Biochem. J. (1978) **174**, 237–256
Printed in Great Britain

The Protonmotive Force in Bovine Heart Submitochondrial Particles

MAGNITUDE, SITES OF GENERATION AND COMPARISON WITH THE PHOSPHORYLATION POTENTIAL

By M. CATIA SORGATO and STUART J. FERGUSON
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.
and DOUGLAS B. KELL and PHILIP JOHN
Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

(*Received* 28 *November* 1977)

1. The magnitude of the protonmotive force in respiring bovine heart submitochondrial particles was estimated. The membrane-potential component was determined from the uptake of $S^{14}CN^-$ ions, and the 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 approx. 145mV and the pH gradient was between 0 and 0.5 unit when the particles were suspended in a P_i/Tris reaction medium. The addition of the permeant NO_3^- ion decreased the membrane potential with a corresponding increase in the pH gradient. In a medium containing 200mM-sucrose, 50mM-KCl and Hepes as buffer, the total protonmotive force was 185mV, comprising a membrane potential of 90mV and a pH gradient of 1.6 units. Thus the protonmotive force was slightly larger in the high-osmolarity medium. 3. The phosphorylation potential ($= \Delta G^{0\prime} + RT \ln [ATP]/[ADP][P_i]$) was approx. 43.1kJ/mol (10.3kcal/mol) in all the reaction media tested. Comparison of this value with the protonmotive force indicates that more than 2 and up to 3 protons must be moved across the membrane for each molecule of ATP synthesized by a chemiosmotic mechanism. 4. Succinate generated both a protonmotive force and a phosphorylation potential that were of similar magnitude to those observed with NADH as substrate. 5. Although oxidation of NADH supports a rate of ATP synthesis that is approximately twice that observed with succinate, respiration with either of these substrates generated a very similar protonmotive force. Thus there seemed to be no strict relation between the size of the protonmotive force and the phosphorylation rate. 6. In the presence of antimycin and/or 2-n-heptyl-4-hydroxyquinoline *N*-oxide, ascorbate oxidation with either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine as electron mediator generated a membrane potential of approx. 90mV, but no pH gradient was detected, even in the presence of NO_3^-. These data are discussed with reference to the proposal that cytochrome oxidase contains a proton pump.

The chemiosmotic hypothesis (Mitchell, 1966) originally envisaged that either the passage of a pair of electrons through one proton-translocating segment of the mitochondrial respiratory chain, or the hydrolysis of 1 molecule of ATP, was linked to the translocation of 2 protons across the inner mitochondrial membrane. Although a good deal of evidence has been obtained that is consistent with this view (Mitchell, 1976*a*), many recent experimental findings indicate that the stoicheiometry of proton translocation may be higher than 2.

Re-examination of the number of protons ejected by mitochondria after an oxygen or substrate pulse has shown that the stoicheiometry of proton-translocation is 3 or even 4 (for a review see Brand, 1977). A study of the number of protons ejected in electroneutral exchange for Ca^{2+} is consistent with the translocation of 4 protons as a pair of electrons pass through a proton-translocating segment of the respiratory chain (Reynafarje & Lehninger, 1977). A stoicheiometry of 2.8 protons has been estimated by Nicholls (1977*a*), on the basis of the number of Ca^{2+} ions taken up by mitochondria respiring on succinate. Hydrolysis of a pulse of ATP by the ATPase in mitochondria is still thought to be coupled to the translocation of 2 protons across the membrane, although

Abbreviations used: ATPase, adenosine triphosphatase (EC 3.6.1.3); EDTA particles, non-phosphorylating particles obtained by sonication of mitochondria at alkaline pH in the presence of EDTA; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate; Mg-ATP particles, phosphorylating particles prepared by sonication of mitochondria in the presence of 15mM-$MgCl_2$ and 1mM-ATP.

the measurements are complicated by the need to allow for, or eliminate, any proton movements that may be associated with the transport of ATP, ADP or P_i across the membrane (Brand & Lehninger, 1977).

Thermodynamic measurements also suggest that more than 2 protons must be translocated per 2 electrons passing through a proton-translocating segment of the respiratory chain. Comparison of the extramitochondrial phosphorylation potential ($= \Delta G^{0\prime} + RT \ln [ATP]/[ADP][P_i]$) with electrochemical gradient of protons [the protonmotive force of Mitchell (1966)] has indicated that the overall process of producing extramitochondrial ATP from added ADP and P_i involves the translocation of 3 protons per molecule of ATP synthesized (Nicholls, 1974; Rottenberg, 1975; Wiechmann *et al.*, 1975). Thus, if the P/O ratio for succinate is 2, these observations mean that the oxidation of 1 molecule of succinate must be associated with the translocation of 6 rather than 4 protons as originally proposed (Mitchell, 1966).

It is clear that with mitochondria the study of respiration- or ATP-driven proton ejection, or the analysis of the thermodynamic relationship between the protonmotive force and the extramitochondrial phosphorylation potential, are complicated by proton movements associated with the transport systems of the inner membrane. Comparison of the phosphorylation potential with the protonmotive force is most probably complicated by the electrogenic nature of the adenine nucleotide translocator (Klingenberg & Rottenberg, 1977), and by proton movements connected with the movement of P_i into or out of mitochondria (Brand, 1977). In view of the inherent uncertainties, and possible experimental discrepancies (Mitchell, 1977), there is a need for additional approaches to the problem of the stoicheiometry of proton translocation.

Submitochondrial particles, which are inside-out relative to mitochondria (Racker, 1970), have an ATP-synthesizing apparatus directly available to added substrates. Thus they provide a system free of the complicating factors that are experienced with intact mitochondria. In particular the adenine nucleotide translocator is not involved in oxidative phosphorylation in submitochondrial particles. The potential advantages of working with submitochondrial particles have so far been exploited to only a limited extent. For example, the number of protons taken up by submitochondrial particles during the passage of a pair of electrons through a proton-translocating segment of the respiratory chain has been shown to be approx. 2 at pH 6.0–6.5 (Hinkle & Horstman, 1971). An uptake of about 2 protons has also been found to be linked to the hydrolysis of 1 molecule of ATP by the particles (Moyle & Mitchell, 1973; Thayer & Hinkle, 1973). However, advantage has not been taken of the properties of submitochondrial particles for comparison of the phosphorylation potential with the protonmotive force. The value of such measurements would be that the number of protons translocated by the ATPase for each molecule of ATP synthesized could be determined and compared with the values obtained by other methods.

The present paper reports measurements of both the phosphorylation potential and protonmotive force in submitochondrial particles. Although the phosphorylation potential in submitochondrial particles is distinctly lower than its extramitochondrial counterpart, it has been argued (Ferguson & Sorgato, 1977) that the phosphorylation potential is not restricted to a low value by the poor coupling properties of the particles. Thus the present work had two main objectives: to determine whether, despite the low phosphorylation potential, submitochondrial particles were able to maintain a protonmotive force of similar magnitude to those found in other energy-coupling membranes, and to estimate the number of protons translocated per ATP molecule synthesized from a comparison of the protonmotive force with the phosphorylation potential.

If the stoicheiometry of proton translocation is higher than was suggested in the chemiosmotic hypothesis, then the original concept of a looped respiratory chain (Mitchell, 1966) cannot account for the full extent of proton translocation. Wikström (1977) has presented evidence that cytochrome oxidase acts as a proton pump, a property that could account for some extra proton translocation. In contrast Mitchell & Moyle (1967) had previously concluded that cytochrome oxidase is not proton-translocating, as the same respiratory proton-translocation stoicheiometry was obtained when ferricyanide was used as oxidant instead of O_2 with intact mitochondria. Furthermore Hauska *et al.* (1977) have also suggested that electron flow through cytochrome oxidase is not coupled to proton translocation, as judged by the apparent absence of ATP synthesis during oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine by submitochondrial particles. A further purpose of the present work was therefore to examine whether electron flow through cytochrome oxidase was coupled to the generation of a protonmotive force.

Experimental

Submitochondrial particles

Mg-ATP bovine heart submitochondrial particles (Löw & Vallin, 1963) were prepared by the method of Ferguson *et al.* (1977) for making ATPase-inhibitor-depleted particles [type II particles in the nomenclature of Ferguson *et al.* (1977)]. The particles were stored at 0°C as a concentrated suspension in the

reaction medium that was to be used in the experiments. (Occasionally the particles were suspended in a medium containing 20mM-Tris/HCl buffer, pH7.4, 225mM-mannitol and 75mM-sucrose. No experimental difference was noted between particles that were suspended in the latter medium, and those that were suspended in the other reaction media.) The experiments were usually completed within 6h of preparing the particles, although very little change in the membrane potential generated by the particles was seen over a period of 18h. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Determination of protonmotive force

Mitchell (1966) has defined the protonmotive force (Δp) at 23°C as:

$$\Delta p = \Delta\psi - 59\Delta\text{pH} \quad (1)$$

where $\Delta\psi$ is the membrane potential in mV, ΔpH the pH gradient across an energy-coupling membrane, and Δp is in mV.

In the present work $\Delta\psi$ was determined from the distribution of $S^{14}CN^-$ across the membranes of submitochondrial particles, with the assumption that the $S^{14}CN^-$ ion becomes distributed so that an electrochemical equilibrium is reached, in which case:

$$\Delta\psi = 59 \log \frac{[SCN^-]_i}{[SCN^-]_o} \quad (2)$$

$[SCN^-]_i$ and $[SCN^-]_o$ represent the concentrations of the ion in the internal (lumen of the particles) and external (medium) phases respectively. Strictly, activities rather than concentrations should be inserted in eqn. (2), but it is assumed that the ratio of activity coefficients for the internal and external phases approaches unity.

Use of eqn. (2) requires that the membranes of submitochondrial particles, which are derived from the inner mitochondrial membrane, are permeable to SCN^-. Evidence that SCN^- can permeate the inner mitochondrial membrane or submitochondrial particles has been given by Mitchell & Moyle (1969), Papa *et al.* (1973*a*,*b*) and Lehninger (1974). In support of this evidence we have found that addition of 50mM-KSCN to submitochondrial particles in the presence of valinomycin does not cause an increase in the fluorescence of added 8-anilinonaphthalene-1-sulphonate. A similar experiment with KCl instead of KSCN did produce a fluorescence increase, which is regarded as being diagnostic of the generation of a K^+ diffusion potential (Azzi *et al.*, 1971; Jasaitis *et al.*, 1971). It seems that SCN^-, unlike Cl^-, can rapidly permeate the membrane, thus preventing the formation of a K^+ diffusion potential.

HSCN has a sufficiently low pK_a (−1.8; Morgan *et al.*, 1965) that the concentration of HSCN will be so low both in the external medium and in the lumen of the particles that the passage of HSCN across the membrane should be insignificant. Further, SCN^- is thus unable to accumulate inside submitochondrial particles in response to a pH gradient (particle lumen acidic).

