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Stoichiometric relationship between energy-dependent proton ejection and electron transport in mitochondria

(oxidative phosphorylation/cation transport/phosphate transport/chemiosmotic hypothesis)

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ABSTRACT **The number of protons ejected during electron transport per pair of electrons per energy-conserving site (the H^+/site ratio) was measured in rat liver mitochondria by three different methods under conditions in which transmembrane movements of endogenous phosphate were minimized or eliminated. (*1*) In the Ca^{2+} pulse method, between 3.5 and 4.0 molecules of 3-hydroxybutyrate and 1.75 to 2.0 Ca^{2+} ions were accumulated per 2 e^- per site during Ca^{2+}-induced electron transport in the presence of rotenone, when measured under conditions in which movements of endogenous phosphate were negligible. Since entry of 3-hydroxybutyrate requires its protonation to the free acid these data correspond to an H^+/site ratio of 3.5–4.0. (*2*) In the oxygen pulse method addition of known amounts of oxygen to anaerobic mitochondria in the presence of substrate yielded H^+/site ratios of 3.0 when phosphate transport was eliminated by addition of *N*-ethylmaleimide or by anaerobic washing to remove endogenous phosphate. In the absence of such measures the observed H^+/site ratio was 2.0. (*3*) In the reductant pulse method measurement of the initial steady rates of H^+ ejection and oxygen consumption by mitochondria in an aerobic medium after addition of substrate gave H^+/site ratios near 4.0 in the presence of *N*-ethylmaleimide; in the absence of the inhibitor the observed ratio was only 2.0. These and other experiments reported indicate that the values of 2.0 earlier obtained for the H^+/site ratio by Mitchell and Moyle [*Biochem. J.* (1967) 105, 1147–1162] and others were underestimates due to the unrecognized masking of H^+ ejection by movements of endogenous phosphate. The results presented here show that the H^+/site ratio of mitochondrial electron transport is at least 3.0 and may be as high as 4.0.**

It is now widely accepted that the flow of electrons from substrate to oxygen along the respiratory chain of mitochondria is accompanied by the ejection of H^+ into the suspending medium (1–4); similarly, photosynthetic electron transport in chloroplasts is accompanied by H^+ absorption (1, 3–5). The electrochemical H^+ gradient so generated provides the driving force for the transport of a number of mineral ions and metabolites across the mitochondrial or chloroplast membrane; it may also be the vehicle for respiratory energy transduction during oxidative and photosynthetic phosphorylation (1, 3, 6).

Some years ago Mitchell and Moyle reported the results of measurements of the stoichiometric relationship between H^+ ejection and electron transport in mitochondria with the oxygen pulse technique (7, 8). In this method a small, known amount of oxygen is added to anaerobic mitochondria supplemented with an electron donor; the resulting acidification of the medium is measured with a glass electrode. In such experiments the outward movement of H^+ is assumed to be electrically compensated by an inward movement of cations such as Ca^{2+} or K^+, in the latter case in the presence of valinomycin, a potassium ionophore. Such oxygen-pulse measurements yielded H^+/site ratios (defined as the number of H^+ ejected per pair of electrons per energy-conserving site) close to 2.0 (8). Such values have also been reported by others (9–11) under similar conditions.

More recently the value of 2.0 for the H^+/site ratio has been brought into some question by other experimental approaches. Rumberg and colleagues (12–14) have reported values between 3.0 and 4.0 H^+ per ATP in the case of photosynthetic electron transport. Moreover, comparison of the electrochemical gradients generated across the energy-transducing membrane by electron transport with the chemical potential against which ATP can be formed from ADP and P_i indicates that an H^+/site ratio of 2.0 is insufficient to account for the known phosphorylation capacity of mitochondria (15–17). These discrepancies call for some resolution.

This paper is a preliminary report of a re-evaluation of the magnitude of the H^+/site ratio of electron transport in rat liver mitochondria; it describes results obtained with three different experimental approaches. The starting point for the design of these experiments is the long-known (2) and recently reaffirmed (18) stoichiometry of respiration-coupled Ca^{2+} transport, the inward movement of 2 Ca^{2+} (i.e., four positive charges) per pair of electrons per site. The point of departure leading to the more refined experiments described here is recognition of the necessity to measure the movements of other ions relevant to H^+ movements, in particular, the movements of phosphate. The three types of measurement described here yield values of the H^+/site ratio of at least 3.0 and approaching 4.0. Moreover, they reveal the basis for the earlier values of 2.0 reported by others (8–11).

