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Stoichiometry of vectorial H^+ movements coupled to electron transport and to ATP synthesis in mitochondria

(electron transport/ATP hydrolysis/oxidative phosphorylation/H^+ transport)

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ABSTRACT **In order to verify more directly our earlier measurements showing that, on the average, close to four vectorial H^+ are ejected per pair of electrons passing each of the three energy-conserving sites of the mitochondrial electron transport chain, direct tests of the $H^+/2e^-$ ratio for sites 2 and 3 were carried out in the presence of permeant charge-compensating cations. Site 2 was examined by utilizing succinate as electron donor and ferricyanide as electron acceptor from mitochondrial cytochrome *c*; the directly measured $H^+/2e^-$ ratio was close to 4. Energy-conserving site 3 was isolated for study with ferrocyanide or ascorbate plus tetramethylphenylenediamine as electron donors to cytochrome *c* and with oxygen as electron acceptor. The directly measured $H^+/2e^-$ ratio for site 3 was close to 4. The H^+/ATP ratio (number of vectorial H^+ ejected per ATP hydrolyzed) was determined with a new method in which the steady-state rates of both H^+ ejection and ATP hydrolysis were measured in the presence of K^+ + valinomycin. The H^+/ATP ratio was found to approach 3.0. A proton cycle for oxidative phosphorylation is proposed, in which four electrochemical H^+ equivalents are ejected per pair of electrons passing each energy-conserving site; three of the H^+ equivalents pass inward to derive ATP synthesis from ADP and phosphate and the fourth H^+ is used to bring about the energy-requiring electrogenic expulsion of ATP^{4-} in exchange for extramitochondrial ADP^{3-}, via the $H^+/H_2PO_4^-$ symporter.**

Earlier reports from this laboratory have provided evidence that close to 12 H^+ are ejected from the matrix to the medium as a pair of electrons pass through the three energy-conserving sites of the mitochondrial respiratory chain to molecular oxygen (1–8). Thus the *average* H^+/site ratio, the number of H^+ ejected per pair of electrons per energy-conserving site, approaches 4. This value of the H^+/site ratio has been observed with four different procedures (2, 3, 8, 9) and has also been observed in mitochondria isolated from different cell types (refs. 3 and 6; also A. Villalobo, J. Benavides, and A. L. Lehninger, unpublished data). The value of 4 for the average H^+/site ratio provides better agreement with thermodynamic data on electron transport, ATP synthesis, and transmembrane electrochemical gradients (4, 5, 10) than does the H^+/site ratio of 2 proposed by Mitchell and Moyle (11) on the basis of oxygen pulse experiments, which have been shown to be subject to serious underestimation (5, 9, 12). Moreover, the average value of 4 for the H^+/site ratio during succinate oxidation very strongly indicates that electron flow through site 3, catalyzed by cytochrome oxidase, also promotes H^+ ejection. However, there are still two major questions open regarding the H^+ stoichiometry of electron transport and oxidative phosphorylation.

With regard to the H^+/site ratio at site 3 of the respiratory chain, Mitchell and Moyle have concluded that no H^+ ejection is coupled to the cytochrome oxidase reaction (13–16); in the "loop" concept proposed in the chemosmotic hypothesis the cytochrome oxidase reaction is assumed to transport only electrons (14–16). Papa (17) also has concluded that site 3 does not participate in H^+ transport. On the other hand, Wikström and Saari (18–20) have provided evidence from direct tests of site 3 that 2 H^+ are ejected per pair of electrons transported from cytochrome *c* to oxygen in rat liver mitochondria and in cytochrome oxidase vesicles. Moreover, Sorgato and Ferguson (21) have demonstrated that electron transport through site 3 develops a transmembrane gradient of both electric charge ($\Delta\Psi$) and H^+ (ΔpH) in inverted submitochondrial vesicles. Although it seems unlikely that the H^+/site ratio for site 3 is intrinsically lower than that for sites 1 and 2, the question requires resolution.