The distribution of the weak base [^{14}C]methylamine between the lumen of the particles and the external medium was used to monitor ΔpH. As the pK_a of methylamine (10.47) is substantially greater than the pH in either the internal or external phases then (Rottenberg, 1975):

$$\Delta\text{pH} = \log \frac{[\text{methylamine}]_i}{[\text{methylamine}]_o} \quad (3)$$

Use of eqn. (3) assumes that the ratio of activity coefficients for the internal and external phases is sufficiently close to unity that insertion of concentration terms into the equation is a reasonable approximation.

Determination of $\Delta\psi$ and ΔpH requires that the internal volume of the particles must be known (see the Results section and Table 1), and that the extent of SCN^- or methylamine uptake can be accurately measured. The usual method for measuring the uptake of a solute into an intramembrane space is to separate the membranes rapidly from the suspending medium either by centrifugation or by filtration, and to analyse the separated membranes for the accumulated solute. However, for small membrane vesicles such as submitochondrial particles use of these methods can be more difficult, and there is the attendant risk of accumulated material being lost from the particles during the separation procedure. It was to overcome these problems that Ramos *et al.* (1976) adopted the flow-dialysis method for following the uptake of solutes into vesicles from *Escherichia coli*.

The flow-dialysis method was also used in the present work. The general principle of this technique (Colowick & Womack, 1969; Ramos *et al.*, 1976) is that solutes in the upper chamber of a flow-dialysis cell diffuse through a dialysis membrane into the lower chamber of the cell at a rate that depends on the concentration of free solute in the upper chamber (Colowick & Womack, 1969). Hence, by constantly monitoring the diffusate from the upper chamber, changes in the concentration of unbound solute in the upper chamber can be followed. The advantage of the flow-dialysis technique is that it permits the accumulation of solutes by submitochondrial particles to be determined without separating the particles from the suspending medium. The uptake (or release) of a solute by submitochondrial particles in the upper chamber is directly reflected by a decrease (or increase) in the concentration of solute in the diffusate from the upper chamber. The total amount of any solute in the upper chamber is essentially constant during a typical experiment lasting

20min; only about 3% of the solute was lost from the upper chamber to the lower chamber of our flow-dialysis cell over the period of our experiments.

A cylindrical flow-dialysis cell that followed the design of Colowick & Womack (1969) was constructed in the workshop of the Botany School, University of Oxford. The volume of the lower chamber was 0.3ml and the upper chamber had a maximum capacity of 2.5ml. Visking dialysis tubing (Gallenkamp), of average pore diameter 2.4nm, was boiled for 1h in 5mM-EDTA (monosodium salt) and stored in water at 4°C before being inserted between the two chambers with Parafilm gaskets to ensure water-tightness. Water was pumped through the lower chamber, from a reservoir, at 2ml/min by means of a Watson–Marlow MHRE peristaltic pump. Fractions (1ml) of the outflow from the lower chamber, which contained the diffusate from the upper chamber, were collected in scintillation-vial inserts containing 2ml of Triton/toluene scintillant (Turner, 1969), held in an LKB Ultrorac fraction collector. The dead volume between the flow-dialysis cell and the fraction collector was 0.3ml. Radioactivity was counted in a Tracerlab Corumatic 200 liquid-scintillation counter. Water-saturated O_2 was blown over the upper chamber of the cell during all experiments.

Materials

All radioactive isotopes were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. KS^{14}CN and [^{14}C]methylamine hydrochloride were made up carrier-free to give stock solutions of 2.08mM and 2.25mM (60 and 55.5mCi/mmol) respectively. [U-^{14}C]Sucrose and ^3H_2O were respectively diluted to specific radioactivities of 5mCi/mmol and 400μCi/ml. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, nigericin and 2,3,5,6-tetramethyl-*p*-phenylenediamine were the respective gifts of Dr. P. G. Heytler (E.I. Du Pont de Nemours and Co., Wilmington, DE, U.S.A.), Dr. R. L. Hammill (Lilly Research Laboratories, IN, U.S.A.) and Professor A. Trebst (Ruhr-Universität Bochum, 463 Bochum, West Germany). Sodium D-isoascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine were from BDH Chemicals, Poole, Dorset, U.K. The *NNN'N'*-tetramethyl-*p*-phenylenediamine was recrystallized from ethanol (Sanadi & Jacobs, 1967). The following reagents and enzymes were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.: freeze-dried NH_4^+-free yeast hexokinase and yeast alcohol dehydrogenase, antimycin, oligomycin, valinomycin, 2-n-heptyl-4-hydroxyquinoline *N*-oxide ATP, ADP, NAD^+, Tris base and Triton X-100. The hexokinase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase and myokinase that were used in the assay for adenine nucleotides were all purchased as suspensions in $(NH_4)_2SO_4$ from Boehringer Corp. (London), Lewes, Sussex, U.K.

Results

Determination of the internal volume of the submitochondrial particles

The internal volume of the particles was estimated from the sucrose-impermeable space. An average

Table 1. *Determination of the internal volume of submitochondrial particles*

Submitochondrial particles were suspended (final concns. were: Expt. 1, 7.54mg/ml; Expt. 2, 3.63mg/ml; Expt. 3, 5.14mg/ml) in 10mM-P_i/Tris (pH7.3)/5mM-magnesium acetate with approx. 0.15μCi of [U-^{14}C]sucrose and 2.5μCi of ^3H_2O to a final volume of 5ml. The mixtures were centrifuged at 100000*g* in a Spinco 50 rotor at 4°C for 1h. Samples (1ml) of the supernatant were counted for radioactivity with 9ml of Triton/toluene scintillant (Turner, 1969). The pellets of the submitochondrial particles were resuspended to a volume of 5ml by homogenization in the original medium (except that the radioactive components were omitted) and centrifuged as above. Samples (1ml) of the resulting supernatant were again counted for radioactivity. This protocol avoids counting radioactivity in the pellet from the first spin, and thus no correction for differential quenching of ^{3}H and ^{14}C counts is required. The channels on the liquid-scintillation counter were set such that ^{3}H registered only in channel A, in which ^{14}C was counted with an efficiency of about 93%. The efficiency of counting of ^{14}C in channel B was approximately 42%. A standard containing only ^{14}C in the same scintillation mixture was run to obtain the factor by which the ^{14}C radioactivity in channel B had to be multiplied to obtain the ^{14}C counts in channel A. The ^{3}H counts in channel A were thus calculated by subtracting the ^{14}C counts in channel A (calculated as described above) from the total counts in channel A. Abbreviation: V_1, specific internal volume of the submitochondrial particles in μl/mg of protein, is given by:

$$V_1 = \frac{1000}{x}\left[\left(\frac{\text{c.p.m. of } ^3\text{H in 2nd supernatant}}{\text{c.p.m. of } ^3\text{H in 1st supernatant}}\right) - \left(\frac{\text{c.p.m. of } ^{14}\text{C in 2nd supernatant}}{\text{c.p.m. of } ^{14}\text{C in 1st supernatant}}\right)\right]$$

where x=mg of protein/ml in the 5ml initial reaction mixture. From the three experiments shown a mean value for the internal volume of submitochondrial particles was taken as 1.3μg/mg of protein.

		Radioactivity (c.p.m.)		V_1
		First supernatant	Second supernatant	(μl/mg of protein)
Expt. 1	^{14}C	56601	1772	1.0
	^{3}H	409365	16041	
Expt. 2	^{14}C	76252	2192	2.1
	^{3}H	723922	26287	
Expt. 3	^{14}C	51949	1314	1.0
	^{3}H	478545	14957	

value of 1.3 μl/mg of protein was obtained in the P_i / Tris / magnesium acetate reaction medium that was used as a routine for measurements of the protonmotive force (Table 1). Papa *et al.* (1973*b*) reported a value of 2.5 μl/mg of protein for the internal volume of Mg-ATP particles using the dextran-impermeable space as a marker for the internal volume. A possible explanation for the smaller internal volume determined in the present work is that dextran, being a considerably larger molecule than sucrose, is excluded from the interface between the particles and the suspending medium, thus giving a larger apparent impermeable space than sucrose. For EDTA-particles an internal volume of 1.4 μl/mg of protein has been reported (Papa *et al.*, 1973*a*), using the dextran impermeable space. However, this value cannot strictly be related to the internal volume of Mg-ATP particles as the different preparative procedures for the two types of particle may well produce particles of different sizes.

The internal volume of the submitochondrial particles is determined under conditions where the particles are not energized, but the internal volume under energized conditions is needed for measurement of $\Delta\psi$ and ΔpH. Energization of submitochondrial particles may cause swelling of the particles owing to the inward movement of H^+ plus any migrating anions into the particles. However, the magnitude of this effect appears to be small, except where relatively high concentrations of permeant ions are present (Papa *et al.*, 1973*a*). Even when significant swelling of submitochondrial particles was demonstrated (Papa *et al.*, 1973*a*), the maximum effect was less than a 2-fold increase in internal volume. It is noteworthy that 2-fold errors in the estimate of internal volume will be reflected (eqns. 2 and 3) by errors of only 18mV in the estimation of $\Delta\psi$ or 59ΔpH. In the present work an internal volume of 1.3 μl/mg of protein has been used throughout unless otherwise indicated. Use of this estimate of the volume means that, if there is a significant energy-linked swelling of the particles under our reaction conditions, our results will err in the direction of overestimating $\Delta\psi$ and ΔpH.