EXPERIMENTAL DETAILS

Mitochondria were isolated in 250 mM sucrose, washed three times, and resuspended in 250 mM sucrose to yield a stock suspension containing 50 mg of protein per ml. Ca^{2+} pulse experiments were carried out as previously described (18). Oxygen- and reductant-pulse experiments were carried out with rapid magnetic stirring in a 2 ml chamber with a restricted opening. In the oxygen pulse experiments a stream of oxygen-free nitrogen was directed on this opening. The outputs from a Clark oxygen electrode and a pH-sensitive combination glass electrode were fed into a dual channel recorder. The response time of the entire system to injected HCl was less than 1 sec. Other details are described in the figure legends. *N*-Ethylmaleimide (NEM) was obtained from Sigma Chemical Co.

RESULTS

Ca^{2+} Pulse Experiments. Earlier experiments (18) in which respiration-coupled uptake of labeled Ca^{2+} and a

Abbreviations: NEM, *N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

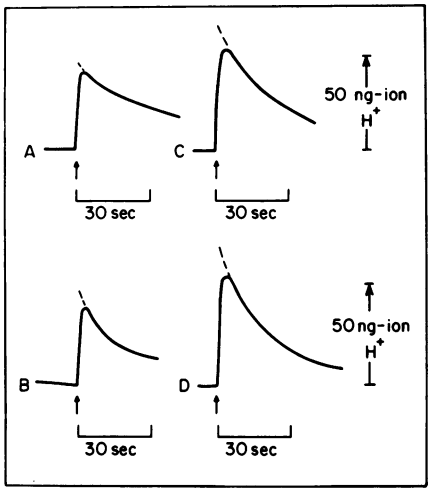

FIG. 1. H^+ ejection by mitochondria pulsed with oxygen. The system during the anaerobic preincubation contained 109 mM KCl, 23 mM sucrose, 2.7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 0.5 mM succinate, 5 μM rotenone, 5.0 mg of mitochondrial protein per ml, and either 17 ng-ion Ca^{2+} per mg of protein (A, C) or 100 ng of valinomycin per mg of protein (B, D). NEM was present at 40 nmol/mg of protein in C and D. The temperature was 28° and the pH was 7.10. H^+ ejection was initiated by addition of 25 μl of air-saturated medium (120 mM KCl, 3 mM Hepes, pH 7.10) where indicated by arrows. Extrapolated H^+/site values were: A, 2.04; B, 2.04; C, 2.95; D, 3.06. The broken lines represent the magnitude of the extrapolation. The total volume of the test systems was 2.0 ml.

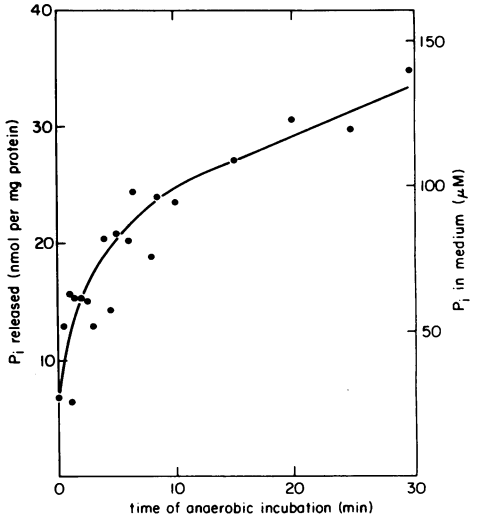

FIG. 2. Appearance of endogenous phosphate in the extramitochondrial medium during anaerobiosis. Mitochondria (5 mg of protein per ml) were incubated in the anaerobic medium (120 mM KCl, 3 mM Hepes, pH 7.1, 28°) until the residual oxygen was consumed (about 1 min). The suspension was then transferred to centrifuge tubes, sealed, and incubated for the times stated. The mitochondria were collected by a 2 min centrifugation in an Eppendorf 3200 centrifuge and the supernatant was assayed for inorganic phosphate (23) after precipitation of remaining protein with 150 mM $HClO_4$.