The second point at issue is the value of the H^+/ATP ratio. The available data (4, 22, 23) indicate that the H^+/ATP ratio for hydrolysis of extramitochondrial ATP is close to 2, although K^+/ATP uptake ratios ranging from about 3 to as high as 7 have been reported (24, 25). To be compatible with an average H^+/site ratio of 4, the H^+/ATP ratio is expected to be 3, as pointed out before (4, 5, 10); the fourth H^+ is required to bring about the energy-requiring electrogenic exchange of ADP^{3-}(out) for ATP^{4-}(in), via the electroneutral H^+-$H_2PO_4^-$ symport process (4, 26, 27). Although an earlier communication from this laboratory reported a determination of the H^+/ATP, with a new rate method, giving values close to 3 (5), some unexpected observations subsequently made on the transmembrane movements of ATP, ADP, and phosphate required resolution before this value could be accepted. These questions have since been resolved, following the discovery of a hitherto unreported membrane transport system for ATP, ADP, and phosphate that is operative during ATP hydrolysis (28).

In this preliminary communication we report individual determinations of the H^+/site ratio at sites 2 and 3 of the respiratory chain and the determination of the H^+/ATP ratio by a new rate procedure. The results, which are compatible with each other and with data reported earlier, fully support the value 4 for the H^+/site ratio and the value 3 for the H^+/ATP ratio; they also allow a quantitative description of a cycle of electrochemical proton equivalents during electron transport and oxidative phosphorylation.

Some of the data reported here were briefly communicated at the annual meetings of the American Society of Biological Chemists, Atlanta, June, 1978 (29, 30), and the Federation of European Biochemical Societies, Dresden, July, 1978 (10, 31). Complete details of these extensive investigations will be presented elsewhere.

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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EXPERIMENTAL DETAILS

Mitochondria were isolated from 0.25 M sucrose homogenates of the livers of male 400-g Charles River (CD) rats fasted overnight before sacrifice. They were washed twice with 0.25 M sucrose. H^+, Ca^{2+}, and K^+ movements were recorded from selective electrodes as described (7, 8). Dual wavelength measurements were carried out in an Aminco DW2 spectrophotometer. Phosphate, ADP, and ATP were determined as described (28).

RESULTS

H^+/Site Ratio for Site 2. Energy-conserving site 2 of the respiratory chain of rat liver mitochondria was isolated for direct study by use of succinate as electron donor in the presence of rotenone to prevent flow of electrons from endogenous NAD-linked substrates and ferricyanide as electron acceptor in the presence of cyanide, added to block electron flow from ferrocyanide via cytochrome *c* and cytochrome oxidase to oxygen. K^+ (plus valinomycin) was used as the permeant charge-compensating cation required to exchange for the ejected H^+, thus maintaining the gross electroneutrality of the matrix, *N*-ethylmaleimide was added to suppress interfering H^+ uptake on the H^+-$H_2PO_4^-$ carrier (3, 5, 8), and ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) was added to prevent Ca^{2+} movements. H^+ ejection was followed with a glass electrode, and ferricyanide reduction was measured spectrophotometrically with the wavelength pair 420/500 nm. Appropriate internal standards were used to calibrate the instrument responses to H^+ and ferricyanide. Under these conditions, ferricyanide, which does not penetrate the membrane, accepts electrons from mitochondrial cytochrome *c*, which is located on the outer surface of the inner membrane (32).

Fig. 1 shows the recorder traces for the ejection of H^+ and the reduction of ferricyanide by succinate as observed in a typical experiment. The initiation of electron flow by addition of succinate to previously de-energized rat liver mitochondria was followed by rapid acidification of the medium and disappearance of ferricyanide. In the absence of succinate there was no significant H^+ ejection or ferricyanide reduction. Both H^+ formation and ferricyanide disappearance proceeded for a sufficiently long period in a nearly linear manner to allow accurate determination of their rates, given in italic numbers along the traces. The ratio *rate of H^+ ejection/rate of ferrocyanide reduction* (in ng-ions or nmol per min per mg of protein) was 157/84.5 = 1.86, equivalent to an $H^+/2e^-$ ratio of 2 × 1.86 = 3.72 for the segment of the respiratory chain between succinate and cytochrome *c*—i.e., site 2.

