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H^+/site, charge/site, and ATP/site ratios in mitochondrial electron transport

(energy transduction/H^+ channel/stoichiometry of H^+ pump)

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ABSTRACT **H^+/site, charge/site, and ATP/site ratios have been determined at coupling sites I, II, and III. Three e^- donors have been used for coupling site III: ferrocyanide, ascorbate + tetramethyl-*p*-phenylenediamine (TMPD), and succinate + TMPD. The H^+/site ratios are 4.0 with ferrocyanide and 6.0 with succinate + TMPD (at pH <7.0); the charge/site ratios are 6.0 with ferrocyanide and with succinate + TMPD (at pH <7.0) and 4.0 with ascorbate + TMPD; the ATP/site ratio is 1.34 with ascorbate + ferrocyanide. These ratios have been obtained in the presence of amounts of antimycin A that provide full inhibition of site II. For coupling sites I and II, ferricyanide has been used as e^- acceptor and succinate or NAD-linked substrates as e^- donors. The H^+/site ratios are 4.0 at sites I and II; the charge/site ratios are 4.0 at site I and 2.0 at site II; the ATP/site ratios are 1.0 at site I and 0.5 at site II. Two major factors affect the stoichiometries: (*i*) dimension of $\Delta\tilde{\mu}_H$ and (*ii*) supply of H^+ from the matrix. There is a correlation between collapse of $\Delta\tilde{\mu}_H$ and increase of H^+/site and charge/site ratios. This indicates that approximation of the phenomenologic stoichiometry of the H^+ pump is obtained when flow ratios are measured at level flow. That charge/site and ATP/site ratios increase when ferrocyanide is e^- donor and decrease when ferricyanide is e^- acceptor is attributed to the localization of the redox couple. This leads to separation of 1 charge/e^- when ferrocyanide is e^- donor and to consumption of 1 charge/e^- when ferricyanide is e^- acceptor. To account for an extrusion of H^+ in excess of that predicted by the loop model, it is proposed that each coupling site contains a channel acting as a H^+ pump.**

The mechanism of H^+ translocation is thought of as involving either a loop arrangement of hydrogen and electron carriers in the respiratory chain (1) or protein patches acting as H^+ channels coupled with electron carriers (2–5). Distinction between the two concepts has been attempted by determination of the topology of the respiratory carriers or measurement of the H^+/e^- ratios. Mitchell and Moyle (6, 7) measured the H^+/site ratios on the entire chain (by using oxygen as e^- acceptor) or on segments (by using ferrocyanide or ferricyanide as e^- donor and acceptors) and found a ratio of 1 H^+/e^- per site with no H^+ extrusion at the level of cytochrome oxidase. However, Mitchell and Moyle's conclusions have been challenged. First, Azzone and coworkers (8–11) found 4 charges/site by measuring cation transport in the steady state [cf. also Cockrell *et al.* (12)]. Second, Lehninger and coworkers (13–16) found 4 charges/site and 3–4 H^+/site by measuring the stoichiometries either with oxygen and reductant pulses or in the steady state. Lehninger and colleagues (14) reported a substantial increase in the H^+/site ratios after abolition of P_i reuptake by *N*-ethylmaleimide (MalNEt). Third, Wikström (17–19) reported H^+ extrusion at the level of site III, with a stoichiometry of $2H^+/2e^-$ and of 4 charges/$2e^-$

Our study has aimed: (*i*) to dissect the stoichiometries at the three coupling sites; (*ii*) to compare the dissected with the overall substrate → O_2 stoichiometries; (*iii*) to compare the H^+/site with the charge/site and ATP/site stoichiometries; and (*iv*) to determine the factors affecting the stoichiometries. We designate H^+/site, charge/site, and ATP/site ratios as the number of H^+ extruded or cation taken up and of ATP synthesized during the passage of 2 e^- per each coupling site. We assume that mitochondria contain three coupling sites corresponding to the respiratory chain segments located in complexes I, III, and IV, respectively.

The data are consistent with the view of an extrusion of four protons at each coupling site. A preliminary account has already been presented (20). Similar conclusions have been reached independently by Alexandre and Lehninger (21).