weak acid anion such as 3-hydroxybutyrate were measured under conditions allowing large net accumulations established the following points: (*a*) The ratio of weak acid anion to Ca^{2+} accumulated was close to 2.0, (*b*) the value of 2.0 for the Ca^{2+}/site ratio observed previously (2) was confirmed, (*c*) four molecules of weak acid were accumulated per site. Since 3-hydroxybutyrate and other weak acid anions enter the matrix only as the undissociated acid, these observations indicate that during uptake of Ca^{2+}, $4H^+$ are ejected per site and are subsequently transported back into the matrix with the weak acid anions.

In order to establish more securely that superstoichiometric Ca^{2+} uptake and H^+ ejection [i.e., that portion coupled to hydrolysis of intramitochondrial ATP (19)] were not compromising the values of the Ca^{2+}/site and H^+/site ratios obtained in our earlier study (18), we have repeated our observations in the presence of the ATPase inhibitor oligomycin. Moreover, the effects of NEM and of atractyloside, inhibitors of phosphate transport and adenine nucleotide transport, respectively, were also studied. None of these inhibitors altered the observed 3-hydroxybutyrate/Ca^{2+} ratio or the Ca^{2+}/site ratio, indicating that movements of phosphate and/or of ADP(ATP), if they took place at all, were irrelevant to the stoichiometry of Ca^{2+} and H^+ transport under the conditions used.

Another possible source of error, the calculation of Ca^{2+}/site ratios from the *extra* oxygen rather than the total oxygen consumed during a Ca^{2+}-induced respiratory jump, was also investigated and eliminated. Our previous results (18) showed that the 3-hydroxybutyrate/Ca^{2+} ratio approached 2.0 even with limiting amounts of Ca^{2+} added. Under these conditions measurement of the extra oxygen uptake yielded a Ca^{2+}/site ratio of 2 and a 3-hydroxybutyrate/site (i.e., H^+/site) ratio close to 4.0. However, if the *total* oxygen consumed during such a respiratory jump was used in the calculation, the Ca^{2+}/site ratio was 1.75, corresponding to an H^+/site ratio of 3.5. This latter figure must, therefore, be taken as the minimum value of the H^+/site ratio in this type of experiment.

Oxygen Pulse Experiments. These experiments were refinements of the approach first described by Mitchell and Moyle (7, 8). Rat liver mitochondria were incubated with succinate and rotenone (to inhibit endogenous electron transport) in a glass cell closed from the atmosphere and equipped with a combination glass electrode. The medium was made anaerobic by flushing with oxygen-free nitrogen, and after addition of mitochondria and equilibration of the system a small amount of dissolved oxygen was added in the form of a known volume of air-saturated medium. A known amount of electron transport ensues after such an oxygen pulse and is complete within seconds. Simultaneously, the H^+ appearing in the medium was recorded. From the traces (Fig. 1) the peak H^+ release was extrapolated by methods described by Mitchell and Moyle (8). Fig. 1 shows a typical result of such an experiment, carried out under conditions similar to those described by Mitchell and Moyle (8). Fig. 1A shows the trace obtained with a small amount of Ca^{2+} added, conditions in which the ejection of H^+ is electrically compensated by uptake of the Ca^{2+}. The observed H^+/site ratio in this experiment was 2.04. Identical results were obtained in the absence of added Ca^{2+}; in this case, the compensating cation moving inward was Ca^{2+} which had leaked out of the mitochondria in the preceding anaerobic preincubation (20–22). In Fig. 1B, the same experiment was carried out with valinomycin present but no added Ca^{2+}, conditions which allow the rapid entry of K^+ into the mitochondria to compensate electrically for the H^+ ejection. The H^+/site ratio was again found to be 2.04. The two types of system thus yielded values in good agreement with those of Mitchell and Moyle (7, 8).