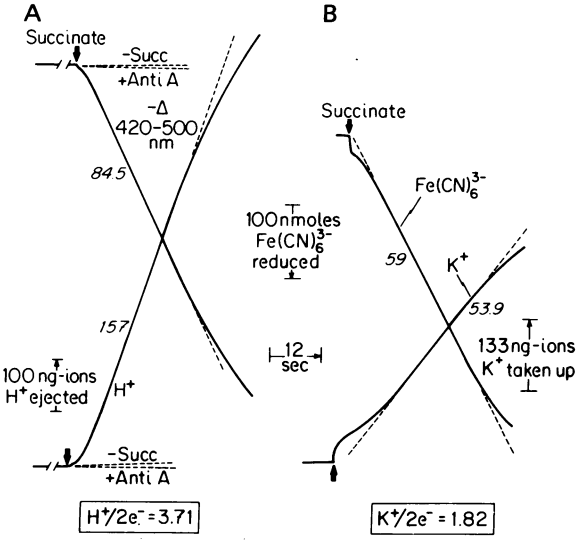

FIG. 1. $H^+/2e^-$ and $K^+/2e^-$ ratios at site 2 with succinate as electron donor and ferricyanide as electron acceptor. The test system (2.6 ml, 25°C) contained 125 mM sucrose, 60 mM KCl, 2 mM Li Hepes at pH 7.15, 3 μM rotenone, 0.15 mM Tris/EGTA, 40 nmol of *N*-ethylmaleimide per mg of protein, and 10 mg of mitochondrial protein. Valinomycin (0.1 nmol per mg of protein) was added at 2 min; 0.5 mM KCN was added at 4 min. After calibrating additions of 600 nmol of ferricyanide, the reaction was initiated with 0.3 mM lithium succinate. In *B* the medium was the same except that 12 mg of mitochondrial protein was used and 60 mM LiCl plus 1.7 mM KCl replaced the 60 mM KCl. Ferricyanide reduction was monitored at 420/500 nm, pH change with a glass electrode, and K^+ uptake with a K^+-selective electrode. Anti A, antimycin A.

Appropriate control experiments showed that addition of antimycin A, which blocks electron flow through site 2, inhibited both ferricyanide reduction and H^+ ejection >96%. Addition of ferrocyanide to the test system evoked no net H^+ ejection. When valinomycin and EGTA were omitted and 100 μM $CaCl_2$ was added, nearly identical results were observed, with an observed $H^+/2e^-$ ratio of 3.86. Thus, either K^+ (plus valinomycin) or Ca^{2+} can serve as the permeant charge-compensating cation. When neither cation was present, the ratio of H^+ ejection was <2% of that observed in the complete system.

To verify such measurements, which show a $H^+/2e^-$ ratio close to 4 for site 2, we have also measured the rate of the simultaneous uptake of the charge-compensating cation, by using selective electrodes. Fig. 1*B* shows the results of such an experiment; the ratio *rate of K^+ uptake/rate of H^+ ejection* is 53.9/59 = 0.91, equivalent to a $K^+/2e^-$ uptake ratio of 1.82 and close to the predicted value, 2.0. This prediction follows from the established fact that succinate is dehydrogenated on the inner side of the inner membrane; the resulting H^+ are delivered into the matrix compartment, where they provide charge-compensation for 2 of the 4 vectorial H^+ ejected from the matrix to the medium. The loss of the other 2 vectorial H^+ from the matrix is compensated for by the entry of 2 K^+ from the medium via valinomycin.

$H^+/2e^-$ Ratio at Site 3. Because the oxidation of succinate by molecular oxygen via sites 2 and 3 shows an H^+/O ratio of 8 and because the experiment in Fig. 1 showed that site 2 tested directly has an $H^+/2e^-$ ratio of 4, site 3 must, by difference, have an $H^+/2e^-$ ratio of 4, rather than the value 2 proposed by Wikström and colleagues (18–20, 33, 34).

Shown in Fig. 2 are results from a typical experiment in which the $H^+/2e^-$ ratio at site 3 was measured by a steady-state rate method. The electron donor was ferrocyanide and oxygen was electron acceptor. The rat liver mitochondria were supplemented with rotenone and antimycin A to prevent electron flow from endogenous substrates into site 3, with K^+ (plus valinomycin) to serve as charge-compensating cation and with *N*-ethylmaleimide to suppress interfering H^+ uptake via the H^+-$H_2PO_4^-$ carrier (3, 5). Ferrocyanide (6 mM) was added last to initiate electron flow. It was rapidly oxidized to ferricyanide, whose appearance was monitored spectrophotometrically with the wavelength pair 420/500 nm. Concurrently, H^+ produced in the medium was recorded spectrophotometrically in a parallel vessel containing phenol red as indicator (measurement at the wavelength pair 560/580 nm). Both ferrocyanide oxidation and H^+ ejection showed nearly linear steady state rates, indicated by italic numbers. The ratio *rate of H^+ ejection/rate of ferrocyanide oxidation* (in ng-ions or nmols per min per mg) was found to be 293/160 = 1.83, equivalent to an $H^+/2e^-$ ratio of 3.66 for site 3.

