Measurement of the Electrochemical Proton Gradient in Submitochondrial Particles

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The pH gradient and membrane potential of submitochondrial particles from bovine heart were estimated by the uptake of [3H]ethylamine and [3H]perchlorate, using filtration through a glass fiber prefiltrof and Milipore filter without washing to separate the vesicles from the medium. An external volume probe of [3H]sucrose was also used. Internal volume of the vesicles was measured by the extent of uptake of glucose, which equilibrates slowly across the membrane.

The electrochemical potential gradient of H+ (ΔμH+) calculated from uptake of ethylamine and perchlorate, assuming the ions taken up were free in solution inside the vesicles, was 23 to 24 kJ/mol of H+ (240–250 mV) during respiration in the absence of ATP. The ratio of the free energy of ATP synthesis (ΔGATP) to ΔμH+ was 2.2 to 2.3 during oxidative phosphorylation and only slightly higher during ATP hydrolysis indicating that the H+-translocating ATPase is close to equilibrium under both conditions. The nonintegral ratio suggests there is a systematic error in the measurement of ΔμH+.

The value of ΔμH+, calculated from ion uptake could be too high if some of the ions taken up are bound to the membrane or concentrated into the electric double layer at the inner membrane-water interface. The effects of vesicle volume (varied osmotically) and permeant ions (which affect internal ionic strength and pH) on the ratio of ΔGATP to ΔμH+ suggested that ion association with the membrane in fact caused significant overestimation of ΔμH+. Association of ethylammonium and perchlorate ions with unenergized submitochondrial particles was measured by centrifugation, in the presence of a high concentration of impermeant salt to minimize association with the external surface. The results were used to estimate the extent of binding during the ion uptake assays, and ΔμH+ was recalculated taking this binding into account. The resulting values were between 19 and 20 kJ/mol of H+ (197–207 mV) during respiration in the absence of ADP, and the ratio of ΔGATP to ΔμH+ was about 3 during oxidative phosphorylation.

Respiration or ATP hydrolysis by mitochondria or submitochondrial particles generates a difference in the electrochemical potential of hydrogen ions across the mitochondrial inner membrane (ΔμH+). According to the chemiosmotic hypothesis of Mitchell (1, 2), this gradient is responsible for the energy coupling between oxidation and phosphorylation. Studies of the energetics of oxidative phosphorylation in whole mitochondria are complicated by transport of nucleotides (3), phosphate (4), and respiratory substrates (5). These complications can be avoided by studying oxidative phosphorylation in inverted submitochondrial particles.

The ΔμH+ across energy-transducing membranes consists of an electrical potential difference (ΔΨ) and a pH difference (ΔpH), which can be measured by their effects on the partition of certain ions across the membrane (6). Ions which cross the membrane electrogenically distribute according to the membrane potential, while ions which cross the membrane electro-neutrally by dissociating or binding a proton reflect the pH gradient.

Ion distribution measurements of ΔμH+ in mitochondria have given values from 140 to 240 mV (7–10). The lowest values (10) were obtained using centrifugation to measure ion uptake, which may have resulted in loss of isotopes from the pellet after oxygen or substrate was exhausted. The highest values (7, 8) assumed an internal volume of 0.4 μl/mg, which is lower than recently determined values (9).

Use of the ion distribution technique with submitochondrial particles is more difficult because of the difficulty in separating the vesicles from the medium without disturbing the ion distribution. Sorgato and co-workers (11–13) have avoided this problem by using the flow dialysis method of Ramos et al. (14) to measure uptake of ions. With succinate as substrate in a HEPES-KCl medium, ΔμH+ was about 240 mV (12). Lower values were obtained with NADH as substrate (12) or in a Tris-phosphate medium (13).

In this paper, we describe a filtration assay for ion uptake by submitochondrial particles and survey possible probes to measure ΔΨ and ΔpH. In addition, we measured ion binding inside the vesicles which must be taken into account in the determination of ΔΨ and ΔpH, and compared values of ΔμH+ with the Gibbs free energy of ATP synthesis in equilibrium with the proton gradient.

MATERIALS AND METHODS†

[3H]Cl was prepared by electrolysis of [3H]NaCl essentially as described by Troubwil (15). [3H]Ethylamine was prepared by treating [3H]ethyl iodide with a large excess of morpholine, treating excess morpholine with an electrolysate made from [3H]NaCl in a way similar to that described by Troubwil.

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ETPH, electron transport particles prepared by sonication of heavy layer bovine heart mitochondria; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; (Me)2DAE, hexamethylenediamine; SDS, sodium dodecyl sulfate.

† "Materials and Methods" is presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard

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line with between-ensemble differences, and purifying the product by repeated extraction between acidic or basic aqueous phases and organic phases. [131] Labelling (ammonium) was prepared by adding 1.5 mCi of [1-14C]ammonium to an exhaust of 0.1 mCi of ammonium and removing the product with charcoal. [132] Labelling (ammonium) was prepared from hexamethylenetetramine from Aminco (Bethesda, MD). The purity of these compounds was confirmed by chromatography on silica media. [133] Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-873, Waverly Press.