Determination of protonmotive force with NADH as substrate

Fig. 1 shows two plots of the radioactivity ($S^{14}CN^-$) in sequentially collected fractions of the outflow from the lower chamber of the flow-dialysis cell. The open symbols represent an experiment in which no submitochondrial particles were added to the upper chamber. The experiment was started by adding 20 μM-$KS^{14}CN$ to the upper chamber of the dialysis cell; simultaneously the collection of the outflow from the lower chamber was begun. After 2.5min (collection of five fractions) a steady-state distribution of $S^{14}CN^-$ across the dialysis membrane was attained (Fig. 1). At this point the rate of entry of $S^{14}CN^-$ ions into the lower chamber almost equalled the rate of loss of $S^{14}CN^-$ from the lower chamber to the fraction collector. In subsequent fractions the amount of radioactivity in the outflow decreased

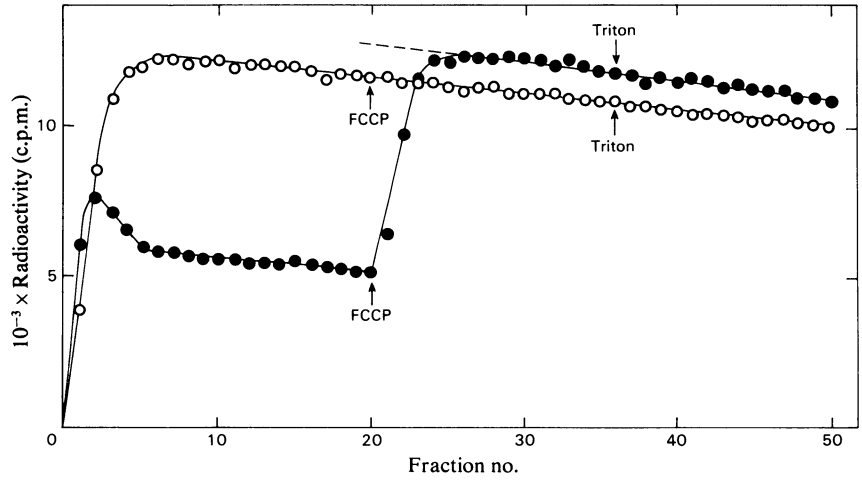


Fig. 1. *Respiration-driven uptake of the SCN^- ion by submitochondrial particles as determined by flow dialysis*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20 μM-$KS^{14}CN$ (60 μCi / μmol), 0.6mM-NAD^+, 1% (v/v) ethanol, 0.05mg of alcohol dehydrogenase, 10mM-P_i/Tris and 5mM-magnesium acetate. Submitochondrial particles (6.25mg of protein) were either included in (●) or omitted from (○) this reaction mixture as indicated. The temperature was 23°C and the pH was 7.3. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (5 μM) and Triton X-100 (0.1%, w/v) were added as shown.

slightly owing to the slow (approx. 3% depletion in 20min) decrease of the $KS^{14}CN$ concentration in the upper chamber. When the $S^{14}CN^-$ concentration in the upper chamber was varied (no submitochondrial particles present) there was a directly proportional variation in the amount of radioactivity in the outflow from the dialysis cell. Thus the concentration of unbound $S^{14}CN^-$ in the upper chamber of the dialysis cell could be reliably determined from the concentration of $S^{14}CN^-$ in the outflow from the lower chamber.

The closed symbols (Fig. 1) show an experiment in which the uptake of $S^{14}CN^-$ by respiring particles was measured. $KS^{14}CN$ (20μM) was added to the upper chamber of the dialysis cell, which also contained submitochondrial particles oxidizing NADH, and the collection of fractions of the outflow was started simultaneously.

The concentration of $S^{14}CN^-$ in the outflow first rose and then fell, before reaching a concentration at which there was an essentially steady-state distribution of $S^{14}CN^-$ across the dialysis membrane. Thereafter the concentration of $S^{14}CN^-$ in the outflow decreased very slowly (Fig. 1). The rise in $S^{14}CN^-$ concentration in the outflow before the fall to the steady-state concentration can be attributed to a relatively slow uptake of $S^{14}CN^-$ into the respiring particles. Mitchell & Moyle (1969) and Lehninger (1974) have shown that SCN^- crosses the inner mitochondrial membrane with a half-time ($t_{\frac{1}{2}}$) between 30 and 60s. Thus in our flow-dialysis experiments (Fig. 1) the concentration of free $S^{14}CN^-$ probably decreased during the approach to a steady-state distribution of $S^{14}CN^-$ across the dialysis membrane, and consequently the concentration of $S^{14}CN^-$ in the outflow 'overshot' (Fig. 1, closed symbols).

The response time of the flow-dialysis apparatus to a change in the $S^{14}CN^-$ concentration in the upper chamber was approx. 2.5min, as judged by the time taken for an approximately steady-state concentration of $S^{14}CN^-$ to be reached in the outflow after addition of 20μM-$KS^{14}CN$ to the upper chamber (open symbols, Fig. 1). The final extent of $S^{14}CN^-$ uptake by the respiring particles must be reached within this period as it can be seen from Fig. 1 (closed symbols) that a steady-state distribution of $S^{14}CN^-$ was reached within 2.5–3min of adding $KS^{14}CN$ to respiring particles. The rate of $S^{14}CN^-$ uptake by respiring particles was very much faster than the rate of $S^{14}CN^-$ dialysis. This is evident from Fig. 1, which shows that the particles took up half the total added SCN^- during a period in which virtually no $S^{14}CN^-$ was lost from the upper chamber of the cell by dialysis.

Addition of an uncoupler of oxidative phosphorylation, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, caused efflux of the $S^{14}CN^-$ accumulated by respiring submitochondrial particles (Fig. 1, closed symbols). The subsequent addition of a lytic amount of Triton X-100 did not cause any extra efflux of $S^{14}CN^-$ (Fig. 1), which indicated that addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone had completely dissipated the membrane potential and thus caused complete efflux of the accumulated $S^{14}CN^-$. Fig. 1 (open symbols) shows that addition of either carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or Triton X-100 had no effect on the rate of $S^{14}CN^-$ transfer across the dialysis membrane when no submitochondrial particles were present in the upper chamber.

$\Delta\psi$ was calculated as a routine from the extent of $S^{14}CN^-$ uptake before uncoupling. The difference between the amount of radioactivity in a given fraction of the outflow during the steady state before uncoupling and, by extrapolation, the amount of radioactivity that would have been in the same fraction had carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone been present throughout, was taken as the measure of $S^{14}CN^-$ uptake (Fig. 1). This procedure was adopted, rather than the alternative of measuring the decrease in radioactivity in the outflow after addition of respiratory substrate, for the following reason. The introduction into the upper chamber of substrates such as NADH, succinate or ascorbate perturbed the rate of $S^{14}CN^-$ transfer across the dialysis membrane, so that the extent of $S^{14}CN^-$ uptake after energization of the particles could not be readily calculated. A plausible explanation of this effect is that diffusion potentials were set up across the dialysis membrane owing to the different diffusion rates of the cation–anion pair added as substrate. We have observed similar effects on adding ATP to *Rhodospirillum rubrum* chromatophores in the upper chamber of the flow-dialysis cell (Kell *et al.*, 1978*a*), and van Dam *et al.* (1978) have reported similar phenomena.

After addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to the respiring particles, the concentration of $S^{14}CN^-$ (closed symbols, Fig. 1) in the outflow from the dialysis cell was slightly higher than in the experiment in which no particles were present (open symbols, Fig. 1). This effect is interpreted as follows. First, the uptake of $S^{14}CN^-$ by the respiring particles resulted in a substantially decreased concentration of free $S^{14}CN^-$ relative to that in the experiment in which no particles were present. Consequently, over a given period, slightly more $S^{14}CN^-$ was lost from the upper chamber in the experiment without particles. Therefore, after uncoupling, the concentration of $S^{14}CN^-$ in a given fraction of the outflow is expected to be slightly higher compared with the corresponding fraction of the outflow from the experiment without particles.

The data in Fig. 1 indicate that the extent of binding of $S^{14}CN^-$ to the non-energized particles is relatively small. The binding of a significant fraction

of the total $S^{14}CN^-$ to the particles would have been reflected by a markedly lower amount of radioactivity in the outflow from the dialysis cell when particles plus uncoupler (closed symbols, Fig. 1) were present compared with the experiment without particles (open symbols, Fig. 1).

If eqn. (2) is to be used to calculate the membrane potential, it is essential that the energy-linked uptake of $S^{14}CN^-$ represents an accumulation of the ion into the lumen of the particles, rather than an energy-dependent binding of $S^{14}CN^-$ to the particles. Discrimination between binding and accumulation can be made by varying the concentration of $S^{14}CN^-$. Binding of $S^{14}CN^-$ should be a saturable process, whereas accumulation of $S^{14}CN^-$ inside submitochondrial particles until the electrochemical equilibrium for $S^{14}CN^-$ is reached requires that the ratio $[SCN^-]_{in}/[SCN^-]_{out}$ (eqn. 2) be independent of the total concentration of $S^{14}CN^-$. When the $S^{14}CN^-$ concentration was varied from 5 to 50 μM, we found that the $S^{14}CN^-$ accumulation ratio was constant, consistent with the notion that $S^{14}CN^-$ uptake does represent an accumulation of $S^{14}CN^-$ to electrochemical equilibrium. The $S^{14}CN^-$ accumulation ratio was also unchanged when, at a fixed added $S^{14}CN^-$ concentration (20 μM), the particle concentration was varied from 1 to 8 mg of protein/ml. This result was again consistent with equilibrium accumulation of $S^{14}CN^-$ inside the particles rather than an energy-linked binding.

When a solute is extensively accumulated within submitochondrial particles, the concentration in the suspending medium in the upper chamber will greatly decrease. In these circumstances the rate of dialysis of the solute across the dialysis membrane will also decrease, but van Dam *et al.* (1978) have suggested that the accumulated solute can act as a buffer of solute so that the concentration of solute in the external medium, and hence the rate of solute dialysis, may not decrease to the 'true' extent. An effect of this kind would mean that, at a fixed total concentration of $S^{14}CN^-$, a higher accumulation ratio, $[SCN^-]_{in}/[SCN^-]_{out}$, would be observed at lower particle concentrations when the proportion of the total $S^{14}CN^-$ taken up would be less. However, as already noted, we found that the accumulation ratio was constant over an 8-fold range of particle concentration, so that it appears that the effect noted by van Dam *et al.* (1978) was not a source of error in our experiments.

From a series of experiments with ten different particle preparations, a value for the membrane potential of 145 ± 5 mV was obtained with NADH as substrate in the standard P_i/Tris reaction mixture. The mean value of 145 mV is included in Table 2 (Expt. 1).

The open symbols in Fig. 2 are a plot of the radioactivity ([^{14}C]methylamine) in sequentially collected fractions of the outflow from the lower chamber of the flow-dialysis cell, in an experiment where the

Table 2. *Magnitude of the components of the protonmotive force measured under various conditions*

$\Delta\psi$ and ΔpH were measured from the extent of $S^{14}CN^-$ or [^{14}C]methylamine uptake by using the flow-dialysis procedure. The upper chamber of the flow-dialysis cell contained in a volume of 1 ml: 10 mM-P_i/Tris, 5 mM-magnesium acetate and submitochondrial particles, plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. In Expts. 1 and 8, 12 mg of submitochondrial particle protein was present; in other experiments approx. 4 mg of particle protein was used. For measurements of $\Delta\psi$ 20 μM-$KS^{14}CN$ (60 μCi/μmol) was added to the upper chamber, and for measurements of ΔpH 20 μM-[^{14}C]methylamine hydrochloride (55.5 μCi/μmol) was added. When NADH was the substrate, 0.6 mM-NAD^+, 1% (v/v) ethanol and 0.05 mg of alcohol dehydrogenase were added. When succinate was the substrate, 10 mM-sodium succinate was 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 of ΔpH or $\Delta\psi$ to the observed ΔpH or $\Delta\psi$. Abbreviation: n.d., not determined. One unit of hexokinase catalyses the phosphorylation of 1.0 μmol of glucose/min at 25°C, pH 8.5.