EXPERIMENTAL

Rat liver mitochondria were prepared according to standard procedures (22). Incubation details are given in the legends. H^+ displacement was measured with a pH-sensitive electrode, and K^+ uptake was measured with a cation-specific electrode; radiometer pH-meters connected to a Texas Instrument recorder were used. Ferrocyanide oxidation and ferricyanide reduction were followed in a dual wavelength spectrophotometer (420/460 nm). Ca^{2+} uptake was measured with murexide in a dual wavelength spectrophotometer. Oxygen consumption was determined with a Clark oxygen electrode. ATP synthesis was measured either on the basis of the amount of oxygen or ferrocyanide consumed during a state 4-to-state 3-to-state 4 transition or directly (23). The reaction was terminated with perchloric acid. The sample was neutralized and the amount of ATP was determined fluorimetrically through the formation of NADPH. Determination of $\Delta\tilde{\mu}_H$ was as described (23). All experiments were performed in thermostated glass cuvettes with magnetic stirring.

RESULTS

Stoichiometries at Site III. The H^+ extrusion after addition of ferrocyanide to antimycin A-inhibited mitrochondria has been attributed by Moyle and Mitchell (24) to a scalar reaction and by Papa *et al.* (25) to operation of the b–c_1 complex (complex III). An alternate interpretation has been provided by Wikström and Kraab (26). We agree with their view on the following evidence. First, the rate of H^+ extrusion is a function of the K^+ permeability and thus of the K^+ and valinomycin concentrations. Second, the effects of antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide are equivalent. Both inhibitors abolish ferrocyanide-induced H^+ extrusion only at concentrations higher than required to inhibit the b–c_1 complex;

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Abbreviations: TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MalNEt, *N*-ethylmaleimide.

at these concentrations they also cause a marked increase of the state 4 respiration and a collapse of $\Delta\psi$.

H^+/e^- ratios of 1 at site III have been measured with ferrocyanide (17–19) or ascorbate + *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as e^- donor (27). These measurements are open to question for a number of reasons. The extrusion involves a small amount of H^+ (3–4 nmol per mg of protein) through a short period of time. Ferrocyanide in low concentrations provides low rates of respiration and H^+ extrusion that may be affected by the H^+ leakage. Ferrocyanide in high concentration causes a large effect on the electrode. TMPD may cause some uncoupling as indicated by the linear increase of the state 4 respiration with the TMPD concentration.

Table 1 shows H^+/O and K^+/O ratios at site III. H^+/O ratios were measured with two e^- donors: ferrocyanide and succinate + TMPD. Ferrocyanide and succinate are oxidized at the outer and inner surfaces of the inner mitochondrial membrane, respectively. Antimycin A was added in both cases to inhibit operation of site II. TMPD is known to bypass the antimycin A inhibition of site II and to transfer reducing equivalents across the membrane from succinate dehydrogenase to cytochrome *c* (28). The experiment was carried out in the following manner: TMPD was oxidized completely to Wurster's blue$^+$ in the absence of succinate; reduction of Wurster's blue$^+$ was then initiated by the addition of succinate. In this system there is no net reduction of Wurster's blue$^+$ during succinate oxidation because the oxidation of TMPD by cytochrome oxidase is much faster than the reduction of Wurster's blue$^+$ by succinic dehydrogenase. The H^+/O ratios were measured at 10°–12°C. H^+ extrusion and oxygen uptake were linear for more than 10 sec. The amount of H^+ extrusion was at least 5 times higher with succinate + TMPD than with ferrocyanide. This is presumably due to liberation of H^+ in the matrix in the former case. The reaction was initiated by adding valinomycin to substrate-supplemented mitochondria.

At pH 7.5 the H^+/O ratio with succinate + TMPD was decreased to 4.4. This was due to a major increase in the rate of oxygen uptake—to 17.0 nmol/mg of protein per min—without a parallel increase in the rate of H^+ extrusion. The rate of K^+ uptake during reduction of Wurster's blue$^+$ by succinate in KCN-treated mitochondria was negligible. At pH 7.5 the K^+/O ratio was decreased to 4.0. Again this was due to an increase in the rate of oxygen uptake without a parallel increase in the rate of K^+ uptake.

The charge/site ratio was also measured with Ca^{2+} as permeant cation and ferrocyanide as e^- donor; values approaching 6.0 were obtained. Furthermore, with ferrocyanide as e^- donor (in the presence of 2 mM ascorbate), an ATP/site ratio of 1.34 was found. With ascorbate + 200 μM TMPD, the charge/site ratio was 4.0 and the ATP/site ratio was 1.0.