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trapped inside the vesicles when \[^{14}\text{C}]\text{sucoose was present at the sonication step was 0.76, 0.73, 0.71, and 0.63 \text{pl/mg in four experiments. Sucrose is not completely impermeant, however, and significant efflux may have occurred while washing the pellet (about 1 h on ice).}

Internal volume measured as the difference between glycerol and sucrose space in centrifuge pellets varied from 0.3 to 1.0 \text{pl/mg at 250 mm osmolarity. The large variation was due to the large volume of interstitial water in the pellet (about 5 \text{pl/mg}), which prevented accurate measurement of the small internal volume. We concluded that the glucose uptake method was probably the most accurate and that measurement should be made on each preparation. A typical value is 0.95 \text{pl/mg at 0.25 M sucrose concentration.}

Papa and co-workers measured internal volumes of about 1.4 \text{pl/mg} for EDTA particles (21) and 2.5 \text{pl/mg} for Mg-ATP particles (22) as the difference between water and dextran space in pellets. Branca et al. (13), using sucrose and water, found the internal volume of Mg-ATP particles to be 0.7 \text{pl/mg}.

Sorgato and Ferguson (12) suggested the higher values measured by Papa’s group were due to exclusion of dextran from a portion of the external pellet volume because of its large radius. Rottenberg et al. (23) found that with chloroplasts inulin, but not sorbitol, was excluded from part of the external pellet volume.

**Indicators of Membrane Potential**—We have measured uptake of four anions, SCN\(^-\), Cl\(^-\), I\(^-\), and C\(_2\)O\(_4\)^{2-}, as possible indicators of \(\Delta\phi\). Chloride uptake was too slow to be useful as an indicator. Iodide uptake during NADH oxidation by ETP\(_H\) was much smaller than that of SCN\(^-\) and C\(_2\)O\(_4\)^{2-}, and was only partially reversed by addition of rotenone and CCCP. Only thiocyanate and perchlorate were investigated in detail. Thiocyanate has been used to measure \(\Delta\psi\) in bacterial chromatophores (24) and in submitochondrial particles (11-13). Perchlorate has not been used before for this purpose, but Gromet-Elhanan and Leiser (25) found that perchlorate and thiocyanate behave as permeant ions and have similar effects on the balance between \(\Delta\psi\) and \(\Delta\phi\) in Rhodospirillum rubrum chromatophores. Lehninger (26) showed that valinomycin causes swelling of mitochondria suspended in KC\(_\text{ClO}_4\), indicating that the inner membrane is permeant to perchlorate as the anion. Dilger et al. (27) have measured the permeability of black lipid membranes to thiocyanate and perchlorate.

When we measured simultaneous uptake of \[^{14}\text{C}]\text{thiocyanate and }[^{35}\text{Cl}]\text{perchlorate under a wide range of conditions, we found that perchlorate was always taken up to a greater extent than thiocyanate. A possible source of this difference was binding of perchlorate to the ETP\(_H\) membranes. Binding of either ion to the unenergized vesicles is small relative to the amount taken up in response to a membrane potential, but binding to sites inside the vesicles would be expected to increase with increasing internal concentration, and so may constitute a significant part of the uptake in response to \(\Delta\phi\). To investigate this possibility, we compared the osmotic sensitivity of the two indicators using sucrose to vary the osmolarity. If the vesicles behaved as ideal osmometers and \(\Delta\phi\) was the same at each sucrose concentration, a plot of uptake expressed as vesicle space versus reciprocal osmolarity should give a straight line. Extrapolation to zero (infinite osmolarity) should give the contribution of bound probe to the uptake. Our results are plotted in this fashion in Fig. 3. The lines are curved, probably due to the nonideal osmotic dependence of internal volume seen in Fig. 2. Interpretation is further complicated because \(\Delta\psi\) cannot be assumed to be constant. High concentrations of sucrose inhibit respiration and so presumably decrease \(\Delta\psi\). (1 m \text{M sucrose inhibits respiration by about 35\% relative to 0.25 m sucrose under these conditions; the uncoupled rate is also inhibited.) The effect of sucrose on \(\Delta\psi\).
should change any uptake due to bound probe by approximately the same factor as it changes uptake due to free probe, but the effect of sucrose on internal volume should only affect uptake due to free probe. From the fact that perchlorate and thiocyanate are taken up in essentially the same ratio over a range of sucrose concentrations that results in a large change in vesicle volume (see Fig. 2), we conclude that if either probe is binding they are binding to essentially the same extent. The difference between perchlorate and thiocyanate uptake is thus not due to binding of perchlorate.

Fig. 4 shows the effect of pH on uptake of perchlorate and thiocyanate in the presence and absence of nitrate, a permeant anion. The agreement between the two indicators is fairly good at pH values above 8, but thiocyanate uptake is less than a third of perchlorate uptake below pH 6. A possible explanation for this effect of pH on the ratio of anion uptakes is that efflux of HSCN from the acidic interior of the vesicles prevents SCN⁻ from coming to equilibrium with the membrane potential. This effect would be greater at low pH because of the greater concentration of HSCN. Perchlorate, being a much stronger acid, would be less susceptible to this problem. Sorgato et al. (11) have concluded that the pKₐ of HSCN (1.8) is low enough to ensure that the concentration of the neutral form is insignificant. The transmembrane distribution of an ion that can cross the membrane in two ionization states depends on the relative permeabilities as well as the relative concentrations of the two forms. Assuming that perchlorate accurately measures the membrane potential, a permeability coefficient of 9.5 × 10⁻⁸ cm/s for SCN⁻ was calculated from the initial uptake rate. It could be shown that a reasonable permeability coefficient for HSCN (0.5 cm/s) would result in the observed difference between thiocyanate and perchlorate uptake (28). This value is not unreasonably high as the permeability of phosphatidylcholine bilayer membranes to HSCN is 2.6 cm/s (29). A significant efflux of HSCN implies that thiocyanate behaves as an uncoupler, increasing the permeability of the membrane to protons, and there have been some reports of uncoupling by thiocyanate at low pH (30, 31, 28). HClO₃ is a much stronger acid than HSCN. Raman and ³¹Cl NMR spectroscopic data show that it is probably at least 91% ionized at a concentration of 12 m (32). This concentration, has a Hammet acidity function value of -8.12 (estimated from the data of Ref. 33), so the pKₐ of HClO₃ is probably below -8. Earlier reports that the pKₐ of HClO₃ is on the order of -2 were based on proton NMR measurements of the extent of dissociation which were shown to be incorrect (32). The initial rate of uptake of perchlorate is about the same as that of thiocyanate (28), and so the permeability coefficient of HClO₃ would have to be unreasonably high for HClO₃ efflux to significantly affect the extent of uptake.