Control experiments showed that in the absence of ferrocyanide no significant absorption changes occurred at either wavelength. When cyanide was added to the complete system

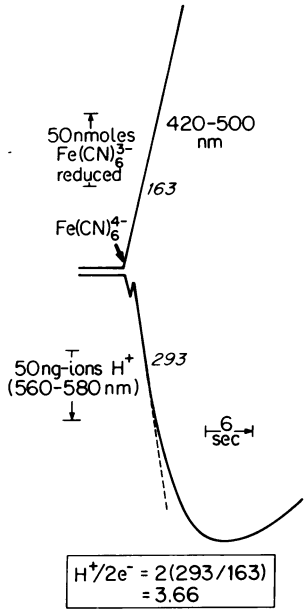

FIG. 2. $H^+/2e^-$ ratio at site 3 with ferrocyanide as substrate. The test system (2.3 ml, 25°C) contained 200 mM sucrose, 22 mM KCl, 1.5 mM K Hepes at pH 7.3, 3.5 μM rotenone, 0.2 nmol of antimycin A per mg of protein, 40 nmol of *N*-ethylmaleimide per mg, and 8.0 mg of mitochondrial protein added at zero time. Valinomycin (0.1 nmol/mg) was added at 30 sec and the reaction was started 4 min later with 6.0 mM potassium ferrocyanide.

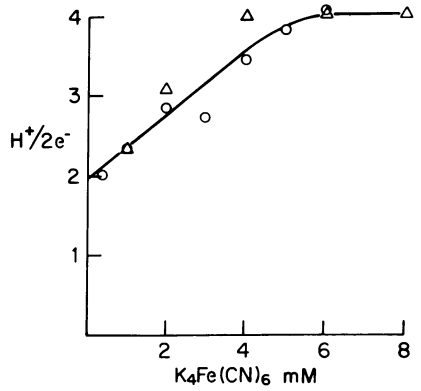

FIG. 3. Effect of ferrocyanide concentration on the $H^+/2e^-$ ratio at site 3. The experimental details were exactly as in Fig. 2. The points shown came from two experiments (△, ○). Each point in turn was the average of two to four determinations.

no significant electron flow or H^+ ejection took place. Replacement of ferrocyanide with ferricyanide (3 mM) resulted in negligible H^+ production. No evidence for significant "extra" H^+ ejection, as described by Moyle and Mitchell (16), could be found; in any case, the rates of "extra" H^+ ejection reported by them are very small compared to the overall rate of ferrocyanide oxidation in experiments such as those in Fig. 2.

We have also observed $H^+/2e^-$ values approaching 4 for site 3 with the use of ascorbate plus ferrocyanide (6–8 mM) and ascorbate plus *N,N,N',N'*-tetramethylphenylenediamine (≈200 μM) as electron donor systems. In the latter cases the rate of electron flow was measured with an oxygen electrode and H^+ output, with a glass electrode; scalar H^+ changes consequent to oxidation of ascorbate to dehydroascorbate were deducted to obtain the vectorial H^+ ejection. The observed $H^+/2e^-$ values for site 3 obtained from spectrophotometric vs. electrode measurements of electron flow were in excellent agreement.