Stoichiometries at Sites I and II. The only data on the stoichiometries at sites I and II are those of Mitchell and Moyle (7) who measured the H^+ extrusion after ferricyanide pulses to mitochondria oxidizing either succinate or β-hydroxybutyrate (cf. also refs. 29 and 30): $4H^+/2e^-$ were found during succinate oxidation (site II) and $6H^+/2e^-$ during β-hydroxybutyrate oxidation (site I + site II). Table 2 shows the $H^+/2e^-$ ratios at sites I and II, carried out in the presence of MalNEt. Whereas the $H^+/2e^-$ ratios in the presence of succinate were similar to those observed by Mitchell and Moyle, the ratios with NAD-linked substrates were higher, being close to 8.0. Other measurements were also carried out during restriction of the reuptake of endogenous P_i by lowering the temperature and omitting MalNEt. Furthermore, stoichiometries were also determined on the total extent of H^+ extrusion (without extrapolation) and on the initial rates of H^+ extrusion and ferricyanide reduction. Under all conditions, the $H^+/2\ e^-$ ratios approached 8.0 with NAD-linked substrates.

Table 2 also shows the charge/$2e^-$ ratios. The ratios were measured on the initial rates of Ca^{2+} uptake and ferricyanide reduction. Similar results were obtained by measuring the stoichiometries on the total amount of Ca^{2+} uptake. When the ratio with NAD-linked substrates (site I + site II) is corrected for the site II ratio, a charge/$2e^-$ ratio approaching 4.0 for site I is obtained. Table 2 also shows the ATP/$2e^-$ ratios with succinate and with NAD-linked substrates. Correction of the

Table 1. Stoichiometries at site III

Exp.	e^- donor	nmol/mg protein per min V_H	V_O	H^+/O
1	Ferrocyanide	46.5 ± 10.7	10.8 ± 1.2	4.3
2	Succinate + TMPD	65.3 ± 0.8	11.8 ± 0.8	5.5
3	Succinate + TMPD (K^+/O)	168.3 ± 18.4	27.6 ± 1.4	6.1

The medium for H^+/O ratios contained 0.12 M sucrose, 40 mM KCl, 2 mM Tris·HCl, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2 μM rotenone, 8 mM $MgCl_2$, 40 pmol of antimycin A per mg of protein, and 4 mg of protein per ml. The medium for K^+/O ratios contained 0.12 M sucrose, 60 mM choline Cl, 10 mM Tris·HCl, 1 mM P_i, 2 mM KCl, 0.1 mM EGTA, 2 μM rotenone, 1 mM $MgCl_2$, 40 pmol of antimycin A per mg of protein, and 2 mg of protein per ml; pH was 7.0 with ferrocyanide and 6.9 with succinate + TMPD. Other conditions were as follows: Exp. 1, after 3 min of preincubation, 10 mM ferrocyanide, 0.5 μg of oligomycin per mg of protein, and 300 pmol of valinomycin per mg of protein were added at 20-sec intervals; Exp. 2, 400 μM TMPD was oxidized to Wurster's blue$^+$ in 3 min, and 0.2 mM succinate, 0.5 μg of oligomycin per mg of protein, and 300 pmol of valinomycin per mg of protein were added at 20-sec intervals; Exp. 3, 400 μM TMPD was oxidized to Wurster's blue$^+$ in 3 min, and then 1 mM succinate, 0.5 μg of oligomycin per mg of protein, and 150 pmol of valinomycin per mg of protein were added at 20-sec intervals. Temperature for the H^+/O ratios was 10–12°C; for the K^+/O ratios, temperature was 25°C. The values are the means (±SD) of four to six independent experiments.

Table 2. Stoichiometries at sites I and II

e^- donor	$H^+/2e^-$	charge/$2e^-$	ATP/$2e^-$
Succinate	3.90 ± 0.18(4)	2.02 ± 0.11(10)	0.41 ± 0.02(6)
NAD-linked substrates	7.52 ± 0.42(7)	5.60 ± 0.24(5)	1.29 ± 0.04(6)