Expt. no.	Substrate	Additions	$\Delta\psi$ (mV)	-59ΔpH (mV)	Δp (mV)
1	NADH	None	145	<30	145 (175)
2	NADH	10 mM-KNO_3	n.d.	90	—
3	NADH	10 mM-KSCN	<40	110	110 (150)
4	NADH	2 mM-KNO_3	90	60	150
5	NADH	10 mM-Potassium acetate, nigericin (0.5 μg/mg of protein)	145	n.d.	—
6	NADH	0.2 mM-ADP	145	n.d.	—
7	NADH	Oligomycin (1 μg/mg of protein)	150	n.d.	—
8	Succinate	None	140	<30	140 (170)
9	NADH	0.3 mM-ADP, 10 mM-glucose, 20 units of hexokinase	125	n.d.	—
10	Succinate	0.3 mM-ADP, 10 mM-glucose, 20 units of hexokinase	130	n.d.	—

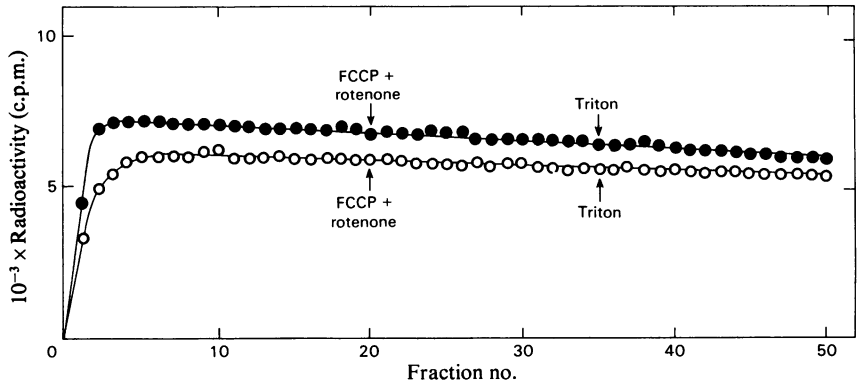


Fig. 2. *Absence of detectable respiration-driven uptake of methylamine into submitochondrial particles as determined by flow dialysis*

The upper chamber of the flow-dialysis cell contained in a final volume of 1 ml: 20 μM-[^{14}C]methylamine hydrochloride (55.5 μCi/μmol), 0.6 mM-NAD^+, 1% (v/v) ethanol, 0.05 mg of alcohol dehydrogenase, 10 mM-P_i/Tris and 5 mM-magnesium acetate. Submitochondrial particles (12.5 mg of protein) were either included in (●) or omitted from (○) this reaction mixture as indicated. The temperature was 23°C and the pH was 7.3. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5 μM), together with rotenone (5 μM), and Triton X-100 (0.1%, w/v) were added as shown.

upper chamber contained [^{14}C]methylamine plus the standard P_i/Tris reaction mixture without submitochondrial particles. The rise in radioactivity over the first five fractions, and the ensuing slow decay in the amount of radioactivity, is similar to, and has the same basis as, that seen with $S^{14}CN^-$ in the upper chamber (Fig. 1).

The closed symbols in Fig. 2 show an experiment in which 20 μM-[^{14}C]methylamine was added to the upper chamber of the dialysis cell, which also contained submitochondrial particles oxidizing NADH. Subsequent addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, together with rotenone, did not cause any efflux of [^{14}C]methylamine from the particles, as judged by the absence of a detectabie increase in the radioactivity in the outflow from the lower chamber. The addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone alone cannot always be guaranteed to decrease $\Delta\psi$ or ΔpH (or their sum) to zero in submitochondrial particles (P. C. Hinkle, personal communication). Therefore rotenone was added with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 2) to inhibit respiration and thus slow the rate of proton translocation across the membranes of the particles. At low rates of proton translocation (rotenone present), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, by carrying protons back across the membrane, would be expected to decrease the steady-state ΔpH or $\Delta\psi$ to a lower value than in the absence of a respiratory inhibitor. The precaution of adding rotenone was taken as the logarithmic nature of eqn. (3) means that the magnitude of ΔpH could be underestimated if carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were to dissipate only partially a small ΔpH. If ΔpH (or $\Delta\psi$) is large, a small residual gradient left after addition of the uncoupler will not cause a significant error, as the amount of [^{14}C]methylamine (or $S^{14}CN^-$) retained in the particles by any residual gradient will be very small relative to the amount of [^{14}C]methylamine (or $S^{14}CN^-$) released on addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. In practice we did not observe any extra efflux of either [^{14}C]-methylamine or $S^{14}CN^-$ on addition of rotenone in addition to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The conclusion that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone effectively dissipated ΔpH or $\Delta\psi$ is supported by the finding that no additional efflux of [^{14}C]methylamine or $S^{14}CN^-$ was seen on adding a lytic amount of Triton X-100 (Figs. 1 and 2).

The open symbols (Fig. 2) show that addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, rotenone or Triton X-100 had no effect on the rate of [^{14}C]methylamine transfer across the dialysis membrane when no submitochondrial particles were present in the upper chamber.

It is concluded from Fig. 2 that respiration generated an essentially insignificant ΔpH. The experiment shown in Fig. 2 was done at a high particle concentration so as to increase the extent of any [^{14}C]methylamine uptake. The reaction conditions were such that an uptake of 3% of the total added [^{14}C]methylamine would have been detected. The particle concentration was 12.5 mg of protein/ml (giving 16 μl of internal volume/ml), and from eqn. (3) it may be calculated that uptake of less than 3% of the methyl-

amine sets an upper limit on ΔpH of 0.5 unit (≡30mV). At an equally high concentration of particles the $S^{14}CN^-$ accumulation ratio was similar to that observed with lower concentrations, which therefore indicated that anaerobiosis of such highly concentrated suspensions of particles was not occurring.

The addition of permeant ions to respiring submitochondrial particles is expected to cause a decrease in $\Delta\psi$ and a compensating increase in ΔpH (Rottenberg & Lee, 1975). Table 2 (Expts. 2 and 3) shows that indeed the presence of either 10mM-KSCN or 10mM-KNO_3 resulted in the appearance of a substantial ΔpH. [Evidence that NO_3^- is a permeant ion has been given by Montal *et al.* (1970) and by Lehninger (1974).] When 10mM-KSCN was present no accumulation of $S^{14}CN^-$ inside the particles was detected in an experiment with sufficient particles to allow a $\Delta\psi$ of 40mV to be detected. Expt. 4 (Table 2) shows that with 2mM-KNO_3 added to the standard reaction mixture (P_i/Tris) both $\Delta\psi$ and ΔpH were detectable. The magnitude of the total protonmotive force was virtually the same as the value of $\Delta\psi$ measured in the absence of permeant ions (Expt. 1). This suggests that the flow-dialysis method was not failing to detect a small but significant ΔpH when only $\Delta\psi$ could be measured (as in Expt. 1, Table 2). The logarithmic nature of eqns. (2) and (3) means that the flow-dialysis method is relatively insensitive to a very small $\Delta\psi$ or ΔpH, but, if a ΔpH of as much as 0.5 unit (≡30mV) had escaped detection in Expt. 1, it might have been anticipated that the total measured protonmotive force would have been greater with 2mM-KNO_3 present (Expt. 4) than under standard conditions (Expt. 1). The basis of this argument would be undermined if 2mM-KNO_3 had an uncoupling effect on the particles, but, as shown below (Table 5), this concentration of KNO_3 did not decrease the magnitude of the phosphorylation potential generated by the submitochondrial particles and hence did not cause any uncoupling.

$\Delta\psi$ was not increased when K^+ plus nigericin were present (Table 2, Expt. 5). Nigericin catalyses an electroneutral exchange of accumulated protons inside the particles for externally added K^+, and so the effect of adding nigericin plus K^+ should be to increase $\Delta\psi$ at the expense of ΔpH (Ashton & Steinrauf, 1970; Montal *et al.*, 1970; see also Table 3). The failure of K^+ plus nigericin to increase $\Delta\psi$ is thus consistent with the absence of a significant ΔpH across respiring submitochondrial particles in the P_i/Tris reaction medium.

Expt. 6 (Table 2) showed that addition of ADP did not change the magnitude of the respiration-dependent $\Delta\psi$. Submitochondrial particles do not phosphorylate all the added ADP (Ferguson & Sorgato, 1977; Table 5), and the conditions in Expt. 6 were such that the final extent of conversion of added ADP into ATP would have been reached in less than 1min. Expt. 6 shows, therefore, that there is no energetic demand on the protonmotive force when there is no net ATP synthesis.

Oligomycin improves, probably by blocking a proton channel (Mitchell & Moyle, 1974), the coupling properties of submitochondrial particles (e.g. EDTA-particles) that are deficient in ATPase molecules. Addition of oligomycin (Expt. 7, Table 2) to the Mg-ATP particles used in the present work resulted in only a slight increase in $\Delta\psi$, consistent with the widely held view that Mg-ATP particles do not lose ATPase molecules during preparation.

Effect of osmolarity and ionic strength on the magnitude of the components of the protonmotive force

The major part of the present work was done with reaction media of both low osmolarity and low ionic strength. However, reaction media of high osmolarity and/or high ionic strength are often used for work with submitochondrial particles, and it was therefore decided to make some determinations of the protonmotive force with reaction conditions similar to those used by other workers. Table 3 shows that, in a Hepes/sucrose/KCl medium similar to that used by Thayer & Hinkle (1973) and by C. L. Bashford & W. S. Thayer (personal communication), the total protonmotive force was approx. 40mV higher than in the P_i/Tris medium (Table 2). It is noteworthy that in medium A (Table 3) both $\Delta\psi$ and ΔpH are of comparable magnitude. The relatively large ΔpH (compare Table 2, Expt. 1) can be attributed to the use of a relatively impermeable buffer (Hepes) and the inclusion of a high Cl^- concentration, which will presumably result in some accumulation of Cl^- ions at the expense of $\Delta\psi$. In calculating $\Delta\psi$ and ΔpH in medium A, an internal particle volume of 1 μl/mg of protein was used. This value was obtained from an experiment similar to those detailed in Table 1 except that medium A (Table 3) was substituted for the P_i/Tris suspending medium.

Inclusion of nigericin in reaction medium A (Table 3) resulted in an increased $\Delta\psi$ at the expense of a decreased ΔpH, as expected in view of the mode of action of nigericin outlined above.

Reaction medium B (Table 3) is similar to that used by Rottenberg & Lee (1975), except that we included Mg^{2+} and P_i, and differs from medium A in that the ionic strength is lower, Cl^- is replaced by the less-permeant acetate ion, and Hepes is replaced by Tris as buffer. The protonmotive force generated in reaction medium B is similar to that developed in the P_i/Tris medium (Table 2, Expt. 1), which was of comparable ionic strength but lower osmolarity.

