$ (+ Uncoupler)	
	Observed	Predicted	Observed	Predicted
Benzyl Viologen radical	-0.68 ± 0.34 (6)	0	1.99 ± 0.25 (11)	2.0
Diquat radical	-1.36 ± 0.34 (8)	0	2.09 ± 0.13 (13)	2.0
Reduced <i>N</i> -methylphenazonium methosulphate	0.48 ± 0.18 (11)	1.0	0.78 ± 0.06 (11)	1.0
Reduced <i>N</i> -methylphenazonium-3-sulphonate	0.73 ± 0.27 (7)	1.0	1.03 ± 0.52 (7)	1.0

Table 3. Nitrate-dependent oxidation of Diquat radical and reduced *N*-methylphenazonium methosulphate in a double quinone-deficient mutant

Growth of cells and assays were as described in the Experimental section and by Jones & Garland (1977). Activities are expressed as means $\pm$ s.d. (number of determinations in parentheses). Units are μmol of Diquat radical oxidized/min per mg of protein and, with *N*-methylphenazonium methosulphate as donor, μmol of NO_2^- produced/min per mg of protein.

Donor	Activity in strain			
	A1002		AN 384 (<i>ubiA</i> ⁻ , <i>menA</i> ⁻)	
	Whole cells	Sonicated cells	Whole cells	Sonicated cells
Diquat	1.22 ± 0.16 (4)	1.90 ± 0.18 (4)	0.49 ± 0.06 (12)	0.97 ± 0.13 (12)
Ascorbate/ <i>N</i> -methylphenazonium methosulphate	0.15 ± 0.03 (8)	0.19 ± 0.03 (7)	0.15 ± 0.07 (7)	0.231 ± 0.05 (7)

zation phase was close to 1.0 in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazine, consistent with the reaction:

Because the acidification phase is followed by an overall alkalization, the correction of the $\rightarrow H^+/2e^-$ stoichiometry used previously with ubiquinol₁ as reductant (Fig. 4) is not applicable. The observed $\rightarrow H^+/2e^-$ stoichiometry was 0.48, which is probably an underestimate. If the stoichiometry is an integral number, the true stoichiometry is probably 1.0. $\rightarrow H^+/2e^-$ and $-\Delta H^+/2e^-$ ratios of 1.0 are accounted for by Scheme 2(c). Similar results were obtained using reduced *N*-methylphenazonium-3-sulphonate as a pulsed reductant (Table 2).

Respiratory carriers involved in the oxidation of non-physiological reductants

The availability of the double quinone-deficient mutant enabled us to determine whether the cytochrome *b*-dependent oxidation of Diquat radical or ascorbate plus *N*-methylphenazonium methosulphate is dependent on endogenous quinones. Table 3 shows that the rates of nitrate reduction by these donors is similar in mutant strain AN384 and

wild-type A1002, indicating that endogenous quinones are not necessary for the nitrate-dependent oxidation of these donors.

We also re-explored the ability of reduced *N*-methylphenazonium-3-sulphonate to reduce nitrate via the nitrate reductase system. We concluded earlier that it could not (Jones & Garland, 1977), but we have subsequently discovered that this mediator interferes with the chemical assay used to measure nitrite production. We therefore designed an alternative assay system in which *N*-methylphenazonium-3-sulphonate acted as a mediator between the non-permeant radical of the bipyridylum compound Morfamquat and the nitrate reductase system in intact spheroplasts. Morfamquat radical is unable to reduce the nitrate reductase system in intact spheroplasts (Jones & Garland, 1977). When 20 μM -*N*-methylphenazonium-3-sulphonate or 20 μM -*N*-methylphenazonium methosulphate was added to the reaction medium, Morfamquat radical was oxidized by nitrate at a rate of 0.75 $\mu\text{mol}/\text{min}$ per mg of protein. This activity was dependent on the presence of functional cytochromes but not of endogenous quinone, as shown by the use of appropriate mutants, and was inhibited by 2-n-heptyl-4-hydroxyquinoline-*N*-oxide.