The medium for the $H^+/2e^-$ ratios contained 0.12 M KCl, 2 mM Hepes (pH 7.2), 2 mM $MgCl_2$, 0.5 mM EGTA, 110 pmol of valinomycin per mg of protein, and 50 nmol of MalNEt per mg of protein. The reaction was initiated by addition of 10 μM ferrocyanide. $H^+/2e^-$ ratios were calculated by extrapolation according to Mitchell and Moyle (6). The medium for the charge/$2e^-$ ratios contained 0.2 M sucrose, 20 mM Tris·HCl (pH 7.3), 2 mM $MgCl_2$, 0.5 mM P_i/Tris, and 50 μM murexide. After 3 min of incubation the reaction was initiated with 100 μM ferrocyanide followed, after 30 sec, by 200 μM $CaCl_2$. Charge/$2e^-$ ratios were measured on initial rates of Ca^{2+} uptake and ferrocyanide reduction. The rates were linear for at least 10 sec. In all samples, 0.5 μg of oligomycin per mg of protein, 1 mM KCN, and 4 mg of mitochondrial protein per ml were added. Where indicated, 2 mM succinate and 3 μM rotenone were added. NAD-linked substrates were endogenous substrates when $H^+/2e^-$ ratios were measured and 1 mM β-hydroxybutyrate when charge/$2e^-$ and ATP/$2e^-$ ratios were measured. The medium for the ATP/$2e^-$ ratio was identical to that for charge/$2e^-$ ratios except that oligomycin was omitted and 30 μM diadenosine 5′-pentaphosphate was also added. Temperature was 20–22°C. The data are shown as mean ± SD; number of experiments is shown in parentheses.

Table 3. Effect of $\Delta\tilde{\mu}_H$ on dimension of stoichiometries during succinate oxidation

K^+, mM	P_i, mM	nmol/mg protein per min V_H	V_O	H^+/O
0.3	0	25.0	6.4	3.91
1.0	0	37.5	7.0	5.36
3.0	0	59.2	10.3	5.75
10.0	0	81.2	10.4	7.88
0.3	1.0	76.5	17.8	4.29*
0.5	1.0	142.9	21.6	6.62*
1.2	1.0	271.3	33.6	8.07*
2.0	0.01	111.5	22.8	4.89*

Conditions for measuring H^+/O and K^+/O ratios were similar to those described in Table 1 except that antimycin A was omitted and succinate, 0.2 or 1 mM, was added. After 3 min of preincubation, succinate, oligomycin, and valinomycin were added in sequence. Temperature was 12 and 25°C for H^+/O and K^+/O ratios, respectively.
* K^+/O.

$ATP/2e^-$ ratio with NAD-linked substrates for the site II $ATP/2e^-$ ratio yields an $ATP/2e^-$ ratio for site I approaching 1.0. The charge/$2e^-$ and $ATP/2e^-$ ratios were also measured during TMPD bypass of antimycin inhibition of β-hydroxybutyrate oxidation, which reflects the operation of site I + site III. An overall charge/$2e^-$ ratio of 7.52 was observed, which is in accord with the separation of 4 charges at site I. The $ATP/2e^-$ ratio was 1.42.

Flexibility of Stoichiometries. Azzone and Massari (9) showed that the K^+/O ratio was affected by the concentrations of K^+ and valinomycin and by the presence of P_i. Increasing K^+ concentration from 0.3 to 10 mM resulted in an increase in the rate of H^+ extrusion (Table 3). Because, in the same range of K^+, the rate of O_2 uptake increased only slightly, there was a parallel increase of the H^+/O ratio from 3.91 to 7.88. Table 3 also shows that increase of K^+ from 0.3 to 1.2 mM resulted in an almost 4-fold stimulation of the rate of K^+ uptake. Because the rate of O_2 uptake in the same range of K^+ was doubled, the K^+/O ratio increased from 4.29 to 8.07. Similar effects were obtained by varying the amount of valinomycin, alone or in combination with the K^+ concentrations. Under conditions such that K^+/O ratios reached 8.0 $\Delta\tilde{\mu}_H$ decreased from 200 to about 120 mV, due to the high rate of permeant cation influx.

The data of Table 3 indicate that a maximal ratio was obtained at lower K^+ concentration in the case of K^+/O compared to H^+/O ratios. This is due to the presence of P_i in the former case. Table 3 also shows that, in the presence of 2 mM K^+, decreasing P_i from 1 mM to 10 μM was accompanied by a depression of the rate of K^+ uptake. Again, because the rate of O_2 uptake was much less depressed, the K^+/O ratio decreased.

DISCUSSION

Determination of the H^+/e^- ratio, albeit a difficult experimental problem, is the most direct means to test whether loops are adequate to account for H^+ transport during e^- transfer. In the case of the H^+/site ratios, a major source of artifact is the uptake of weak acids. H^+ extrusion, tightly coupled to cation uptake, leads to formation of ΔpH. Since weak acids distribute according to ΔpH, there is a decrease in the net H^+ extrusion proportional to the extent of uptake of weak acids that are in the medium or have leaked from the mitochondria.