Rottenberg & Lee (1975) reported that, in reaction medium C (Table 3), $\Delta\psi$ was probably very small, but ΔpH was very substantial. We could not detect $\Delta\psi$ in this medium, but the extent of respiration-

Table 3. *Magnitude of the components of the protonmotive force measured in reaction media of different osmolarity and ionic strength*

Submitochondrial particles (approx. 4mg of protein) were incubated in one of the reaction mixtures listed, together with 0.6mM-NAD^+, 1% (v/v) ethanol and 0.05mg of alcohol dehydrogenase. The total volume was 1ml and the temperature was 23°C. For measurement of $\Delta\psi$ 20μM-$KS^{14}CN$ (60μCi/μmol) was added to the upper chamber, and for measurement of ΔpH 20μM-[^{14}C]methylamine hydrochloride (55.5μCi/μmol) was added. $\Delta\psi$ and ΔpH were calculated from the extents of thiocyanate and methylamine uptake determined from flow-dialysis measurements. The symbol < and the values of Δp in parentheses have the significance described in the legend to Table 2. Volatile amines were removed from medium C by evaporation under reduced pressure.

	Reaction medium	Addition	$\Delta\psi$ (mV)	-59ΔpH (mV)	Δp (mV)
A	200mM-Sucrose 10mM-Hepes/NaOH 50mM-KCl 5mM-KH_2PO_4/KOH, pH7.5 2mM-$MgCl_2$, pH7.5	None	90	95	185
		Nigericin (0.5μg/mg protein)	135	<40	135 (175)
B	180mM-Sucrose 30mM-Tris/acetate 5mM-Magnesium acetate 10mM-P_i/Tris, pH7.5	None	135	<40	135 (175)
C	100mM-Choline chloride 1.5mM-Tris/HCl, pH7.5	None	<50	100	100 (150)

dependent [^{14}C]methylamine uptake indicated a ΔpH· of 1.6 units (≡100mV). The appearance of ΔpH was attributed (Rottenberg & Lee, 1975) to the presence of a high concentration of Cl^- and the virtual absence of a buffer.

Rottenberg & Lee (1975) determined ΔpH from the quenching of 9-aminoacridine fluorescence, obtaining values of 2.2 units under conditions similar to those of medium B (Table 3) and 3.6 units in medium C (Table 3). These values for ΔpH are much larger than those obtained in the present work (Table 3). Although part of this discrepancy may be because Rottenberg & Lee (1975) used a different type of particle preparation from that used in the present work, it seems that the 9-aminoacridine-fluorescence-quenching method gives larger values for ΔpH than the methylamine-uptake procedure. We made some measurements using the 9-aminoacridine method, and found ΔpH to be 2.8 units in medium A, 2.4 units in medium B and 3.0 units in the standard P_i/Tris reaction mixture plus 10mM-KNO_3. Comparison with results obtained from the extent of [^{14}C]methylamine uptake (Tables 2 and 3) shows that the 9-aminoacridine method gives values that are larger by about 1.5 units. In our standard P_i/Tris reaction mixture the extent of respiration-linked 9-aminoacridine-fluorescence quenching was very small (less than 1% of the total fluorescence) and so comparison of the two procedures for measuring ΔpH was not possible.

The estimation of ΔpH in the present work relies on methylamine uptake, rather than on 9-aminoacridine-fluorescence quenching, because doubts have been raised about the validity of the fluorescence method (Fiolet *et al.*, 1974, 1975; Kraayenhof *et al.*, 1976), although we recognize that there is some persuasive evidence that in sonicated phospholipid vesicles the procedure is reliable (Deamer *et al.*, 1972; Casadio & Melandri, 1977). Searle *et al.* (1977) reported that 9-aminoacridine binds, with fluorescence quenching, as a cation to the surface of chloroplast thylakoid membranes, and this may add weight to the reservations noted above.

Protonmotive force with succinate as substrate

Submitochondrial particles generate the same phosphorylation potential with either NADH or succinate as substrate (Ferguson & Sorgato, 1977), and Table 2 (Expts. 1 and 8) shows that both substrates also established a $\Delta\psi$ of similar magnitude. A value of 0.5 unit (≡30mV) for ΔpH has been given as an upper limit as no [^{14}C]methylamine uptake was detected under conditions where a ΔpH as low as 0.5 unit would have been detected. The protonmotive force with succinate as substrate was measured only in the standard P_i/Tris reaction medium.

Protonmotive force with ascorbate plus an electron mediator as substrate

The purpose of the experiments summarized in Table 4 was to determine the magnitude of the membrane potential that could be generated by electron flow through the cytochrome *c*/cytochrome aa_3 segment of the respiratory chain. In general, oxidation of ascorbate plus either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine generated a lower potential (Table 4) than was observed with NADH or succinate as sub-

strate. However, there was a greater variability in the size of the membrane potential linked to ascorbate oxidation, and in two sets of experiments ascorbate oxidation generated a potential as high as 130mV with either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine as electron donor.

Uptake of SCN^- driven by oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine was measured in the presence of either antimycin or 2-n-heptyl-4-hydroxyquinoline *N*-oxide, which were added to inhibit any electron flow through the cytochrome *b*–cytochrome c_1 section of the electron-transport chain. This ensured that electron flow was restricted to the cytochrome *c*–cytochrome aa_3 region of the chain. The titres of antimycin and 2-n-heptyl-4-hydroxyquinoline *N*-oxide necessary to inhibit electron flow between cytochromes *b* and c_1 were determined by titrating the rate of succinate oxidation with the two inhibitors. It is important to use the minimum titre of these two inhibitors, as when present in significant excess they can act as uncouplers (see e.g. Wikström, 1978). [The experiment with 2,3,5,6-tetramethyl-*p*-phenylenediamine as electron carrier (Table 4) was done with a slight excess of antimycin present.] Measurements of $\Delta\psi$ were made with either antimycin or 2-n-heptyl-4-hydroxyquinoline *N*-oxide present, as it has been suggested (Eisenbach & Gutman, 1975; Mitchell, 1976*b*; Papa *et al.*, 1978*a*,*b*) that these two inhibitors may have different modes of action despite the indications that they share a common binding site (Brandon *et al.*, 1972; van Ark & Berden, 1977). The combined effect of both antimycin and 2-n-heptyl-4-hydroxyquinoline *N*-oxide was also studied, as Papa *et al.* (1978*a*,*b*) have suggested that only when these two inhibitors are added together is electron flow through the cytochrome *b*–cytochrome c_1 region of the respiratory chain completely inhibited. Table 4 shows that with *NNN'N'*-tetramethyl-*p*-phenylenediamine as electron donor very similar values of $\Delta\psi$ were found with either or both of the inhibitors present.

Oxidation of ascorbate plus either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine did not generate a detectable ΔpH when the mitochondrial particles were suspended in the standard P_i/Tris reaction mixture. Moreover, whereas with NADH or succinate as substrate ΔpH was formed when the permeant NO_3^- ion was added to the standard reaction mixture (Table 2), no ΔpH linked to oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine was detected even when 5mM-KNO_3 was included in the same reaction mixture. The latter experiment was done under conditions where a ΔpH of 0.5 unit could have been detected, and in a separate experiment it was shown that 5mM-KNO_3 did not inhibit the oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine. Attempts to detect a ΔpH linked to ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation with 50mM-KCl and valinomycin (0.5μg/mg of particle protein) added to the standard P_i/Tris reaction mixture were also unsuccessful under conditions where a pH gradient of 0.5 unit would have been detected. Valinomycin and KCl were added to allow full expression of the protonmotive force as ΔpH (Thayer & Hinkle, 1973). Oxidation of ascorbate (a 2 electron plus 1 proton donor at pH7.0) at neutral pH can result in an alkalinization of the reaction medium, followed by an acidification due to hydrolysis of dehydroascorbate. In our experiments with a well-buffered reaction medium no pH changes were detected during ascorbate oxidation, so that the failure to detect a ΔpH (expected to be more acid inside the particles) was not due to a decrease in the external pH.

Comparison of the phosphorylation potential with the protonmotive force

According to the chemiosmotic hypothesis of energy coupling (Mitchell, 1966), the protonmotive force can be equated with the free energy stored in ATP, if it is assumed that the ATPase reaction is

Table 4. *Membrane potentials generated by ascorbate oxidation in submitochondrial particles*

Submitochondrial particles were incubated (approx. 6mg of protein) in a reaction mixture that contained the following components in the upper chamber of the flow dialysis cell: 10mM-P_i/Tris, 5mM-magnesium acetate, 10mM-sodium D-isoascorbate and 20μM-$KS^{14}CN$ (60μCi/μmol). Respiratory-chain inhibitors were added as indicated. The reaction was started by adding either *NNN'N'*-tetramethyl-*p*-phenylenediamine (0.1mM) or 2,3,5,6-tetramethyl-*p*-phenylenediamine (0.1mM). The pH, measured potentiometrically, remained at 7.3 throughout the experiments.

Electron carrier	Respiratory-chain inhibitor	Concentration (μg/mg of protein)	$\Delta\psi$ (mV)
NNN'N'-Tetramethyl-*p*-phenylenediamine	Antimycin	0.2	90
	2-n-Heptyl-4-hydroxyquinoline *N*-oxide	2	80
	Antimycin plus 2-n-heptyl-4-hydroxyquinoline *N*-oxide	0.2 + 2	75
2,3,5,6-Tetramethyl-*p*-phenylenediamine	Antimycin	1	95

Table 5. *Comparisons of the protonmotive force with the phosphorylation potential in submitochondrial particles*
Parallel experiments were done with the same preparation of submitochondrial particles to determine both the protonmotive force and the phosphorylation potential under the same set of conditions. For determination of the protonmotive force, submitochondrial particles (approx. 4–8 mg of protein) were incubated in one of the reaction mixtures listed, together with 0.2 mM-ADP. The total volume was 1 ml, and the temperature was 23°C. For measurement of $\Delta\psi$ 20 μM-KS^{14}CN (60 μCi/μmol) was added; for measurements of ΔpH 20 μM-[^{14}C]methylamine hydrochloride (55.5 μCi/μmol) was added. When NADH was the substrate, 0.6 mM-NAD^+, 1% (v/v) ethanol and 0.05 mg of alcohol dehydrogenase were added; when succinate was the substrate 10 mM-sodium succinate was added. The phosphorylation potential was determined by incubating submitochondrial particles (approx. 1.5 mg of protein) in the appropriate reaction mixture to which were added 0.2 mM-ADP and 20 μM-KSCN. The presence of 20 μM-KSCN did not decrease the phosphorylation potential but as a routine it was included; 20 μM-methylamine hydrochloride (in either the presence or absence of 20 μM-KSCN) was also found not to decrease the phosphorylation potential but as a routine was omitted. The temperature was 23°C, and the total volume 3 ml. The experiments were done in a cell that was fitted with a Clark-type oxygen electrode. The reaction mixtures were open to the atmosphere and the response of the electrode showed that they did not become anaerobic during the 5 min period in which the particles were allowed to phosphorylate added ADP. At the end of this period [which has been previously shown to be sufficient to allow the maximum extent of ADP phosphorylation to be reached (Ferguson & Sorgato, 1977)], 2 ml of the reaction mixture was added to 0.2 ml of ice-cold 40% $HClO_4$. The acid extracts 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 as described by Bergmeyer (1970). In calculating ΔG_p a value for $\Delta G^{0'}$ of 30.1 kJ/mol (7.2 kcal/mol) was used (Rosing & Slater, 1972). Under conditions in which a pH gradient could not be detected the value of Δp given comprises $\Delta\psi$ alone. For these experiments a value of Δp assuming ΔpH equivalent to 30 mV (or in one case 40 mV), which we regard as upper limits, is also shown in parentheses. Similarly the values for H^+/ATP shown in parentheses also include an estimate of 30 mV (or 40 mV) for ΔpH in the total protonmotive force.