Effect of Ferrocyanide Concentration on the $H^+/2e^-$ Ratio. We have found (ref. 9; also unpublished data) that, in the 120 mM KCl used by Wikström (18, 19), initiation of electron flow in the presence of valinomycin causes rapid osmotic swelling of mitochondria, which is not reversed during the time span of the $H^+/2e^-$ experiments. This swelling is due to increased membrane permeability, allowing influx of Cl^- together with K^+, as reported by others (35, 36); moreover, swelling is also accompanied by an increased leak of H^+. It can be prevented by replacement of most of the KCl by the nonpermeant solute sucrose or LiCl (3, 9). To observe maximal H^+/site ratios at site 3, it is also necessary to use relatively high concentrations (6–8 mM) of ferrocyanide as electron donor compared to the relatively low concentrations (<1 mM) used by Wikström (18, 19). At ferrocyanide concentrations of 1 mM or less, we found the rate of H^+ ejection to be very low, whereas at 6–8 mM ferrocyanide the rate of H^+ ejection is many-fold higher. Moreover, as shown in Fig. 3, we found the $H^+/2e^-$ ratio to be about 2.0 at 1 mM ferrocyanide, increasing to close to 4 at 6 mM (see also Fig. 2). The 20-fold higher rate of H^+ ejection in the experiments with 6–8 mM ferrocyanide minimizes the negative error caused by ΔH^+ back-decay in the initial linear phase of the test.

The $H^+/2e^-$ ratio of close to 4 at site 3 was verified by measurements of the uptake of the charge-compensating cation. Fig. 4 shows the results of a typical experiment in which Ca^{2+} uptake was measured during the oxidation of the couple ascorbate/tetramethylphenylenediamine via site 3. The ratio *rate of* Ca^{2+} *uptake/rate of oxygen consumption* (in ng-ions or ng-atoms per min per mg) was found to be 2.72, equivalent to 5.44 positive charges, close to the value 6 expected for site 3 under the conditions described. Four positive charges must enter to replace the 4 H^+ ejected from the matrix to the medium and two additional positive charges are required to compensate for the 2 H^+ absorbed from the matrix as an atom of oxygen is reduced to yield H_2O.

H^+/ATP Stoichiometric Ratio for Hydrolysis and Synthesis of Extramitochondrial ATP. Earlier measurements of

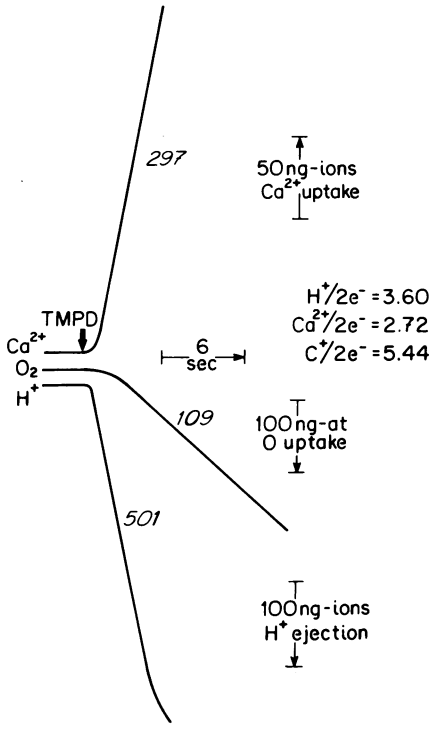

FIG. 4. $Ca^{2+}/2e^-$ uptake ratio at site 3 during oxidation of ascorbate plus tetramethylphenylenediamine (TMPD). The test system (2.6 ml, 25°C) contained 200 mM sucrose, 22 mM KCl, 3 mM K Hepes at pH 7.15, 3 mM K ascorbate, 4 μM rotenone, 0.2 nmol of antimycin A per mg of protein, 40 nmol of *N*-ethylmaleimide per mg, and 10 mg of mitochondrial protein. $CaCl_2$ (370 μM) was added 2 min after the mitochondria, and the reaction was started 2 min later by addition of 220 μM TMPD. Oxygen consumption was measured with the oxygen electrode, H^+ with the glass electrode, and Ca^{2+} with a Ca^{2+} selective electrode.

the H^+/ATP_{out} (hydrolysis) ratio (4, 22, 23) used the ATP pulse technique (22).

However, estimation of the H^+/ATP ratio by such pulse methods is inherently unsatisfactory. Because the concentration of ATP added in such experiments is well below the K_m for ATPase activity, the ATPase reaction approaches completion at an increasingly low rate, at a time when the rate of ΔH^+ back-decay is relatively high. Thus, estimation of the total amount of H^+ ejected becomes somewhat arbitrary. We have developed a much simpler and more direct steady-state rate method. In this procedure, a relatively high concentration of ATP, well above the K_m for the ATPase activity, is added to respiration-inhibited, de-energized rat liver mitochondria, supplemented with diadenosine pentaphosphate to inhibit adenylate kinase activity (37) and with K^+ (plus valinomycin) as the charge-compensating cation. A rapid sampling method is used to obtain colorimetric measurements of the inorganic phosphate released from the ATP at 2.5-sec intervals. No arbitrary corrections are thus necessary. The ratio *rate of H^+ ejection/rate of ATP hydrolysis* (ng-ions or nmol per min per mg) gives the number of H^+ ejected per molecule of ATP hydrolyzed.