All of our results are consistent with the difference between perchlorate and thiocyanate uptake being due to HSCN efflux, and so we feel that perchlorate is the better ion for measuring...
the membrane potential. At pH 7.0 and above, however, the difference in Δψ measured by the two probes is less than 20 mV.

**Indicators of ΔpH**—We have investigated the use of methyamine, ethylamine, hexylamine, and N-methylmorpholine for measuring ΔpH in submitochondrial particles. Methyamine has been used for measuring pH gradients in several systems since its introduction for this purpose by Rottenberg et al. (23). Ethylamine and hexylamine have been used for measuring ΔpH in chloroplasts (34, 35). N-Methylmorpholine was chosen for its hydrophilic nature and low pK, of 7.41 (36). Ethanolamine was also found to be taken up in response to a pH gradient, but was not studied in detail. When uptake of these amines was measured under the same conditions and expressed as vesicle space of the protonated form, methyamine and ethylamine were taken up to about the same extent. Methylmorpholine uptake was slightly greater and hexylamine uptake was significantly greater, the magnitude of the discrepancy depending on conditions.

Portis and McCarty (34) found that thylakoids take up more hexylamine than ethylamine, and that decreasing pH inhibits ethylamine uptake more than hexylamine uptake. They concluded that ethylamine fails to reach equilibrium because of efflux of the protonated form, which becomes more significant at acid pH because of the low concentration of the neutral form of the amine. This does not seem to be the cause of the discrepancy in submitochondrial particles, where all the amines show approximately the same pH dependence (Fig. 5). Depressor of the uptake due to permeability of the protonated form should vary with the ratio of protonated to neutral form of the amine, and the pH at which it becomes significant should depend on the pK, of the amine and the ratio of permeabilities of the two forms. The fact that all the uptakes show the same pH dependence thus makes it unlikely that any of the uptakes are significantly affected by efflux of the protonated form. The pH dependence of uptake thus presumably reflects the pH dependence of ΔpH.

An alternative explanation of the difference between hexylamine and ethylamine uptake in submitochondrial particles is that part of the hexylamine taken up in response to a pH gradient is bound to the membrane. Several investigators (36, 37) have concluded that the high values of ΔpH calculated from 9-aminoacridine uptake are incorrect because most of the amine taken up is bound to the membrane. Because of its positive charge and hydrocarbon chain, it would not be surprising if hexylamine also binds to the membrane. The osmotic dependence of the uptakes is consistent with this interpretation. As Fig. 6 shows, hexylamine uptake was proportionately less sensitive to sucrose than ethylamine, methyamine, or methylmorpholine uptake. The ratio of ethylamine to hexylamine vesicle space, for example, decreased from 0.5 at low sucrose to 0.2 at high sucrose concentration. Quantitative analysis of the results is complicated by the inhibition of respiration by high sucrose concentration. (1 m sucrose inhibits respiration by 50% relative to 0.25 m sucrose under these conditions.) Qualitatively, however, the lesser sensitivity of hexylamine uptake to sucrose may be taken as evidence that a significant fraction of the hexylamine is bound, and so, osmotically insensitive.

The pH gradient is decreased by permeant amines, because influx of the free base form increases internal pH. At a given concentration, hexylamine was more effective than ethylamine at inhibiting uptake of either amine, as expected from the greater extent to which hexylamine is taken up. While ethylamine inhibited both uptakes by almost the same fraction, hexylamine inhibited its own uptake more than that of ethylamine, so that the ratio of ethylamine uptake to hexylamine uptake increased from 0.35 at 6 μm hexylamine to 0.5 at 0.5 mm hexylamine. This could be accounted for by saturable binding of hexylamine to the membrane, as such binding would become less significant at high amine concentrations.

As shown later, binding is probably significant with all of the amines. If permeability of the protonated forms is insignificant, as indicated by the fact that all the amines show the same pH dependence, it seems reasonable to assume that the differences in uptake are due to binding, and thus, that the amines that give lowest uptakes are most likely to be accurate indicators of ΔpH. By this criterion, ethylamine and methyamine are the most desirable of the indicators we have studied. Methylamine binds to the glass fiber filters more than ethylamine does, so we have chosen ethylamine for routine use.

**Simultaneous Measurement of ΔpH and Δψ Using Ethylamine and Perchlorate**—From the results of the preceding sections, we chose to use perchlorate to measure Δψ and ethylamine to measure ΔpH. The measurements can be made simultaneously using [3H]perchlorate and [14C]ethylamine, with [3H]sucrose to correct for filter volume. Fig. 7 shows the time course of NADH-driven uptake of these two probes in the absence and presence of the permeant anion nitrate. The steady state extent is reached in 2 to 5 min, the longer time being required when one probe is taken up to a very large concentration ratio. In the sucrose K-MOPS medium without nitrate, perchlorate uptake was about 100-fold greater than ethylamine uptake. Nitrate (10 mM) stimulated ethylamine uptake and inhibited perchlorate uptake. In both cases, the uptakes were reduced nearly to zero by rotenone and CCCP.
The gradients calculated from the extent of uptake at 5 min and the internal volume of 0.95 pl/mg are 6.67 and 16.96 kJ/mol for ΔpH and Δψ in the presence of nitrate and 12.68 and 8.54 for ΔpH and Δψ in the absence of nitrate.