However, direct measurements of the phosphate concen-

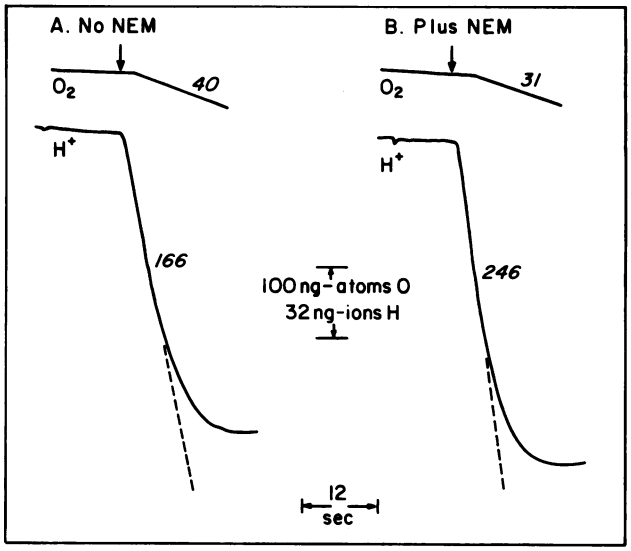

FIG. 3. Oxygen uptake and H^+ ejection evoked by addition of succinate. Mitochondria (2.5 mg of protein per ml) were preincubated for 2 min at 25° in 2 ml of medium containing 120 mM LiCl, 10 mM KCl, 3 mM Hepes, pH 7.2, 4 μM rotenone, and 100 ng of valinomycin per mg of protein. Where indicated NEM was present at 30 nmol/mg of protein. Respiration was initiated by addition of 1.0 mM sodium succinate (arrows). The H^+/site ratio was 2.08 in the absence of NEM (A) and 3.98 in its presence (B). The numbers shown are rates of O uptake in ng-atoms O min^{-1} mg^{-1} and H^+ ejection in ng-ion H^+ min^{-1} mg^{-1}

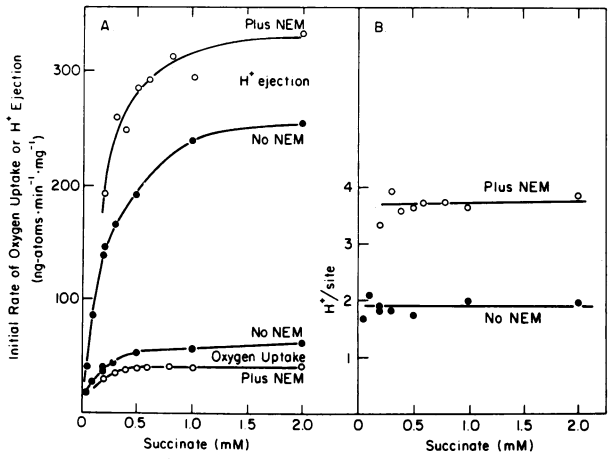

FIG. 4. (A) Initial rates of oxygen uptake and H^+ ejection as a function of succinate concentration in the presence and absence of NEM. The medium was identical to that in Fig. 3 except that succinate concentration was varied as shown. (B) H^+/site ratios derived from the data of (A).

tration of the suspending medium during the anaerobic preincubation revealed that phosphate was lost from the mitochondria during the preincubation period before the oxygen pulse was added. The phosphate reached a concentration of 100 μM in the suspension medium in 10 min (Fig. 2). It appeared quite probable that re-uptake of this phosphate by phosphate-hydroxide exchange after addition of oxygen and subsequent alkalinization of the matrix might affect the magnitude of the observed H^+/site ratio. A simple way of eliminating the phosphate movements was to include in the test system NEM, a potent inhibitor of the transport system responsible for the movement of phosphate across the mitochondrial inner membrane, the phosphate-hydroxide antiporter. Addition of NEM in an amount (40 nmol/mg of protein) known to inhibit phosphate transport essentially completely (24, 25) caused the observed H^+/site ratios in both the Ca^{2+}-dependent and valinomycin-K^+-dependent systems to increase to values of about 3.0 (Fig. 1C and D). Even without any extrapolation values of 2.4 were routinely measured with NEM present. H^+/site ratios of 3.0 have been consistently observed in a large number of such experiments with very little deviation. Values of 3.0 have also been observed with the 3-site substrates 3-hydroxybutyrate or malate plus glutamate. The ratios obtained in the presence and absence of NEM were independent of variables such as amount of oxygen added (below a critical value), succinate concentration, and pH between 6.5 and 7.5.