Substrate	Reaction medium	Δp (mV)	ΔG_p [kJ/mol (kcal/mol)]	H^+/ATP
NADH	10 mM-P_i/Tris, 5 mM-magnesium acetate, pH 7.3	145 (175)	43.1 (10.3)	3.1 (2.6)
NADH	10 mM-P_i/Tris, 5 mM-magnesium acetate, 2 mM-KNO_3, pH 7.3	150	43.1 (10.3)	3.0
Succinate	10 mM-P_i/Tris, 5 mM-magnesium acetate, pH 7.3	140 (170)	42.2 (10.1)	3.1 (2.6)
NADH	200 mM-Sucrose, 10 mM-Hepes/NaOH, 50 mM-KCl, 5 mM-KH_2PO_4/KOH, 2 mM-$MgCl_2$, pH 7.5	185	43.1 (10.3)	2.4
NADH	180 mM-Sucrose, 30 mM-Tris/acetate, 5 mM-magnesium acetate, 10 mM-P_i/Tris, pH 7.5	135 (175)	43.5 (10.4)	3.3 (2.6)

poised against the protonmotive force so that:

$$\Delta G_p = -zF\Delta p \qquad (4)$$

where ΔG_p is the phosphorylation potential ($= \Delta G^{0'} + RT \ln [ATP]/[ADP][P_i]$), F is the Faraday constant, z the number of protons that are translocated across the particle membrane for each molecule of ATP synthesized (i.e. H^+/ATP ratio), and Δp is the protonmotive force.

Data from experiments in which ΔG_p was compared with Δp under several different sets of reaction conditions are given in Table 5. In the standard P_i/Tris reaction medium oxidation of either NADH or succinate generated similar values for both ΔG_p and Δp. The magnitude of ΔG_p generated by the particles used for experiments shown in Table 5 was slightly lower than was found, under similar conditions, in earlier work (Ferguson & Sorgato, 1977). A lower ΔG_p was not due to the presence of either 20 μM-KSCN or 20 μM-methylamine, both of which had no effect on ΔG_p. Some preparations of particles gave higher values of ΔG_p [up to 44.3 kJ/mol (10.6 kcal/mol)], but an unchanged Δp, so that there appeared to be some variation in the exact value of ΔG_p between different preparations of particles.

Substitution of the values of Δp and ΔG_p into eqn. (4) gives a value of 3.1 for the H^+/ATP ratio with either NADH or succinate as substrate in the P_i/Tris reaction medium. No ΔpH was detected under these conditions (Table 2), when the lower limit of detection for ΔpH was about 0.5 pH unit, which is equivalent to 30 mV. The values shown in parentheses for Δp and the H^+/ATP ratio (Table 5) thus represent respectively upper limits on Δp and lower limits for H^+/ATP.

When the particles were suspended in a sucrose/Tris/acetate reaction medium, the values for Δp and ΔG_p were very similar to those obtained in the P_i/Tris reaction medium (Table 5). The lower limit of detection for ΔpH in the experiment with a sucrose/Tris/acetate reaction medium was judged to be 0.65 pH unit, and so the values of Δp and H^+/ATP in parentheses were obtained by including a contribution of 40 mV for the ΔpH.

Table 2 showed that addition of 2 mM-KNO_3 to the

P_i/Tris reaction medium resulted in a decrease in the value of $\Delta\psi$ with a compensating increase in ΔpH. Table 5 shows that particles respiring with NADH as substrate in the presence of 2mM-KNO_3 generated a ΔG_p of the same magnitude as in the absence of 2mM-KNO_3. The H^+/ATP ratio in the presence of 2mM-KNO_3 was thus 3.0, and under those reaction conditions there was no uncertainty about a failure to detect a very small $\Delta\psi$ or ΔpH as both these variables have values (Table 2) that are well above the lower limit of detection afforded by the flow-dialysis method. As ΔG_p was not lowered by the addition of 2mM-KNO_3, and as Δp ($\Delta\psi - 59\Delta$pH) in the presence of KNO_3 was very similar to the Δp ($\Delta\psi$ only) in the absence of KNO_3, it seems probable that, as suggested above, $\Delta\psi$ is the sole component of Δp in the P_i/Tris reaction medium.

Although Δp was as much as 35mV larger in the Hepes/sucrose/KCl reaction medium (Tables 3 and 5), the magnitude of ΔG_p generated under these conditions was no larger than in the P_i/Tris reaction medium. Hence the H^+/ATP ratio was lower in the Hepes/sucrose/KCl medium.

Ascorbate oxidation with either *NNN'N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine as mediator did not generate a significant ΔG_p; virtually all the added ADP was recovered as AMP. A rather low value of ΔG_p is expected with ascorbate as substrate since a Δp of 80–90mV means that, in comparison with NADH or succinate as substrate, ΔG_p should be decreased by approximately one-half to 5.1kcal/mol. Presumably, when ascorbate is being oxidized, the ATPase is able to hydrolyse the ATP that is produced by the adenylate kinase reaction together with AMP. Hence the final product is AMP. However, *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated ascorbate oxidation did support the synthesis of ATP with a glucose and hexokinase trap present (the rate was 50nmol/min per mg of protein), and so it appears that the protonmotive force on the ATPase is sufficient to drive ATP synthesis at only very low prevailing phosphorylation potentials. This contrasts with the situation in rat liver mitochondria, where ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine generate an extramitochondrial phosphorylation potential of very similar magnitude to that generated by either NADH or succinate (van Dam *et al.*, 1978). van de Stadt *et al.* (1973) indirectly showed that the oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine in submitochondrial particles maintained a lower protonmotive force than either NADH or succinate oxidation, as they found that activation of the ATPase by the protonmotive force ('energy pressure' in their terminology) was markedly less with ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine as substrate.

Rate of ATP synthesis and magnitude of protonmotive force

Submitochondrial particles catalyse NADH-driven ATP synthesis at about twice the rate of succinate-driven ATP synthesis (see, e.g., Thayer & Hinkle, 1975; Ferguson & Sorgato, 1977). As a possible relationship between the protonmotive force and the rate of ATP synthesis is of interest (Portis & McCarty, 1974, 1976; Ferguson, 1977; Schönfeld & Neumann, 1977), the magnitude of the protonmotive force was determined during ATP synthesis driven by either NADH or succinate. Table 2 (Expts. 9 and 10) shows that $\Delta\psi$ was similar with both substrates, but was about 15mV lower than the value when no ATP synthesis was occurring. Thus in submitochondrial particles ATP synthesis puts an energetic demand on the protonmotive force just as in other systems such as mitochondria (Nicholls, 1974; Rottenberg, 1975) and chloroplasts (Pick *et al.*, 1973). Assuming that only a very small or zero ΔpH is present during ATP synthesis driven by either NADH or succinate oxidation, it is concluded that, although the rate of ATP synthesis is lower with succinate than with NADH as substrate, the protonmotive force does not decrease on replacing NADH by succinate.

Discussion

Respiring submitochondrial particles have been shown to generate a phosphorylation potential of about 44.3kJ/mol (10.6kcal/mol). It has been argued that this relatively low value is not because these submitochondrial particles are poorly coupled relative to systems capable of generating a higher phosphorylation potential (Ferguson & Sorgato, 1977). The phosphorylation potential generated by submitochondrial particles is of similar magnitude to the intramitochondrial phosphorylation potential, and thus it has been suggested that the maximum phosphorylation potential observed with particles reflects the limit to which the mitochondrial ATP-synthesizing apparatus can drive the formation of ATP (Ferguson & Sorgato, 1977), and that the difference between the intra- and extra-mitochondrial phosphorylation potentials is due to the energy expended in adenine nucleotide (and perhaps phosphate) translocation (Brand, 1977; Brand & Lehninger, 1977; Ferguson & Sorgato, 1977; Klingenberg & Rottenberg, 1977). This suggestion requires that the protonmotive force (which, according to the chemiosmotic hypothesis, drives ATP synthesis) in submitochondrial particles is not markedly lower than in other energy-coupling systems, as the relationship between the protonmotive force and phosphorylation potential (eqn. 4) shows that a low protonmotive force alone would account for a low phosphorylation potential.

The present work shows that the protonmotive

force in submitochondrial particles is in the range 145–185mV, the exact value depending on the osmolarity and ionic composition of the medium in which the particles are suspended. A protonmotive force in this range is comparable with some of the estimates that have been made for other systems. For example, Padan & Rottenberg (1973) and Wiechmann *et al.* (1975) have obtained values of 160mV and 175mV respectively for respiring rat liver mitochondria. Further, we have determined, with the same techniques as are described in the present paper, that chromatophores from *Rhodospirillum rubrum* and phosphorylating vesicles from *Paracoccus denitrificans* generate protonmotive forces of 100mV and 145mV respectively (Kell *et al.*, 1978*a*,*b*). Yet, although the protonmotive force in submitochondrial particles is similar to its counterpart in other systems, the phosphorylation potential is distinctly lower than with either the chromatophores or the bacterial vesicles, for which ΔG_p values of between 54 and 59kJ/mol (13 and 14kcal/mol) have been found (Kell *et al.*, 1978*a*,*b*). Hence it appears that the phosphorylation potential generated by submitochondrial particles is not restricted to a low value by an unusually low protonmotive force, thus supporting the arguments of Ferguson & Sorgato (1977).

Comparison of the protonmotive force with the phosphorylation potential enables a value to be estimated for the number of protons (H^+/ATP ratio) that would be translocated across the mitochondrial membrane via the ATPase for each molecule of ATP synthesized, provided that a chemiosmotic mechanism is operating, and assuming that equilibrium is reached between the protonmotive force and the phosphorylation potential. Values for the H^+/ATP ratio in the range 2.4 and 3.1 are obtained in this way (Table 5). Chemical propriety suggests that H^+/ATP ratio should be an integer, and thus the present work indicates that the value of this ratio is 3, whereas previous work is consistent with a value of 2. The evidence for an H^+/ATP ratio of 2 is, briefly, as follows.