Fig. 8 shows the effect of NO$_3^-$ and NH$_4^+$ ions on the gradients measured by perchlorate and ethylamine. KNO$_3$ reduced Δψ and increased ΔpH, while (NH$_4$)$_2$SO$_4$ had the opposite effects. These effects were expected because uptake of nitrate should partially collapse Δψ, allowing respiration to build up a larger pH gradient, while NH$_4^+$ uptake (as NH$_3$) should partially neutralize the pH gradient, allowing respiration to increase Δψ.

Table I shows the calculated values of ΔpH, Δψ, and Δψ$^\text{NH}_4^+$ with NADH, succinate, or ATP as substrates, in the presence or absence of 10 mM KNO$_3$. Each substrate, Δψ was larger than ΔpH in the absence of KNO$_3$ and ΔpH was larger in the presence of KNO$_3$. ATP and succinate gave slightly lower proton gradients than NADH. These values are probably overestimated since, as will be discussed later, part of the ions taken up in response to Δψ and ΔpH are bound to the membrane. The relative values are probably correct, however. It is significant that Δψ$^\text{NH}_4^+$ was larger with NADH as substrate than with succinate, as Sorgato and Ferguson (12) found the opposite to be the case and concluded that NADH increases the proton permeability of the membrane. The difference between our results and theirs may be due to differences in conditions: we used low protein concentrations with NADH added directly; they used high protein concentration and NADH generated by alcohol dehydrogenase. Because of the relatively positive midpoint potential of the ethanol/acetaldehyde couple, it is questionable whether alcohol dehydrogenase can keep NADH reduced for 10 min under these conditions.

Simultaneous Measurement of Δψ$^\text{H}^+$ and ΔG$_{\text{ATP}}$—Under conditions of equilibrium of the proton-translocating ATPase, the ratio of the free energy of ATP synthesis (ΔG$_{\text{ATP}}$) to Δψ$^\text{H}^+$ should be equal to the H$^+$/ATP stoichiometry of the ATPase. While it may not be possible to obtain complete equilibrium of the ATPase, the equilibrium ratio can be bracketed between the ratios measured during ATP synthesis.
and ATP hydrolysis. Fig. 9 shows the time course of $\Delta G_{\text{ATP}}$ and $\Delta \mu_{\text{H}^+}$ during oxidative phosphorylation measured by perchlorate and ethylamine uptake without correction for binding. $\Delta G_{\text{ATP}}$ and $\Delta \mu_{\text{H}^+}$ increase and then decrease in parallel, so that the ratio remains close to 2.3. In other studies where the initial concentration of ADP was varied from 0.25 to 2 mM and P, was 5 or 10 mM, the ratio $\Delta G_{\text{ATP}}/\Delta \mu_{\text{H}^+}$ varied only from 2.14 to 2.26.

Branca et al. (13) observed values of $\Delta G_{\text{ATP}}/\Delta \mu_{\text{H}^+}$ close to 2 with succinate as substrate in a medium containing Hepes and KCl. Because the ratio was close to 3 in a medium containing Tris-phosphate, they concluded that the ATPase was not close to equilibrium in the Hepes-KCl medium. In our medium, which is similar to their Hepes-KCl medium, nearly the same ratio was reached during ATP hydrolysis (Fig. 10), so the ATPase was probably close to equilibrium.

Fig. 11 shows that essentially the same ratios are obtained when the absolute values of $\Delta G_{\text{ATP}}$ and $\Delta \mu_{\text{H}^+}$ are lowered by inhibiting succinate oxidation with fumarate or by adding ADP during ATP hydrolysis. Fig. 11 also illustrates the fact that the system is close to, but not at, equilibrium. When ATP was the driving force, the values of $\Delta G_{\text{ATP}}$ were about 2 kJ/mol higher than when respiration was the driving force at the same value of $\Delta \mu_{\text{H}^+}$. The equilibrium ratio of $\Delta G_{\text{ATP}}/\Delta \mu_{\text{H}^+}$ must lie between the values measured during ATPase and during ATP synthesis driven by respiration since the reaction of the proton transport ATPase is going in different directions under these two conditions.

The nonintegral ratio of $\Delta G_{\text{ATP}}$ to the measured value of $\Delta \mu_{\text{H}^+}$ in these experiments suggests that there is some systematic error in the measurement of $\Delta \mu_{\text{H}^+}$. If the actual ratio is 2, then $\Delta \mu_{\text{H}^+}$ must be underestimated by about 2.7 kJ/mol. If the ratio is 3, then $\Delta \mu_{\text{H}^+}$ must be overestimated by about 5.7 kJ/mol. Systematic errors which could cause underestimation of $\Delta \mu_{\text{H}^+}$ include loss of probes from the vesicles during filtration, permeability of the wrong ionization state of the probes, and presence of vesicles which contribute to the measured internal volume but do not develop a membrane potential and pH gradient.

Filtration of the vesicles is not likely to disturb the ion distribution until after flow through the filter stops, as particles on the filter are continually being washed with medium containing substrates. The agreement between our measurements with succinate as substrate and those of Sorgato and Ferguson (12) support this conclusion, as these authors used the flow dialysis method which does not involve separation of the vesicles from the medium. In addition, perchlorate uptake measured by a perchlorate electrode was only slightly greater than uptake measured by the filtration method (not shown).