The role of phosphate in yielding low values (i.e., near 2.0) of the H^+/site ratio has also been demonstrated by incubating mitochondria anaerobically, separating and washing them free of released phosphate (and Ca^{2+}) anaerobically, and then testing the H^+/site ratio. Such washed mitochondria yielded H^+/site ratios approaching 3.0, which were not further increased on addition of NEM. When 100 μM phosphate was added to such depleted mitochondria the H^+/site ratios decreased to near 2.0; subsequent addition of NEM returned the H^+/site ratio to near 3.0.

Reductant Pulse Experiments. In these experiments mitochondria were first preincubated with rotenone to block endogenous electron transport, in the presence of either valinomycin plus K^+, or Ca^{2+}. This preincubation results in oxidation of all carriers beyond energy-conserving site 1 and depletes the mitochondria of energy stores of the mitochondria. To initiate electron flow to oxygen a pulse of succinate was then added and the initial steady-state rates of both oxygen consumption and H^+ ejection were extrapolated from the recorder traces made at high chart speeds. Instead of measuring the total amounts of H^+ ejected and oxygen consumed, as in the oxygen pulse experiments, the steady-state rates of H^+ ejection and oxygen consumption were determined and expressed as the H^+/O rate ratio. From the latter the H^+/site ratio was obtained, assuming 2.0 energy-conserving sites for succinate oxidation. Fig. 3 shows typical traces of an experiment in a LiCl medium with 10 mM KCl and valinomycin present, in the absence and presence of NEM. The initial velocities of both oxygen consumption and H^+ ejection were linear for at least 8–10 sec in either case. Fig. 4A shows a plot of the initial rates of the H^+ ejection and oxygen consumption and Fig. 4B a plot of the H^+/site ratios, both as a function of succinate concentration. In the absence of NEM the rate of H^+ ejection was close to four times the rate of oxygen consumption, as would be expected for a 2-site substrate and an H^+/site ratio of 2.0. However, in the presence of NEM the rate of H^+ ejection was nearly eight times the rate of oxygen consumption, corresponding to an H^+/site ratio of about 3.8. Similar results were observed when valinomycin was not added and the mitochondria were allowed to utilize inward movement of added Ca^{2+} to compensate electrically for the respiration-dependent ejection of H^+. In such experiments added phosphate depressed the ratio [rate of H^+ ejection]/[rate of oxygen uptake], whereas the addition of NEM increased it.

In these reductant pulse experiments it is important to emphasize that addition of succinate to rotenone-inhibited mitochondria initiates H^+ ejection and O_2 uptake; there are no preexisting electrochemical gradients to compromise the rate ratios observed. Measurement of K^+/site ratios after addition of valinomycin to already energized mitochondria, as

utilized by Pressman (26) and by Azzone and Massari (27), gives values which are not free of this criticism (28).

DISCUSSION

All three experimental methods described here yield values of the H^+/site ratio of mitochondrial electron transport that are in unmistakable excess over the value of 2.0 originally reported by Mitchell and Moyle (7, 8). The Ca^{2+} pulse and reductant pulse procedures gave values approaching 4.0, the oxygen pulse procedure gave values of 3.0. The common denominator among these three sets of values for the H^+/site ratio, which distinguishes them from earlier measurements (7–11), is the exclusion of the movements of endogenous or added phosphate. This is accomplished by measuring H^+/site ratios under conditions in which large amounts of Ca^{2+} and weak acids such as 3-hydroxybutyrate are accumulated, so that movement of endogenous phosphate becomes quantitatively unimportant (Ca^{2+} pulse experiments), by removing endogenous phosphate by anaerobic washing (oxygen pulse experiments), or by addition of NEM to inhibit phosphate-hydroxide antiport (all three types of experiment).