Discussion

Topography of the nitrate reductase system

Kemp *et al.* (1975) using FMNH₂ as a non-permeant reductant, showed that nitrate reductase has an oxidoreduction site accessible from the cytoplasmic aspect of the cytoplasmic membrane. Covalent labelling (Boxer & Clegg, 1975; MacGregor & Christopher, 1978) and immunological studies (Graham & Boxer, 1978) showed that the α -subunit is accessible at the inner surface of the membrane. The studies of Boxer & Clegg (1975) also revealed that the γ -subunit, which is assumed to be the apo-cytochrome $b_{556}^{\text{NO}_3^-}$, is accessible at the periplasmic aspect of the membrane. Identification of this cytochrome as the electron acceptor from the non-permeant Diquat radical (Jones & Garland, 1977) confirms the accessibility of the cytochrome $b_{556}^{\text{NO}_3^-}$ at the periplasmic face of the membrane. In addition, we have now shown that the protons consumed in the nitrate reductase reaction (eqn. 9) come from the cytoplasmic side of the membrane. The site of nitrate reduction is believed also to be at the cytoplasmic aspect of the membrane because chlorate, an analogue of nitrate that is reduced by nitrate reductase, is reduced 5-fold more rapidly after cell breakage (Kristjansson & Hollocher, 1979) and also because azide, a competitive inhibitor of nitrate reductase (Forget, 1974), exerts its effect at the cytoplasmic side of the membrane (Jones *et al.*, 1978*b*). It should be emphasized once again that the site of nitrate reduction is irrelevant to proposed schemes for proton translocation (e.g. Garland *et al.*, 1975*a,b*; Jones & Garland, 1978; Jones *et al.*, 1978*a*) so long as the site of proton consumption is at the cytoplasmic aspect of the membrane (see below). This point has been misinterpreted in recent literature (Kristjansson & Hollocher, 1979; Wientjes *et al.*, 1979).

These topographical considerations are inevitably of interest in relation to the question of how the appropriate polypeptides come to be located in the membrane in this manner (Lodish & Rothman, 1979). They are also important in relationship to the mechanism of respiratory-driven proton translocation because they show that the oxidoreduction pathway is transmembranous, extending from the periplasmic site, where Diquat radical or reduced *N*-methylphenazonium-3-sulphonate reduce cytochrome $b_{556}^{\text{NO}_3^-}$ to the site at the cytoplasmic face where nitrate reductase is reduced by Benzyl Viologen radical or FMNH₂. We have not identified directly the site of ubiquinol₁ oxidation, but this is presumably at the periplasmic site. *N*-Methylphenazonium-3-sulphonate is considered to be non-permeant (Hauska, 1972), so is presumably oxidized at the periplasmic aspect of the membrane. By analogy, the lipid-soluble *N*-methylphenazonium methosul-

phate might be expected to act at this site. In support of this is the observation that *N*-methylphenazonium methosulphate is unable to mediate proton and electron transfer across artificial lipid vesicles in the absence of tetraphenylboron (Hinkle, 1973; Miller *et al.*, 1979). This is presumably because the charge on the *N*-methylphenazonium cation (PMS⁺) makes the oxidized species hydrophilic and unable to penetrate the membrane. Tetraphenylboron may form a permeant complex with this cation (Hinkle, 1973; Miller *et al.*, 1979). To mediate electron transfer from Morfamquat radical to the nitrate reductase system *N*-methylphenazonium methosulphate, when used at low concentrations (20 μM), must therefore be re-oxidized at the periplasmic aspect of the membrane, where it can be reduced again by non-permeant Morfamquat radical.

The mechanism of proton translocation by the nitrate reductase system

Scheme 1 shows two fundamentally different schemes for proton translocation driven by the respiratory nitrate reductase system. Each scheme accounts for the $\rightarrow \text{H}^+ / 2\text{e}^-$ stoichiometry of 2.0 during oxidation of ubiquinol₁ and also for the cytoplasmic source of the protons that are used in the reduction of nitrate to nitrite. One (Scheme 1*a*) can be described as directly chemiosmotic in that it

Scheme 1. Possible mechanisms of a protonmotive oxidation of ubiquinol₁ by nitrate
See the text for details.

is based on the proposals of Mitchell (1972) in which proton translocation occurs as a direct consequence of the chemistry and vectorial organization of the membrane-bound respiratory carriers. The other (Scheme 1b) can be described as conformational because it postulates that proton translocation is driven by conformational changes of polypeptides in response to valency changes at the oxidoreduction centres. In the chemiosmotic scheme the two protons derived from ubiquinol₁ appear in the extracellular bulk phase, whereas in the conformational scheme these two protons must of necessity be released into the cytoplasm (otherwise $\rightarrow\text{H}^+ / 2\text{e}^-$ would be 4.0). The chemiosmotic scheme predicts that if ubiquinol₁ is replaced by a hydride donor, reduced *N*-methylphenazonium methosulphate or *N*-methylphenazonium-3-sulphonate, the $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichio-

metry will fall to 1.0 (Scheme 2c) and if replaced by an electron donor, Diquat radical, to zero (Scheme 2b). By contrast, the conformational scheme predicts that $\rightarrow\text{H}^+ / 2\text{e}^-$ (as measured in the bulk phase) would be independent of the chemistry of the donor. Figs. 3, 7 and 8 and Tables 1 and 2 show clearly that the predictions of the chemiosmotic model are met while those of the conformational scheme are not.