As discussed previously, the strong acid nature of perchloric acid makes it highly unlikely that permeability of HClO₄ affects the distribution of perchlorate. The fact that uptake of ethylamine, methylethylamine, hexylamine, and methylmorpholine have the same pH dependence makes it unlikely that permeability of the protonated form affects distribution of the amines.

The submitochondrial particles may contain some vesicles which do not develop membrane potentials and pH gradients, or whose membrane potentials and pH gradients are much smaller than those in typical vesicles or which are of opposite polarity. These vesicles would have to contribute 40% of the measured internal volume to cause underestimation of $\Delta \mu_{\text{H}^+}$ by 2.7 kJ, however, and this is unlikely. The cytochrome c oxidase activity of the vesicles is stimulated 5- to 8-fold by deoxycholate. This and the fact that greater than 90% of the vesicles have F₁-ATPase molecules on the external surface (as seen in electron micrographs) make it unlikely that 40% of the vesicles are inverted. There may be some outer membrane vesicles present, but they would not contribute to the internal volume measured by glucose or ribitol uptake because of their high permeability to small molecules.

$\Delta \mu_{\text{H}^+}$ could be overestimated because of association of the probe ions with the inner surface of the vesicle membrane, which would result in the internal activity being lower than calculated from the total amount of the ion inside the vesicle and the internal volume. Such association could be either simple binding or accumulation of the ions in the electric double layer adjacent to the membrane surface. As described in the discussion of Figs. 3 and 8, association of an ion with the membrane should make uptake of that ion less sensitive to medium osmolality. Experiments such as those of Figs. 3 and 8 can only indicate a difference in the degree to which two probes are associated with the membrane, however. The absolute extent of association cannot be determined because there is no way to maintain a constant $\Delta \mu$ or $\Delta \phi$ while varying osmolality. Because the ATPase equilibrates readily, however, it should be possible to use the value of $\Delta G_{\text{ATP}}$ to monitor changes in $\Delta \mu_{\text{H}^+}$, and thus, in the sum of $\Delta \mu$ and $\Delta \phi$.

Fig. 12 shows an experiment in which $\Delta G_{\text{ATP}}$ and uptake of ethylamine and perchlorate were measured during oxidative phosphorylation at different sucrose concentrations. The effect of osmolality on internal volume (glucose uptake) has been replotted from Fig. 2 for comparison. Ethylamine and
perchlorate uptake are both less sensitive to osmolarity than is uptake of glucose. This could indicate a “bound” component in the uptake of one or both probes, or could indicate an increase in $\Delta\mu_H^+$ with increasing osmolarity. The latter is unlikely, as $\Delta G_{\text{ATP}}$ decreased with increasing osmolarity (middle). $\Delta\mu_H^+$ was calculated from uptake of perchlorate and ethylamine and the appropriate internal volume, and the ratio of $\Delta\mu_H^+$ to $\Delta G_{\text{ATP}}$ is plotted at the top of Fig. 12. To ensure that the high osmolarity did not prevent equilibration of the ATPase, the experiment was repeated with ATP hydrolysis rather than oxidative phosphorylation, and the results are also plotted at the middle and top of Fig. 12. In both cases, the ratio of $\Delta G_{\text{ATP}}$ to $\Delta\mu_H^+$ decreased at high osmolarity. This is probably because association of the probe ions with the membrane becomes more significant as the internal volume, and thus, the amount of probe free in solution inside the vesicle decreases.

Changes in $\Delta G_{\text{ATP}}$ can reflect changes in $\Delta\mu_H^+$, but not changes in the relative magnitudes of $\Delta\psi$ and $\Delta\mu_H$. The experiment of Fig. 12, thus, cannot indicate whether ethylamine or perchlorate is associated with the membrane, but only that at least one is. Changing experimental conditions in a way that should have the opposite effect on association of perchlorate and ethylamine with the membrane may indicate which of the probes is involved. Because ethylammonium and perchlorate have opposite charge, changing the surface charge of the inner membrane surface should increase association of one ion and decrease association of the other ion. The surface charge can be changed by varying the internal pH. This can be achieved by using a permeant ion such as perchlorate to vary the pH gradient at constant pH. Fig. 13 shows the effect of KClO$_4$ on the measured ratio of $\Delta G_{\text{ATP}}$ to $\Delta\mu_H^+$ during oxidative phosphorylation and ATP hydrolysis. KClO$_4$, like KNO$_3$ in Fig. 8, decreased perchlorate uptake while increasing ethylamine uptake by a smaller ratio, so that $\Delta\mu_H^+$ calculated from the uptakes decreased. $\Delta G_{\text{ATP}}$ decreased by a proportionately smaller amount, so that the ratio of $\Delta G_{\text{ATP}}$ to the measured $\Delta\mu_H^+$ increased with increasing KClO$_4$ concentration.

Ethylamine perchlorate also increased the ratio of $\Delta G_{\text{ATP}}$ to $\Delta\mu_H^+$ calculated from ion uptakes (Fig. 14). With ethylamine perchlorate, the decrease in the measured value of $\Delta\mu_H^+$ occurs at lower concentrations than with KClO$_4$. Because there is no significant effect on $\Delta G_{\text{ATP}}$ in this concentration range, it is unlikely that $\Delta\mu_H^+$ actually decreased, so the decrease presumably reflects a change in some error associated with the measurement of $\Delta\mu_H^+$ by ion distribution, such as in the extent of association of the probe ions with the membrane.