Phosphate creates special problems in any quantitative accounting of mitochondrial ion movements. Since phosphate may exist in three ionic species ($H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}) differing in degree of protonation, a quantitative accounting of proton movements and equilibria across the mitochondrial membrane in the presence of phosphate becomes very difficult, particularly because the absolute pH of the mitochondrial matrix, which becomes alkalinized during electron transport, cannot be measured with reliability. In addition, phosphate may pass through the mitochondrial membrane by two different carriers. The phosphate-hydroxide carrier promotes an electrically neutral $H_2PO_4^-$–OH^- antiport (or $H_2PO_4^-$–H^+ symport), whereas the dicarboxylate carrier promotes exchange of HPO_4^{2-} with a dicarboxylate^{2-} anion (see ref. 29). Thus when $H_2PO_4^-$ enters mitochondria as counteranion with Ca^{2+} or K^+ its entry is accompanied by exit of OH^- (or entry of H^+), a process that obviously results in decrease of the apparent H^+/site ratio. Moreover, phosphate may also precipitate with Ca^{2+} in the alkaline mitochondrial matrix to yield amorphous tricalcium phosphate (see ref. 30), a reaction in which protons are liberated:

$$3Ca^{2+} + 2H_2PO_4^- \longrightarrow Ca_3(PO_4)_2 + 4H^+$$

$$3Ca^{2+} + 2HPO_4^{2-} \longrightarrow Ca_3(PO_4)_2 + 2H^+$$

Clearly, phosphate movements can introduce considerable complexity into the quantitative analysis of H^+ exchanges across the mitochondrial membrane. Our observation that phosphate does in fact pass out of de-energized mitochondria and accumulates in the suspending medium at concentrations of more than 100 μM during the anaerobic preincubation required in oxygen pulse experiments demonstrates the importance of avoiding or minimizing the participation of phosphate in the ion movements coupled to electron transport. Because the rate of phosphate transport is so high, even at 0° (25), the low-temperature experiments of Mitchell and Moyle (8) do not suffice to eliminate the effect of inward phosphate movement on the H^+/site ratio.

When large amounts of Ca^{2+} are being transported the phosphate problem is avoided by the addition of some counteranion other than phosphate; among the various anions that may be accumulated with Ca^{2+} during respiration are those of weak monocarboxylic acids, which are capable of only a single protonation step (31). The use of 3-hydroxybutyric acid or certain other lipophilic aliphatic acids as the anion donor thus overcomes the phosphate problem, particularly when the aliphatic anion is present in high concentration and endogenous phosphate movements are inhibited by NEM. Moreover, since these weak lipophilic acids probably pass through the mitochondrial membrane by simple unmediated diffusion as the undissociated acid they carry a precisely measureable number of protons into the matrix when their anions are accumulated with Ca^{2+}.

Our experiments also show that when phosphate movements are inhibited, oxygen pulse experiments of the type originally described by Mitchell and Moyle yield H^+/site ratios of 3.0. These values obtained by the oxygen pulse method are significantly lower than the H^+/site ratios approaching 4.0 given by the Ca^{2+} pulse and reductant pulse experiments. The reason for this discrepancy is not entirely clear. It is possible that the true H^+/site ratio is in fact 4.0, but that one of the protons ejected is utilized or bound in some other mitochondrial process peculiar to the conditions of the oxygen pulse experiments and is thus not sensed by the glass electrode.

In carrying out these experiments we have been very much aware of other possible ion movements that might influence the validity of H^+/site measurements, in particular movement of ions that occur in rather high concentrations in mitochondria, such as K^+, Mg^{2+}, ADP^{3-}, ATP^{4-}, and the anionic metabolites involved in substrate oxidation. Systematic experiments have been carried out to exclude or minimize movements of such ions and will be described in detail elsewhere.

The experiments reported here should not be construed as invalidating the chemiosmotic hypothesis for oxidative phosphorylation (1, 3, 6), which in its present form postulates H^+/site and H^+/ATP ratios of 2.0. Our observations do, however, bring this stoichiometry into question and indicate that the values of 2.0 for the H^+/site ratio measured previously have been underestimates due to movements of endogenous ions, particularly phosphate. On the basis of our experiments we propose that the true stoichiometry of the primary process of H^+ ejection coupled to electron transport in mitochondria is at least 3.0 and possibly 4.0 H^+/site.

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