Apart from the artifactual masking of the H^+ extrusion due to weak acid uptake, two major factors affect the stoichiometries: (*i*) dimension of $\Delta\tilde{\mu}_H$, and (*ii*) supply of weak acids. The higher the rate of ion fluxes, induced by increase of K^+ and valinomycin, the smaller is the dimension of $\Delta\tilde{\mu}_H$ and the higher the stoichiometry. Similarly, at equivalent K^+ and valinomycin concentrations, weak acids induce higher rates of ion fluxes and higher stoichiometry. The higher ratios at high K^+, valinomycin, or weak acids are due to the establishment of thermodynamic conditions far from those of static head. This is not unexpected. Because the stoichiometries are measured on the flows of H^+ extrusion (or of cation uptake) and of e^- transport, the flow ratio corresponds to the phenomenologic stoichiometry of the H^+ pump only at level flow (31):

$$\left(\frac{J_1}{J_2}\right)_{lf} = qz$$

in which J_1 and J_2 are the flows of H^+ and e^- at level flow (*lf*), q is the degree of coupling, and z is the phenomenologic stoichiometry. When q is close to 1, as with intact mitochondria oxidizing succinate, the flow ratio is an approximation of the phenomenologic stoichiometry. However, this is not the case when flow ratios are measured far from level flow and close to static head—i.e., at low K^+ and valinomycin or when the H^+ supply from the matrix is restricted.

The larger H^+ flows at conditions approaching level flow reflect the smaller gradient against which H^+ is transported, although the output power of the H^+ pump (product of the flow for the force) remains identical. The use of conditions far from level flow is the main reason for discrepancies in the reported stoichiometries.

We find, during the passage of 2 e^- through site I, the extrusion of $4H^+$, the separation of 4 charges, and the synthesis of 1 ATP. These ratios are consistent with the uptake of $6Ca^{2+}$ (32, 33) and $12K^+$ (8, 9) during oxidation of NAD-linked substrates. Mitchell and Moyle (7) have reported the extrusion of $6H^+/2e^-$ during oxidation of NAD-linked substrates by ferricyanide. H^+ extrusion was presumably underestimated due to endogenous P_i reuptake. Furthermore, the correction used by Mitchell and Moyle—$2H^+/2e^-$—is questionable because it is based on the assumption of a ferricyanide-dependent oxidation (either directly or indirectly) of a H carrier at the C surface.

We find, during the passage of $2e^-$ through site II, the extrusion of $4H^+$, the separation of 2 charges, and the synthesis of 0.5 ATP. The extrusion of $4H^+$ is in accord with Mitchell and Moyle's observations during ferricyanide pulses (7). The Q cycle (34) also predicts the extrusion of $4H^+$ and the separation of 2 charges in the b-c_1 complex. However, a stoichiometry of $4H^+$ and 2 charges may also be explained by assuming that 2 of the 4 charges separated during H^+ translocation are lost during ferricyanide reduction at the C surface. This assumption is supported by the correlation between charge/site and ATP/site ratios. If the loop mechanism were correct, charge/site and ATP/site ratios at sites I, II, and III should be identical. However, ATP/site ratios are 1, 0.5, and 1.5 at sites I, II, and III, respectively, and charge/site ratios are 4.0, 2.0, and 6.0 at sites I, II, and III, respectively.

During the passage of $2e^-$ through site III, we find the extrusion of $4H^+$, the separation of 6 charges, and the synthesis of 1.5 ATP. Our data are consistent with the uptake of $4Ca^{2+}/O$ and $8K^+/O$ in the span succinate → oxygen. They are also consistent with the synthesis of 2 ATP in the span succinate → oxygen. Mitchell and Moyle (6) found $4H^+$ during aerobic succinate oxidation. The uptake of $4Ca^{2+}$ is not denied but is assumed to be accompanied by the neutralization of only 4 charges (1 charge/Ca^{2+}); $4H^+$ and $2Ca^{2+}$ are attributed to site II and no H^+ and $2Ca^{2+}$, to site III. This conclusion is in contrast to two groups of observations. First, there is no consistent evidence that Ca^{2+} is transported only with 1 charge; the uptake of $4Ca^{2+}/O$ is paralleled by that of $8K^+/O$ (8, 9); Ca^{2+} is taken up by mitochondria in exchange for $2K^+$ and $2H^+$ (35–37);