KClO$_4$ and ethylamine perchlorate could affect the ionic association of ethylamine and perchlorate and the membrane in three ways: by increasing $\Delta\psi$ and thus making the membrane inner surface more positive, by increasing internal ionic strength and thus collapsing the electric double layer or local...
ionic binding, and by competing for saturable binding sites. The effect on pH would presumably be larger with KClO₄ than with ethylamine perchlorate, while the effect on internal ionic strength and saturation of binding sites would be greater with ethylamine perchlorate. Because ethylamine perchlorate is more effective than KClO₄ at low concentrations, it is likely that the increase in ionic strength and competition for ion binding sites are most important. Because these effects would decrease binding of both cations and anions, these experiments do not indicate whether binding of ethylammonium or perchlorate is more important.

**Direct Measurement of Ion Association with the Submitochondrial Membrane**—Association (or binding) of ethylammonium and perchlorate ions with the vesicle membrane can be measured directly by suspending submitochondrial particles in a medium containing the radioactive ions and glycerol (as a measure of total water volume) and then centrifuging to separate the vesicles from the medium. The filtration assay is not sensitive enough to measure binding to membranes because of the large filter volume. With no respiratory substrates or ATP and with an uncoupler added, Δµᵢ⁻ can be assumed to be zero. If the free concentrations (or activities) of permeant ions inside and outside the vesicles are unequal, there will be an electrical (Donnan) potential across the membrane and an equal but opposite pH gradient. The major ions in the preparation medium (Cl⁻, SO₄²⁻, Mg²⁺, Mn²⁺, and Tris⁺) are lost on washing the vesicles or on resuspending in medium containing EDTA, so that little impermeant salt is trapped inside the interface.

As expected, increasing pH decreases association of perchlorate and increases association of ethylammonium with the membrane. The binding in Fig. 15 is reported in units of microliters of external medium which is equivalent to nanomoles bound divided by the millimolar concentration of the probe. Thus, a horizontal line on the plot represents nonsaturable binding. For both ions, there appear to be two components to the observed binding, a saturable component which is pH dependent and a nonsaturable component.

These experiments measure association of the ions with both faces of the membrane. Association of ions with the external face does not present a problem for measurement of Δψ and ΔpH, as shown by the fact that the amount of ion uptake without substrate is generally insignificant relative to uptake with substrate. The amount of association with the inner face, on the other hand, is a function of the internal concentration and increases on energization. The unenergized control is therefore not relevant.

In order to estimate the amount of association of ethylamine and perchlorate with the internal surface, we used an impermeant salt at a high concentration to minimize association with the external surface. Fig. 16 shows the effect of (Me)₃DAE on association of ethylamine and perchlorate with submitochondrial membranes. Perchlorate binding at pH 5.6 is only partially inhibited by the impermeant salt. The remaining binding (3.5 µl/mg) was then eliminated by addition of the permeant salt ethylamine perchlorate. Similarly, ethylamine binding was decreased to about 0.5 µl/mg by the impermeant salt, (Me)₃DAE-imp. The component of ethylamine and perchlorate binding which is insensitive to the impermeant salt probably represents binding to the internal surface of the vesicles.

**Correction of Measurements of Δµᵢ⁻ for Ion Binding**—Calculation of Δµᵢ⁻ requires knowledge of the free concentrations of the ethylamine and perchlorate probes inside the vesicles. The values measured in uptake experiments are the total amount inside (free + bound). The relationship between free probe concentration and total probe taken up can be calculated from the data of Fig. 17 and the internal volume of the vesicles. This relation is plotted in Fig. 18, assuming an internal volume of 0.96 µl/mg. This figure can be used to estimate the internal concentration of ethylamine and perchlorate, and thus, ΔpH and Δψ from the total amount of uptake. This is valid, however, only if the internal conditions during the uptake measurement are similar to those during the binding experiment. The amount of ethylamine and perchlorate bound presumably depends on the internal pH, ionic strength, and concentration of the ion in question. The binding data of Fig. 18 covers the pH range 6.3-6.8, and are fairly pH independent within that range. These data can thus be applied to uptake experiments at pH 7.5 if the pH gradient is 0.7 to 1.2 pH units. The binding data were obtained with ethylammonium perchlorate constituting most of the internal ionic strength and with ethylammonium and perchlorate at equal concentrations. In general, the internal ionic strength is not known in the uptake experiments, and the internal concentrations of ethylammonium and perchlorate are not the same. When the probe ions are present in the external medium at relatively high concentrations, as in the experiment of Fig. 14, massive uptake results in the probes’ contributing most of the internal ionic strength. Because the center of the vesicle must be electrically neutral (except at very low ionic strength when the membrane double layer may extend to the center), the ethylammonium concentration will approximately equal the perchlorate concentration at the center of the vesicles. It is
therefore reasonable to apply the binding data to the uptake experiment of Fig. 14 in order to calculate $\Delta \phi$ and $\Delta \psi$ with allowance for binding. From Fig. 17, ethylamine binding was within experiment error of 0.3 $\mu$l/mg under all the conditions tested. When these conditions apply, therefore, internal ethy- 

al take experiments due to the short time period, presence of 

ethylamine and perchlorate free in solution inside the vesicles at each concentration at internal pH 6.3 (A), 6.5 (B), 

and 6.8 (C) were measured as described under "Materials and Methods." The medium contained (Me),,DAE-

mucate at varied concentrations. For the points at ionic strength 400 mM and below, 1 mM ethylamine perchlorate was present and (Me),,DAE-mucate was varied between 0 and 100 mM. For ionic strengths greater than 400 mM, 100 mM (Me),,DAE-mucate was present and ethylamine perchlorate was varied between 1 

and 50 mM.