The problem of nitrate transport

Kristjansson *et al.* (1978) have shown that nitrate transport by *Paracoccus denitrificans* is accompanied by proton uptake with a 1:1 stoichiometry. Such a mechanism of nitrate uptake in *E. coli* would lead to an underestimation of $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries. However, Fig. 6 shows that the rate of proton uptake is substantially slower than the absorbance change due to nitrate-dependent oxidation of Benzyl Viologen radical, which is inconsistent with an $\text{H}^+ / \text{NO}_3^-$ symport of 1:1 stoichiometry. A possible mechanism of nitrate uptake by *E. coli* is a $\text{NO}_3^- / \text{NO}_2^-$ antiport, functioning either directly or as a $\text{H}^+ / \text{NO}_3^-$ symport taking up nitrate and a $\text{H}^+ / \text{NO}_2^-$ symport exporting nitrite, which together would appear as an effective $\text{NO}_3^- / \text{NO}_2^-$ antiport (Kristjansson & Hollocher, 1979).

Is ubiquinone₁ an accurate model of endogenous ubiquinone₈?

Because of the differing side chains it seems inevitable that ubiquinone₁ will exhibit some different biochemical properties from the endogenous ubiquinone₈. The real problem, however, is not that such differences may occur, but whether ubiquinone₁ faithfully takes the place of ubiquinone₈ in the mechanism by which substrate dehydrogenases are functionally connected to the nitrate reductase system and the associated proton translocation. The evidence available (Fig. 1) shows that ubiquinone₁ is able to reconstitute a proton-translocating formate to nitrate pathway in the double quinone-deficient mutant AN384. Ubiquinone₁ has also been shown to reconstitute oxygen-dependent aetbrin fluorescence quenching with NADH as electron donor in a ubiquinone-deficient mutant (Haddock & Downie, 1974) and aerobic active transport of proline with lactate as electron donor in a double quinone-deficient mutant (Stroobant & Kaback, 1979). Stroobant & Kaback (1979) showed that, in contrast to ubiquinone₁, exogenous ubiquinone₈ was ineffective in reconstituting active transport of proline, but this is probably due to the fact that exogenous ubiquinone₈ is not readily incorporated into the membrane, for Futami *et al.* (1979) have reported that long-chain quinones can be incorporated into liposomes only during their formation,

Scheme 2. Oxidation of non-physiological electron donors by the nitrate reductase system of *E. coli*

This scheme shows the sites of proton release and uptake when the following are oxidized by the nitrate reductase pathway: (a) Benzyl Viologen radical (BV^+) to its oxidized form (BV^{++}); (b) Diquat radical (DQ^+) to its oxidized form (DQ^{++}); (c) reduced *N*-methylphenazonium methosulphate (PMSH) to the oxidized species (PMS^+). See the text for details.

but when added as an ethanolic solution to pre-formed liposomes ubiquinone, was not incorporated into the membrane.

The role of ubiquinone in proton translocation by E. coli

Downie & Cox (1978) have proposed that the proton-translocating activity of the aerobic respiratory chain of *E. coli* arises solely from a protonmotive Q-cycle. There seems to be little basis on which to propose such a mechanism, especially in the nitrate reductase system. The measured $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries (Lawford & Haddock, 1973; Garland *et al.*, 1975b; Cox & Haddock, 1978) are readily explained if ubiquinone acts as a mobile hydrogen carrier, able to carry protons and electrons across the membrane as the plastoquinone of chloroplasts is believed to do (Tiemann *et al.*, 1979). Hauska (1977) and Futami *et al.* (1979) have shown that both ubiquinone and plastoquinone behave in this way in artificial lipid vesicles.

Ubiquinone₁ has been used as a pulsed oxidant in mitochondria (Lawford & Garland, 1972) but is unsuitable in *E. coli* spheroplasts because the resulting proton translocation is too slow to measure $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries (Haddock *et al.*, 1974; Downie, 1974; R. W. Jones, unpublished work). Nevertheless, reduction of ubiquinone₁ by NADH (Haddock *et al.*, 1974; Downie, 1974) and formate (R. W. Jones, unpublished work) does appear to drive proton translocation. Using hydrogen as a pulsed reductant, Jones (1980) showed that hydrogen-dependent reduction of ubiquinone₁, or the menaquinone analogue menadione, drove proton translocation with an $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometry of approx. 2.0. Since $\rightarrow\text{H}^+ / 2\text{e}^-$ stoichiometries for nitrate respiration with NADH (Garland *et al.*, 1975b), formate (Gutowksi, 1976; R. W. Jones, unpublished work) and hydrogen (Jones, 1980) are close to 4, by implication the stoichiometries for NADH- or formate-dependent reduction of ubiquinone₁ might also be expected to be 2. This is in contrast to the claim of Brookman *et al.* (1979) that NADH dehydrogenase is not proton-translocating. This was based on the observation that addition of menadione to a suspension of haem-deficient cells grown on mannitol, an NADH-linked substrate, caused proton translocation but did not do so in an isogenic uncoupled mutant. Brookman *et al.* (1979) thus attributed the proton translocation to hydrolysis of ATP formed at the substrate level. However, the failure of menadione to elicit proton translocation in the uncoupled mutant could be due to other factors. It is possible that the NADH dehydrogenase could differ in the two strains, as two forms of the enzyme have been detected in *E. coli* (Owen & Kaback, 1979). Also, non-proton-translocating NADH dehydrogenase activities can occur under appro-