Fig. 5 shows a typical experiment carried out on rat liver mitochondria at 15°C. The steady-state rate of H^+ ejection was 72.8 ng-ions of H^+/min per mg and the rate of ATP hydrolysis was 23.2 nmol/min per mg. The H^+/ATP ratio was thus $72.8/23.2 = 3.13$. Many such experiments have given an average value of 2.9 with little deviation.

Under the conditions outlined in Fig. 5 the ATP is hydrolyzed only to ADP and phosphate. ATP hydrolysis and coupled H^+ ejection are completely inhibited by atractyloside and by oligomycin. Moreover, no significant endogenous changes occur in the absence of added ATP; added ADP is not hydrolyzed. In the absence of valinomycin, little or no hydrolysis ensues. Under the experimental conditions used, the scalar H^+ is retained in the alkalinized matrix (cf. 28) and does not appear in the medium; thus, no correction is required for its formation. Either K^+ (plus valinomycin) or Ca^{2+} can be used as charge-compensating cation, with identical results. *N*-Ethylmaleimide is not required in the medium; the H^+/ATP rate ratio is 3.0 in its absence or presence. These data, taken together with our earlier reports (28) that 3 K^+ are taken up per ATP hydrolyzed under these conditions and that the total transport processes involving ATP, ADP, and phosphate during ATP hydrolysis are electroneutral and thus do not require charge compensation by K^+, thus verify that the H^+/ATP ratio is close to 3.0.

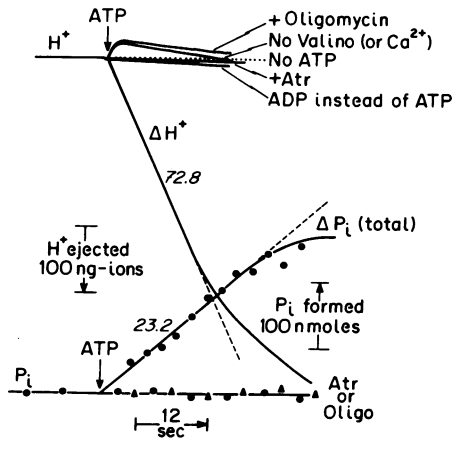

FIG. 5. Steady-state rates of ejection of H^+ and ATP hydrolysis by respiration-inhibited rat liver mitochondria. The test system (8.0 ml) contained 125 mM sucrose, 40 mM LiCl, 20 mM KCl, 3.0 mM K Hepes at pH 7.05, 0.5 mM EGTA, 20 μM diadenosine pentaphosphate, 2 μM rotenone, 40 nmol of *N*-ethylmaleimide per mg of protein, rat liver mitochondria (2.0 mg of protein per ml) at time zero, valinomycin (100 ng/mg) at 2 min, and ATP (200 μM) at 6 min. The incubation was carried out at 15°C. H^+ formation in the medium was monitored with the glass electrode. Total phosphate determinations were performed on 0.3-ml aliquots taken at the indicated intervals. Atr, atractyloside; Oligo, oligomycin; Valino, valinomycin.

DISCUSSION

The key experiments described here verify and extend measurements of the H^+/site ratio of mitochondrial electron transport reported earlier from this laboratory. The observed H^+/site ratios of close to 4.0 obtained for both sites 2 and 3, each measured individually, are in full agreement with our earlier measurements indicating that the *average* H^+/site ratio is close to 4 for sites 1 + 2 + 3 (1–8) and with recent measurements in Azzone's laboratory (38). Our data thus strongly indicate that all three energy-conserving sites in the respiratory chain have a $H^+/2e^-$ ratio of 4.