Fig. 17 (center). Binding of ethylammonium and perchlorate ions to submitochondrial membranes in the presence of (Me),,DAE-mucate. Binding of perchlorate (A) and ethylammonium (B) at pH 6.3 (A), 6.5 (B), and 6.8 (C) were measured as described under "Materials and Methods." The medium contained (Me),,DAE-

mucate to minimize binding to the external surface of the membranes. The incubation mixture contained 14 mg/ml 

ETPn, 250 mM sucrose, 100 mM (Me),,DAE-mucate, 5 mM choline-MES, 0.4 mM K-MOPS, 0.2 mM choline-EDTA, 

0.35 nmol of CCCP/mg of protein, 0.35 $\mu$g valinomycin/mg of protein, $[^{3}H]$glycerol, $[^{14}C]$ethylamine, K$^{+}$ClO$_4$ and 

the indicated concentrations of ethylammonium perchlorate. The pH indicated was measured in the supernatant after 

centrifuging.

Fig. 18 (right). The relation between internal concentration of ethylamine perchlorate and the total amount of ethylamine and perchlorate inside the vesicles. The values were calculated assuming the amount of each ion bound at a given internal concentration is equal to the amount bound at the same (external) concentration in the experiment of Fig. 17. An internal volume of 0.95 $\mu$l/mg was used to calculate the amount of ethylamine and perchlorate free in solution inside the vesicles at each concentration at internal pH 6.3 (A), 6.5 (B), 

and 6.8 (C).

Perchlorate binding was $pH$ dependent and partly saturable. $\Delta \psi$ was therefore calculated from the internal perchlorate concentration determined from total uptake by interpolation from Fig. 18. The internal $pH$ for this interpolation was determined from the external $pH$ and the $pH$ gradient calculated as described above.

If ethylamine and perchlorate were the only ions inside the vesicles, their concentration at the center of the vesicles would be equal, $\Delta \phi$ and $\Delta \psi$ would be equal, and the same internal ethylamine perchlorate concentration could be calculated by dividing the total 

uptake by the internal volume plus 0.3 $\mu$l, that is, by using an "effective internal volume" of 1.25 $\mu$l for ethylamine. The resulting values of $\Delta \phi$ are lower by 0.12 $pH$ unit (0.68 kJ/mol) than values calculated without correcting for binding.

Perchlorate binding was $pH$ dependent and partly saturable. The values of $\Delta \phi$, $\Delta \psi$, and $\Delta G_{ATP}$ calculated from the data of Fig. 15 using the procedure outlined above are shown in Fig. 19. The values for $\Delta G_{ATP}$ were roughly one-third those of $\Delta G_{ATP}$.

There would be very useful if we could estimate the error introduced by binding when the probes are at tracer concentrations, as in most of our experiments. The internal ionic strength in the absence of permeant ions is probably 5 to 10 mM. If binding is unsaturable and depends only on ionic 

strength and $pH$, then Fig. 17 can be applied, taking the horizontal axis as ionic strength instead of ethylamine perchlorate concentration. The $pH$ gradient is usually 7 to 8 kJ/mol (uncorrected) in the absence of permeant ions. Again taking ethylamine binding as 0.3 $\mu$l/mg gives corrected values of $\Delta \phi$ of 6.3 to 7.3 kJ/mol, and internal $pH$ values of 6.2 to 6.4. From the $pH$ 6.3 perchlorate binding curve of Fig. 17, perchlorate binding should be between 2.5 and 3 $\mu$l/mg. Together with the ethylamine binding, this gives a total correction of -3.9 to -4.2 kJ/mol to be applied to $\Delta G_{ATP}$. The actual correction may be much higher, however, if there is specific binding of ethylamine or perchlorate.

Applying a correction of -4 kJ/mol to the values in Table I gives $\Delta G_{ATP}$ values of 19.7 (NADH) and 19.22 (succinate) kJ/mol during respiration in the absence of ADP, and 18.1 kJ/mol during respiration in the presence of external $K^+$, and perhaps action of the cation/hydrogen exchanger (39).
mol during ATP hydrolysis. The values during oxidative phosphorylation with succinate as respiratory substrate are about 15 (Fig. 14) to 17 (Figs. 9 and 13) kJ/mol. This is about one-third the value of $\Delta G_{\text{ATP}}$ (44–48 kJ/mol). It may be that specific, saturable binding of ethylammonium or perchlorate to a small number of sites increases the fraction bound at low concentrations. Another possibility is that a slight permeability of the (Me)$_6$DAE-mucate used in Fig. 17, or permeability of a minor impurity, results in the internal ionic strength being significantly higher than calculated. This would be most significant at low ethylammonium perchlorate concentrations and may account for the fact that the “corrected” ratio of $\Delta G_{\text{ATP}}$ to $\Delta \mu_{\text{H}^+}$ decreases sharply at low ethylammonium perchlorate concentrations (Fig. 19).