priate growth conditions (Poole & Haddock, 1975; Haddock & Jones, 1977).

More general aspects of proton translocation driven by oxidoreductions

Although the evidence for chemiosmotic mechanisms of proton translocation by photosynthetic systems is strong, both in bacterial chromatophores (Crofts *et al.* 1975, 1977; Jones, 1977; Crofts & Bowyer, 1978) and chloroplasts (Trebst, 1974; Trebst *et al.*, 1975; Hauska & Trebst, 1977; Auslander & Junge, 1978; Reeves & Hall, 1978; Witt, 1979), this is not so for mitochondrial systems (Brand, 1977; Wikström & Krab, 1979). Bacterial respiratory systems have been investigated in depth only more recently and there are now at least three well-documented cases where a transmembranous oxidoreduction drives proton translocation: the nitrate reductase system of *E. coli*; the H_2 -fumarate pathway of *E. coli* (Jones, 1980); and the formate-fumarate pathway of *Vibrio succinogenes* (Kroger, 1977, 1978; Kroger *et al.*, 1980). Also the electron transfer reactions involved in the oxidation by oxygen of Fe^{2+} by *Thiobacillus ferrooxidans* are reported to span the membrane and, although not proton-translocating, are electron-translocating and provide a chemiosmotic mechanism (Ingledew *et al.*, 1977) for establishing a difference in electrochemical proton activity across the membrane (Cox *et al.*, 1979).

Until recently, cytochrome *c* oxidase was frequently advanced as an example, perhaps the only example, of vectorial organization of a mitochondrial respiratory system (Racker, 1979), but the work of Wikström (1977, 1978; Krab & Wikström, 1978; Wikström & Krab, 1978, 1979; Wikström & Saari, 1977) and of Casey *et al.* (1979) indicates that cytochrome *c* oxidase has a proton-pumping activity for which a conformational proton pump cannot be excluded (but see Lorusso *et al.*, 1979; Mitchell & Moyle, 1979; Moyle & Mitchell, 1978a,b for a different view). Furthermore, recent estimates of $\rightarrow\text{H}^+ / 2\text{e}^-$ ratios of 3–4/classical oxidative phosphorylation site (Brand *et al.*, 1976a,b) and charge/ 2e^- stoichiometries of 4, 6 and 8 for oxidation of isoascorbate, succinate and 3-hydroxybutyrate respectively (Brand *et al.*, 1978) are not readily explained by present knowledge of the respiratory carriers if proton translocation is supposed to be effected by a chemiosmotic mechanism.

It would seem unlikely that there exists a fundamental difference between proton translocation in photosynthetic and mitochondrial respiratory systems. The demonstration of a chemiosmotic mechanism for respiratory-driven proton translocation in bacteria shows that there is no basic difference between respiratory and photosynthetic systems. The problem of the mechanism of respi-

ratory-driven proton translocation in mitochondria remains, but three possibilities exist. One is that the mechanism is indeed chemiosmotic and that what is lacking is a sufficiently detailed knowledge of the oxidoreduction centres, the pH-dependencies of these centres and their topographies. Another is that conformational mechanisms occur in addition to chemiosmotic ones. This additional complexity might be reflected in the relatively large number of polypeptides found in mitochondrial (Eytan *et al.*, 1975; Ragan, 1976; Pentilla *et al.*, 1979) as opposed to some bacterial systems (Enoch & Lester, 1974, 1975; MacGregor, 1975a,b; Clegg, 1976; Dickie & Weiner, 1979; Sone *et al.*, 1979). The third possibility is that in mitochondria the $\rightarrow\text{H}^+ / 2\text{e}^- / \text{site}$ stoichiometry is 2.0 (Lawford & Garland, 1972, 1973; Downie & Garland, 1973; Moyle & Mitchell, 1978b; Mitchell & Moyle, 1979) and that cytochrome *c* oxidase is not a proton pump (Moyle & Mitchell, 1978a; Lorusso *et al.*, 1979; Mitchell & Moyle, 1979).

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