Perhaps the most crucial point is the finding that the $H^+/2e^-$ ratio of site 3 is close to 4, determined with three different substrate systems. This value contrasts sharply with the conclusion of Mitchell (13–15) that no H^+ ejection is associated with the cytochrome oxidase reaction. However, the latter conclusion is based on pulse-type experiments (13), which exhibit a number of inherent technical problems leading to underestimation of the H^+/site ratio. The most important of these is caused by very rapid interfering uptake of H^+ together with phosphate, on the H^+-$H_2PO_4^-$ symporter (cf. refs. 1, 3, 5), which is much too fast to be corrected for by the extrapolation procedure used by Mitchell and Moyle (11). Although these authors have recently denied (39) that uptake of H^+ plus phosphate is a factor leading to underestimation and have concluded that *N*-ethylmaleimide increases the observed H^+/site ratios spuriously through its capacity to evoke H^+ ejection coupled to oxidation of endogenous substrates, these arguments had already been tested and rejected in earlier communications from this laboratory (3, 5, 12). Moreover, it may be emphasized again that *N*-ethylmaleimide is not required for the observation of H^+/site ratios close to 4; simple removal of phosphate from the test medium (3) or measurement of $K^+/2e^-$ uptake ratios (8) allows observation of $H^+/2e^-$ ratios close to 4 in the absence of *N*-ethylmaleimide.

Of greater importance and relevance, particularly because rather similar experimental approaches were used, is the difference between the $H^+/2e^-$ ratio of 2 for site 3 observed by Wikström and colleagues (18–20, 33, 34) and the value of close to 4 reported here. However, the considerations and data reported here, particularly those in Fig. 3, strongly suggest that these discrepancies may be a reflection of significant differences in the precise experimental conditions used.

The measured H^+/ATP ratio of close to 3.0, briefly reported earlier (5), has been verified by data in this paper and by quantitative examination of the transport pathways and stoichiometry involved in the transmembrane movements of ATP, ADP, and phosphate during ATP hydrolysis (28). The newer stoichiometric data may be placed into a revised "proton cycle" (Fig. 6) for the formation and utilization of energy units equivalent to the ejection of H^+ coupled to electron transport and the uptake of H^+ coupled to ATP synthesis; this scheme is an updating of one described earlier (4). It proposes that an electrochemical gradient equivalent to 4 H^+ per pair of electrons is generated by electron flow through each site. The electrochemical equivalent of 3 H^+ is used to bring about synthesis of ATP from ADP and phosphate in the matrix, and the fourth H^+ equivalent is used to drive the electrogenic ex-

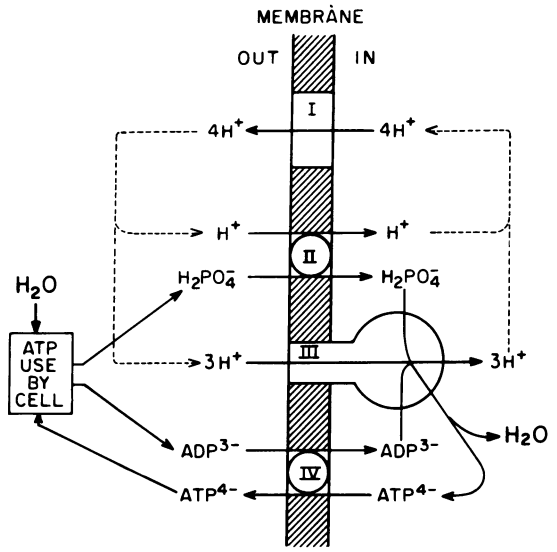

FIG. 6. Revised proton cycle for the formation of electrochemical energy units coupled to electron transport and the utilization of such proton equivalents in ATP synthesis and transport.

plusion of ATP^{4-} from the mitochondria on the atractyloside-sensitive ADP-ATP carrier in exchange for incoming ADP^{3-}, via the action of the electroneutral H^+-$H_2PO_4^-$ symporter. The stoichiometry of the proton cycle shown in Fig. 6 must be accounted for by any valid hypothesis for the mechanism of oxidative phosphorylation, whether or not an electrochemical gradient of H^+ is the primary vehicle for respiratory energy transduction.

The data reported here are not compatible with the original loop hypothesis of Mitchell (40), which allows a maximum ratio of 2.0; alternative mechanisms have been suggested (5). These findings also make unnecessary the assumption in the chemosmotic hypothesis that the allocation of respiratory energy between ATP synthesis and ATP transport is flexible, depending upon the extramitochondrial phosphorylation potential.

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