**DISCUSSION**

In this study, we have tried to systematically investigate each parameter in the measurement of the electrochemical proton gradient of submitochondrial particles. Several methods were used to measure the internal volume, which was found to decrease at high external osmolarities but to increase only a limited amount at low osmolarities because of the small size of the vesicles. Various radioactive probe ions for $\Delta \psi$ and $\Delta \phi$ were surveyed. Differences between amine $\Delta \psi$ probes were minor or, in the case of hexylamine, were due to binding to the membrane. Of the two likely $\Delta \psi$ probes, perchlorate and thiocyanate, the lower uptake of thiocyanate was shown to be caused by permeability of the protonated form. Conventionally, calculation of $\Delta \mu_{\text{H}^+}$ based on perchlorate and ethylamine uptake, however, yielded nonintegral values of the ratio of $\Delta G_{\text{ATP}}$ to $\Delta \mu_{\text{H}^+}$. Looking further for possible errors, we discovered significant association (or binding) of the probe ions with the membrane depending upon the pH and ionic strength. This binding was not apparent from earlier studies of the effect of changing internal volume by high sucrose media. Because of secondary effects of the sucrose and to be measured directly, the binding of probes is probably more significant in submitochondrial particles than other systems because they are prepared by sonication in a low ionic strength medium and contain few ions. Direct measurement of probe binding and correction for it gave typical values for $\Delta \mu_{\text{H}^+}$ of 15 to 19 kJ/mol (155 to 197 mV). The ratio of the H$^+$/ATPase ($F_0 - F_1$) indicated by the corrected value of $\Delta \mu_{\text{H}^+}$ and $\Delta G_{\text{ATP}}$ was close to 3, the equilibrium position of the reaction being bracketed by measurements with and without respiration. Previous studies of $\Delta \mu_{\text{H}^+}$ in submitochondrial particles, using flow dialysis to measure uptake of thiocyanate and methylamine, showed ratios of $\Delta G_{\text{ATP}}$ to $\Delta \mu_{\text{H}^+}$ of 1.89, 2.84, and 3.09 in three different media (13). Assuming that ion binding causes overestimation of $\Delta \mu_{\text{H}^+}$, the ratios obtained seem consistent with a ratio of 3 H$^+$/ATP.

This laboratory previously reported direct measurements of the H$^+$/ATP ratio in submitochondria by ATP pulse methods originally indicated a value of 2 (42) which was confirmed in the presence of N-ethylmaleimide to inhibit phosphate efflux (43). More recently, Andre et al. (44) used a rate method in the presence of N-ethylmaleimide and obtained a value of 3.1, although only half of the phosphate efflux was actually inhibited (45) and it is not clear that the value of 3 represents proton transport by $F_0 - F_1$ ATPase alone.

Van Dam et al. (46), Azzzone et al. (47), and Holian and Wilson (9) reported ratios of $\Delta G_{\text{ATP}}$ to $\Delta \mu_{\text{H}^+}$ between 3 and 4 in whole mitochondria under conditions favorable for oxidative phosphorylation. The slightly lower ratios reported by Nicholls (8) can be attributed to his use of a lower internal volume. Azzzone et al. (47) and Holian and Wilson (9) were able to obtain much higher ratios by various treatments that decreased $\Delta \mu_{\text{H}^+}$ but had a proportionately smaller effect on $\Delta G_{\text{ATP}}$. It is uncertain, however, whether the ATPase reaction is close to equilibrium under these conditions. In particular, it is possible that the ATPase is inhibited by the endogenous inhibitor at low $\Delta \mu_{\text{H}^+}$ (48), and $\Delta G_{\text{ATP}}$ is maintained by substrate level phosphorylation and adenylate kinase.

Because the phosphate and adenosine nucleotide transport reactions are believed to be coupled to translocation of one proton (3, 4), the H$^+$/ATP ratio for external ATP synthesis by whole mitochondria is expected to be one greater than that in submitochondrial particles. Our results together with those of Branca et al. (13) indicate a ratio of 3 in submitochondrial particles, and so we expect a ratio of 4 in whole mitochondria.
The lower ratios measured in mitochondria could be due to poor equilibration of the ATPase, or to ion binding such as we found in submitchondrial particles. The ATPase and ATP/ADP exchanges may fail to equilibrate with the proton gradient because the ADP concentration becomes very low at the high phosphate potentials achieved during mitochondrial oxidative phosphorylation. Lack of equilibration during oxidative phosphorylation due to a low level of external ATPase activity would result in $\Delta Q_{\text{ATP}}/\Delta \mu_{\text{H}}^+$ ratios lower than the equilibrium ratio. On the other hand, Van Dam et al. (46) reported a ratio of 3.46 in whole mitochondria during ATP hydrolysis. During ATP hydrolysis, poor equilibration would make the ratio higher than the equilibrium ratio, so this is incompatible with a ratio of 4 unless some other error caused underestimation of the ratio.

The internal ionic strength is higher in mitochondria than in ETPH submitochondrial particles, and so ion binding might be expected to be less of a problem. As shown in Fig. 17, however, ion binding in submitochondrial particles is significant even at high ionic strength. In addition, the high concentration of protein in the mitochondrial matrix is a much larger internal volume would make a large error in the calculated membrane potential and pH gradient.

The $H^+$ to ATP ratio of 4 for ATP synthesis in whole mitochondria would result in P/O ratios of 1/site" if the respiratory chain transports 4 $H^+$/pair of electron/site, as some recent measurements have indicated (44, 48–50). Wikström et al. (51), however, have provided evidence that during oxidation the $H^+/2e$ ratio is only 6. This would result in a P/O ratio of 1.5 with succinate as substrate, slightly higher than values measured in this laboratory (52). Equilibrium studies of the P/O ratio at the first site (53), which we have confirmed, show that the $H^+/2e$ ratio of the first site must be greater than the $H^+/ATP$ ratio in submitochondrial particles. An $H^+/ATP$ ratio of 3 is thus consistent with an $H^+/2e$ ratio of 4 for the first site. The $H^+/2e$ ratio for NADH-linked respiration is therefore likely to be 10, and the P/O ratio in mitochondria to be 2.5. Such a scheme seems to be most consistent with the data at the present time (54).

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