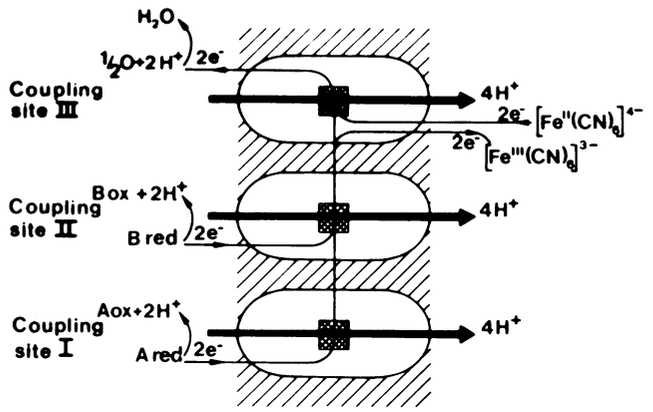

FIG. 1. Proton extrusion and charge separation at the three coupling sites of the mitochondrial respiratory chain, showing the polarity of the three complexes catalyzing e^- transfer and H^+ extrusion. The squares indicate that the mechanism for coupling of e^- transfer to H^+ extrusion is unknown.

furthermore distribution of Ca^{2+}, except in the range of highest $\Delta\psi$, corresponds to $[Ca^{2+}]_i^2/[Ca^{2+}]_o^2 = [K^+]_i/[K^+]_o$; transport of Ca^{2+} with 1 charge would lead to a Ca^{2+} distribution 2 or 3 orders of magnitude different from that actually found (38, 39); and finally, only 1 $Ca^{2+}/2e^-$ is taken up at site II (Table 2) whereas an uptake of 2 $Ca^{2+}/2e^-$ is predicted. Second, Mitchell's view requires an identical ATP/site ratio at sites II and III. However, the ATP/site ratio is less than half at site II as compared to site III (28, 29). Finally, a stoichiometry of $2H^+$/site implies that ATP synthesis be driven only by $1H^+$, because another H^+ is needed to export ATP from the matrix. This is highly unlikely.

Wikström (17, 19) has reported the extrusion of $2H^+$ and the separation of 4 charges at site III (cf. also ref. 27). This leads to an overall stoichiometry of $6H^+$ and 6 charges in the span succinate → oxygen. These data are therefore in contrast with the uptake of $4Ca^{2+}$ and $8K^+$ during succinate oxidation. We attribute the lower ratios to measurements of the H^+/O ratios in short and rapid cycles and of the charge/O ratios in the presence of MalNEt and absence of weak acids.

It has been noted that ferricyanide, a powerful oxidant of ubiquinone for NADH-Q reductase and for NADH oxidation in submitochondrial particles, is unable to bypass the antimycin A block of succinate oxidation (40). The more lipophilic Wurster's blue$^+$ does so, indicating that the reduction of Wurster's blue$^+$ occurs in a lipid environment and not at the surface. The stoichiometries with succinate + TMPD show a marked pH dependence. The higher ratios at acidic pH and the lower ratios at alkaline pH suggest oxidation of a H carrier in the former and of an e^- carrier in the latter case.

Fig. 1 shows a diagram of e^- and H^+ flow through the three coupling sites of the mitochondrial respiratory chain. Coupling sites I and II (corresponding to complex I and III) accept e^- at the matrix side and donate e^- at the cytoplasmic side; coupling site III (corresponding to complex IV) accepts e^- at the cytoplasmic side and donates e^- at the matrix side. Each coupling site (or complex) possesses a H^+ pump that separates 4 charges ($4H^+$ out and $4OH^-$ in). With NADH or succinate the H^+ liberated during substrate oxidation is balanced by the H^+ used for H_2O formation. With ferrocyanide as e^- donor, 2 more charges are separated during e^- transfer from the cytoplasmic to the matrix side through cytochrome oxidase. With ferricyanide as e^- acceptor, 2 charges are neutralized during donation of e^- from cytochrome *c* to ferricyanide.

A stoichiometry of $4H^+$/site at the three coupling sites suggests that, in alternation or in combination with the loops, H^+ transport involves H^+ channels. A stoichiometry of $4H^+$/site can be accounted for both by local control (41–46) or delocalized (1) mechanisms for the role of H^+ in energy transduction.

While the present manuscript was under revision, a paper reaching similar conclusions appeared by Alexandre *et al.* (47). However, close examination suggests that the agreement is accompanied by some experimental discrepancies.

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