Thayer & Hinkle (1973) measured the H^+ uptake into submitochondrial particles that followed the addition of a pulse of ATP to the particles at a pH (6.2) at which no H^+ release was associated with the ATP hydrolysis reaction. It was found that the H^+/ATP ratio approached 2, and so the results of kinetic experiments would seem to be at variance with the present data, which were thermodynamic measurements. A possible explanation for this discrepancy is that the H^+/ATP ratio is lower at the pH of 6.3 used by Thayer & Hinkle (1973) than at pH7.3, which was used in our work. It is noteworthy that studies on active transport by bacterial membrane vesicles have indicated a dependence on pH of the H^+/substrate ratio (Ramos & Kaback, 1977). However, Moyle & Mitchell (1973) also obtained an H^+/ATP ratio of 2 using a similar experimental approach to that of Thayer & Hinkle (1973) except that the measurements were made with rat liver mitochondrial particles and the pH was 7.3, which meant that pH changes due to the scalar hydrolysis of ATP had to be corrected for.

An H^+/ATP ratio of 2 for the mitochondrial ATPase is also indicated by the work of Brand & Lehninger (1977), who carefully redetermined the value of this ratio in experiments in which pulses of ATP were added to rat liver mitochondria under conditions where any complicating transmembrane movements of phosphate were inhibited. Nevertheless, the determination of the H^+/ATP ratio from experiments with whole mitochondria is complicated by the necessary involvement of the adenine nucleotide carrier in the overall process of hydrolysing added ATP, and thus there may be proton movements, associated with ATP hydrolysis by mitochondria, that arise from processes other than the simple hydrolysis of ATP.

Agreement between the values of the H^+/ATP ratio estimated from kinetic and thermodynamic experiments would be expected if energy coupling proceeds via a purely chemiosmotic mechanism. However, if membrane-bound protons (Williams, 1977) were also to participate in coupling, measurements of the protonmotive force might underestimate the true thermodynamic potential of the energized state, and so lead to an overestimate of the H^+/ATP ratio. Kinetic measurements of H^+ uptake, on the other hand, would presumably measure the total number of protons moved into or across the membrane, and thus give the number of protons that participate in the ATPase reaction.

Returning to the widely held view that coupling does occur via a chemiosmotic mechanism (Mitchell, 1977), we must consider that an H^+/ATP ratio of 2 for the ATPase would become consistent with the results of the present work if either the protonmotive force had been underestimated or our value for the phosphorylation potential overestimated. The latter is unlikely, as the value of the phosphorylation potential depends on measuring concentrations of ATP, ADP and P_i, all of which can be done with relative accuracy. In addition a value for $\Delta G^{0\prime}$ must be known. We have used the data of Rosing & Slater (1972), which is one of the lowest estimates in the literature; another determination (Guynn & Veech, 1973) gave a value for $\Delta G^{0\prime}$ that was approx. 2.5kJ/mol (0.6 kcal/mol) higher than the value of Rosing & Slater (1972). Thus it is possible that our values of the phosphorylation potential (Table 5) should be increased by this amount [but see discussion of Slater (1976)] which would result in a small increase in the H^+/ATP ratio.

Underestimation of the protonmotive force could arise from use of a value for the internal volume that

is too high (see eqns. 2 and 3). For instance, if our value of 1.3 μl/mg of protein were a 2-fold overestimate, then the values for both $\Delta\psi$ and ΔpH (Tables 2–5) would have to be increased by 18mV. The addition of an extra 36mV to the protonmotive force measured in the sucrose/Hepes/KCl reaction medium (Table 5) would raise the protonmotive force to 220mV and lower the H^+/ATP ratio to approx. 2. The H^+/ATP ratio with 2mM-KNO_3 present would also be decreased, from 3.0 to 2.4. Thus, for all our data to become consistent with an H^+/ATP ratio of 2, the error in overestimating the internal volume would have to be of the order of 3-fold. Failure of the flow-dialysis technique to measure the full extent of $S^{14}CN^-$ or [^{14}C]methylamine uptake accurately would also lead to an underestimation of the protonmotive force, but we have argued above why we consider that the full extent of uptake was measured in our experiments.

An additional source of error in our work would arise if the assumptions made in using eqns. (2) and (3) were invalid. Portis & McCarty (1973) have pointed out that at pH 7.3 the concentration of the unprotonated form of methylamine may be so low that the assumption of a much higher membrane permeability towards the uncharged rather than the charged form may no longer be justified. However, Casey *et al.* (1977) have shown that the uptake of [^{14}C]methylamine into non-energized chromaffin granules leads to an estimate of the intragranular pH that is very close to the value obtained from observation of pH-dependent chemical shifts of the ^{31}P n.m.r. signals from ATP molecules inside the granules (Ritchie, 1975). As the external pH at which the granules were suspended was 6.5, it may be concluded that the presence of only a very small fraction of the methylamine as the free base does not introduce significant errors into this method for determining pH gradients.

Our evidence that $S^{14}CN^-$ reaches an equilibrium distribution with the membrane potential is that the accumulation ratio $[S^{14}CN^-]_{in}/[S^{14}CN^-]_{out}$ is independent of the total $S^{14}CN^-$ concentration. It is also significant in view of the different assumptions that underlie the use of eqns. (2) and (3) that almost quantitative conversion of $\Delta\psi$ into ΔpH can be achieved by increasing the concentration of KNO_3 or KSCN (Table 2). Failure to demonstrate complete quantitative interconversion was probably due to the chaotropic SCN^- and NO_3^- ions damaging the submitochondrial particles at the concentrations required (>10mM), as judged by the lowered phosphorylation potential that was observed with either 10mM-KSCN or -KNO_3. However, we have found that with *Rhodospirillum rubrum* chromatophores addition of 10mM-KSCN results in a quantitative conversion of $\Delta\psi$ into ΔpH (Kell *et al.*, 1978*a*), an observation that provides support for the contention that $S^{14}CN^-$ and [^{14}C]methylamine uptake are reliable indicators of $\Delta\psi$ and ΔpH in membrane vesicles.

Our estimate of the H^+/ATP ratio might also be overestimated if there was a significant degree of heterogeneity in the particle preparations. Substantial amounts of mitochondrial particles that had retained the same orientation as the parent mitochondria would have contributed to the total sucrose-impermeable space, but would not have taken up $S^{14}CN^-$, thus leading to an underestimate of $[SCN^-]_{in}$. The presence of such particles is improbable, as it is known that sonication of mitochondria produces a population of completely inverted particles (Racker, 1970), and, consistent with this view, we found that our particle preparations did not oxidize potassium ferrocyanide, which is a non-penetrant reductant for cytochrome *c*.

Assuming that all the membranes of the submitochondrial particles used in the present work were oriented in the sense that the ATPase faced the suspending medium, we can consider whether a significant fraction of uncoupled, and therefore non-phosphorylating, particles was present. By contributing to the sucrose-impermeable space such particles could again lead to an underestimation of $[SCN^-]_{in}$. However, the ATPase activity of a significant population of these putative uncoupled particles would also be expected to lower the phosphorylation potential, as the particles used in the present work had a relatively high intrinsic ATPase activity and are deficient in the ATPase-inhibitor protein (Ferguson *et al.*, 1977). As discussed by Ferguson & Sorgato (1977), the phosphorylation potential is not increased in experiments in which either the rate of ATP synthesis is lowered or adenylyl imidodiphosphate is added to inhibit the ATPase activity of any uncoupled particles. These observations are taken to indicate the absence of a significant fraction of uncoupled vesicles.

Finally we consider the possibility that the particles consisted of a heterogeneous population that developed a range of Δp values. If only those particles with a Δp above a certain threshold were to synthesize ATP, and, if the remaining particles were unable to equilibrate with the phosphorylation potential, then comparison of Δp with ΔG_p would lead to an overestimate for the H^+/ATP ratio. There is evidence that this type of behaviour might occur in thylakoids (Junge, 1977), where a certain threshold energized state may be required before the ATPase is activated. To our knowledge there is no evidence for such a threshold, which is not expected on thermodynamic grounds, in submitochondrial particles, and, in contrast with thylakoids, the ATPase of the particles used in the present work is activated in the absence of membrane energization. Thus we think that the contribution of particles generating a low Δp would also be reflected in a lowered ΔG_p, so that H^+/ATP would not be overestimated.

Unless we have substantially underestimated the effect of the most likely sources of error, the present work indicates that the H^+/ATP ratio for the mitochondrial ATPase is 3, and indeed there are some cogent reasons for supposing that the ratio is 3. Rottenberg & Gutman (1977) have concluded that an H^+/ATP ratio of 3 together with an $H^+/2e$ site ratio of 4 would best explain their data on the energetics of ATP-driven reversed electron flow from succinate to NAD^+ in submitochondrial particles. Indications that the $H^+/2e$ site ratio could be as high as 4 have been obtained (Brand, 1977; Brand *et al.*, 1976*a*,*b*; Reynafarje *et al.*, 1976; Reynafarje & Lehninger, 1977), and the possibility has been recognized that the H^+/ATP ratio for the mitochondrial ATPase might be 3, with one proton being used to drive energy-linked transport of substrates across the mitochondrial membrane (Brand, 1977; Brand & Lehninger, 1977).

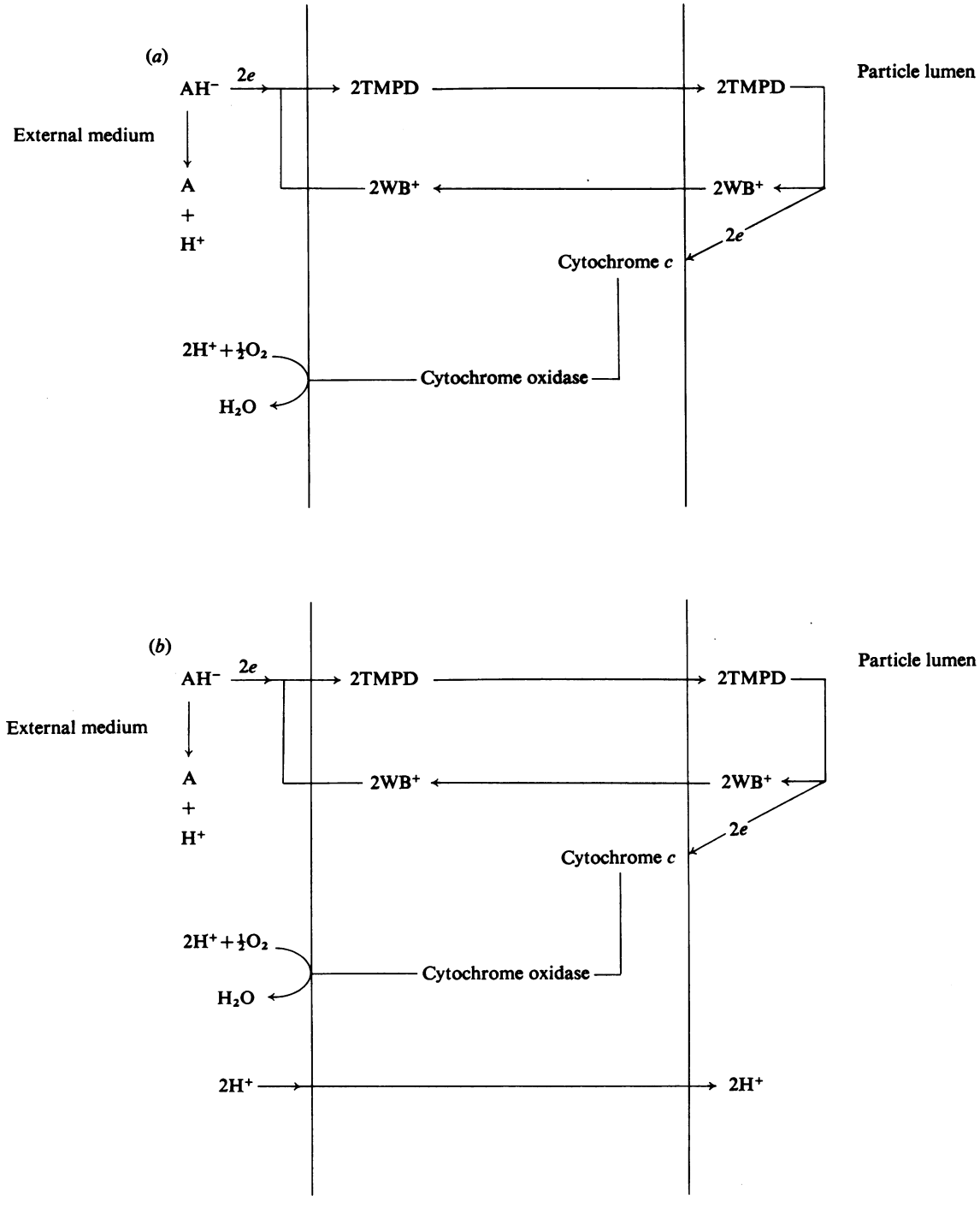
The ATPase of the thylakoid membrane in chloroplasts is structurally closely related to the mitochondrial ATPase, and considerable evidence has accrued that the H^+/ATP ratio for the thylakoid ATPase is 3, as judged by several independent methods (Avron *et al.*, 1976; McCarty & Portis, 1976; Junge, 1977). McCarty & Portis (1976) have suggested that the discrepancy between an H^+/ATP ratio of 3 for thylakoid ATPase and 2 for mitochondrial ATPase is disquieting. An H^+/ATP ratio of 3 for the mitochondrial ATPase seems attractive to us in view of the foregoing considerations, although, if this is the case and if the overall H^+/ATP ratio for synthesis of extramitochondrial ATP is also 3 (Nicholls, 1974; Wiechmann *et al.*, 1975), then accommodating the charge movement that is associated with the adenine nucleotide translocator (Klingenberg & Rottenberg, 1977) poses a problem.

A very similar protonmotive force is generated when either NADH or succinate is oxidized by submitochondrial particles (Table 2). As the rate of succinate oxidation is only approx. 66% of the NADH oxidation rate in these particles (Ferguson & Sorgato, 1977), and allowing for the smaller number of protons that are translocated for each molecule of succinate oxidized, it is evident that the same protonmotive force can be maintained over a 2.5-fold range of proton-pumping rates. This suggests that the conductance of the membrane in submitochondrial particles is non-ohmic at higher respiratory rates, just as has been shown for the inner membrane of mitochondria from rat liver and adipose tissue (Nicholls, 1974, 1977*b*). The rate of ATP synthesis by submitochondrial particles depends on whether NADH or succinate is substrate, and typically the rate with NADH is twice that found with succinate (Thayer & Hinkle, 1975; Ferguson & Sorgato, 1977). As the size of the protonmotive force does not depend on whether NADH or succinate is driving ATP synthesis (Table 2), there does not seem to be a simple relationship between the size of the protonmotive force and the rate of ATP synthesis. This result contrasts with the reports of a close relationship between the protonmotive force and the rate of ATP synthesis in thylakoids (Gräber & Witt, 1976; McCarty & Portis, 1976; Portis & McCarty, 1976; Schönfeld & Neumann, 1977).

Hauska *et al.* (1977) have pointed out that, as *NNN'N'*-tetramethyl-*p*-phenylenediamine is an electron carrier, oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine should not be able to drive energy-linked reactions via a protonmotive force in submitochondrial particles, provided that the terminal segment of the respiratory chain is organized by the generally held view (Scheme 1*a*). However, earlier work has indicated that oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine can be coupled both to the generation of a membrane potential (Grinius *et al.*, 1970), and to the synthesis of ATP (Tyler *et al.*, 1966). It has been suggested that, in the experiments of Grinius *et al.* (1970), oxidation of ascorbate was not responsible for the membrane potential, as the measurements were made in the presence of succinate and antimycin, so that *NNN'N'*-tetramethyl-*p*-phenylenediamine might have been acting as an internal by-pass of the antimycin-binding site and thus the membrane potential could have been linked to a *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated oxidation of succinate (Hauska *et al.*, 1977). From a re-examination of the antimycin-sensitivity of ascorbate-plus-*NNN'N'*-tetramethyl-*p*-phenylenediamine-driven ATP synthesis (Hauska *et al.*, 1977) it has also been suggested that the entry of electrons into the respiratory chain at the level of cytochrome *b* is responsible for the observed ATP synthesis, and that electron flow from cytochrome *c* to oxygen is not coupled to ATP synthesis (Hauska *et al.*, 1977).

Doubt has been cast on the conclusions of Hauska *et al.* (1977) by Wikström (1978), who has pointed out that the concentrations of antimycin used to inhibit ascorbate-driven ATP synthesis were in excess of those needed to inhibit electron flow between cytochrome *b* and cytochrome c_1, and were likely to inhibit ATP synthesis by a secondary uncoupling effect. Our data (Table 4) also suggest that the conclusion of Hauska *et al.* (1977) is not correct, as we clearly observe a membrane potential linked to the oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine in the presence of sufficient 2-n-heptyl-4-hydroxyquinoline *N*-oxide or antimycin to block electron flow between cytochromes *b* and c_1. Thus there is good reason to believe that electron flow from cytochrome *c* to oxygen is coupled to the production of a protonmotive force, and that Scheme 1(*a*) must be modified.

Wikström (1977, 1978) has presented evidence that



Scheme 1. *Oxidation of ascorbate mediated by NNN'N'-tetramethyl-p-phenylenediamine in submitochondrial particles, assuming (a) no proton translocation linked to electron flow between cytochrome c and cytochrome oxidase and (b) a proton pump activity associated with cytochrome oxidase*

Abbreviations: AH^-, ascorbate; A, dehydroascorbate, WB^+, Wurster's Blue; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.

cytochrome oxidase has a proton-pumping activity, and incorporation of this feature into the terminal segment of the respiratory chain is shown in Scheme 1(*b*). It can be seen that the oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine is expected to generate a protonmotive force according to Scheme 1(*b*), and so our data would appear to support the concept of proton-pumping activity associated with cytochrome oxidase. Scheme 1(*b*) predicts that the oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine should be linked to the production of $\Delta\psi$ and/or ΔpH, the relative magnitudes of these two components depending on the reaction conditions. However, an enigmatic feature of our experiments was that we were unable to observe a pH gradient linked to ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation even under conditions (presence of 5mM-KNO_3 or 50mM-KCl plus valinomycin) that should have increased ΔpH at the expense of $\Delta\psi$. Hence, although Wikström & Saari (1977) have found evidence for proton pumping associated with oxidation of ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine in submitochondrial particles, our experiments do not directly confirm this proposal, although we do not exclude the possibility that a small ΔpH escaped detection in our experiments. Further work designed to detect a ΔpH linked to ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation is reported in Sorgato & Ferguson (1978).

The generation of a membrane potential linked to ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation could become consistent with Scheme 1(*a*) if preparations of *NNN'N'*-tetramethyl-*p*-phenylenediamine were contaminated with a demethylated derivative (P. C. Hinkle, personal communication). Data in Table 4 were obtained with recrystallized *NNN'N'*-tetramethyl-*p*-phenylenediamine, although no difference in the magnitude of the membrane potential was noted when unpurified *NNN'N'*-tetramethyl-*p*-phenylenediamine was used. Thus we do not think that our results can be explained on the basis that the *NNN'N'*-tetramethyl-*p*-phenylenediamine contained substantial amounts of demethylated *NNN'N'*-tetramethyl-*p*-phenylenediamine that could act as a proton-plus-electron carrier in an analogous manner to 2,3,5,6,-tetramethyl-*p*-phenylenediamine (Hauska *et al.*, 1977). A membrane potential could also be generated if the positively charged Wurster's Blue were accumulated inside the particles, but this seems unlikely as the potential was dissipated by an uncoupler, which is expected to reverse a proton gradient.

Papa *et al.* (1978*a*,*b*) have suggested that the proton-pumping activity assigned to cytochrome oxidase (Wikström, 1977, 1978; Wikström & Saari, 1977) is really associated with the cytochrome bc_1 region of the respiratory chain. This proposal was prompted by the finding that 2-n-heptyl-4-hydroxyquinoline *N*-oxide, but not antimycin, inhibited proton movements during oxidation of ferrocyanide by mitochondria, and thus it was proposed that ferrocyanide donated electrons not only to cytochrome *c*, as assumed by Wikström (1977, 1978), but also to redox carriers in the cytochrome bc_1 region of the respiratory chain. Our experiments showed that addition of sufficient 2-n-heptyl-4-hydroxyquinoline *N*-oxide to inhibit electron flow from cytochrome *b* to cytochrome c_1, in either the presence or absence of antimycin, did not inhibit the generation of a membrane potential linked to ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation. Thus it is unlikely that *NNN'N'*-tetramethyl-*p*-phenylenediamine was donating electrons to a putative proton-pumping redox carrier in the cytochrome bc_1 region of the respiratory chain. This conclusion is supported by the work of Jasaitis *et al.* (1972), who showed that, in reconstituted cytochrome oxidase vesicles with internal cytochrome *c*, *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated ascorbate oxidation was linked to generation of a membrane potential.

After completion of most of the experimental work described in the present paper we learned that van Dam *et al.* (1978) were making some very similar measurements of the protonmotive force in submitochondrial particles. With NADH as substrate they report a $\Delta\psi$ of 179mV with no detectable ΔpH. A comparison of their data with ours will be of value later when full details of their experiments are available.

D. B. K. thanks the Science Research Council (London) for a research studentship. M. C. S. is a Research Fellow of the Consiglio Nazionale delle Ricerche, and was supported in Oxford by a long-term Fellowship from the European Molecular Biology Organization. We thank Dr. C. L. Bashford, Dr. S. Papa, Dr. W. S. Thayer, Dr. K. van Dam and Dr. M. K. F. Wikström for sending us copies of manuscripts before publication.

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