Stoichiometry of H⁺ Ejection during Respiration-dependent Accumulation of Ca²⁺ by Rat Liver Mitochondria*

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We have investigated the energy-dependent uptake of Ca²⁺ by rat liver mitochondria with succinate as respiratory substrate and with rotenone added to block NAD-linked electron transport. In the presence of 3-hydroxybutyric or other permeant monocarboxylic acids Ca²⁺ was taken up to extents approaching those seen in the presence of phosphate. The quantitative relationship between cation and anion uptake was determined from the slope of a plot of 3-hydroxybutyrate uptake against Ca²⁺ uptake, a method which allowed determination of the stoichiometry without requiring ambiguous corrections for early nonenergized or nonstoichiometric binding events. This procedure showed that 2 molecules of 3-hydroxybutyrate were accumulated with each Ca⁺⁺ ion. Under these conditions close to 2 Ca²⁺ ions and 4 molecules of 3-hydroxybutyrate were accumulated per pair of electrons per energy-conserving site of the respiratory chain. Since 3-hydroxybutyrate must be protonated to pass the membrane as the undissoociated free acid, it is concluded that 4 protons were ejected (and subsequently reabsorbed) per pair of electrons per energy-conserving site, in contrast to the value 2.0 postulated by the chemiosmotic hypothesis.

The 3-hydroxybutyrate/Ca²⁺ ratio was constant over a wide range of concentrations of Ca²⁺ and of 3-hydroxybutyrate and was independent of time of incubation and of extent of uptake. A number of other monocarboxylic acids showed the same stoichiometry demonstrating that the effect was not specific for 3-hydroxybutyrate. Mitochondria isolated from malignant cells exhibited the same behavior under these conditions as those from normal liver.

The quantitative relationships between the transport of cations and the passage of electrons down the respiratory chain have been the object of much attention in the last decade, particularly with respect to Ca²⁺ and to H⁺ transport. The number of Ca²⁺ ions taken up per pair of electrons passing each energy-conserving site (the Ca²⁺/electrons ratio) normally appears to be close to 2 during respiration (1-5), although under special conditions "superstoichiometric" uptake is observed in which the Ca²⁺/electrons ratio may reach very high values (6-13). The number of protons ejected per Ca²⁺ ion taken up (the H⁺/Ca²⁺ ratio) has often been reported to be 0.85 to 1.0 (1, 7, 8, 14, 15), although higher values approaching 2.0 have been observed (16, 17). The H⁺/Ca²⁺ ratio depends in part on the nature of the anions in the system; for example, in the presence of acetate the H⁺/Ca²⁺ ratio falls to about 0.2 (15). These variations, together with the possible importance of the H⁺/Ca²⁺ ratio in the mechanism of oxidative phosphorylation, have led us to reinvestigate the problem of the stoichiometry of H⁺ ejection during Ca²⁺ uptake.

Large amounts of Ca²⁺ may be accumulated by mitochondria together with certain anions, such as phosphate (18-20), acetate and anions of other monocarboxylic acids (15, 21-25), or bicarbonate (25, 26). Formally, each of these anions enters with a proton (26-30), in response to the alkalinity generated in the matrix by electron transport. Uptake of these anions, after correction for residual proton ejection, should be a measure of the number of protons ejected (and then largely carried back into the mitochondria) during uptake of Ca²⁺.

With phosphate as the permeant anion P₇/Ca²⁺ accumulation ratios of 0.6 have been observed (2, 3), corresponding to accumulation within the mitochondrial matrix of insoluble calcium phosphates. With bicarbonate as anion source the CO₃²⁻/Ca²⁺ accumulation ratio is 1.0 (26), corresponding to accumulation (and presumably precipitation) of 2 molecules of CaCO₃ per pair of electrons per site (26). Since phosphate and carbonate may occur in different ionic species depending upon the pH, calculation of the intrinsic H⁺/Ca²⁺ ratio from data on these anions is hazardous since it is necessarily dependent upon assumptions about the intramitochondrial pH. On the other hand, simple monocarboxylic aliphatic acids are not subject to these complications; they have but a single ionizing group, their Ca²⁺ salts are extremely water-soluble and thus do not precipitate from solution in the matrix, and (in the presence of rotenone) their oxidative metabolism is negligible.

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In this paper we describe experiments using 3-hydroxybutyric and a number of other lipophilic acids as a source of counteranions for the respiration-dependent accumulation of Ca^{2+}. We have found that when nonspecific Ca^{2+} binding and other effects are eliminated, the weak acid/Ca^{2+} accumulation ratio is close to 2.0, equivalent to an H^+/Ca^{2+} ratio of 2.0 and an overall H^+/~ ratio of 4.0 for electron transport under conditions in which Ca^{2+} is being accumulated.

**EXPERIMENTAL PROCEDURE**

Liver mitochondria from male Charles River rats which had been fasted overnight were isolated in 250 mM sucrose with three washes. AS-307 rat lipoatous ascites tumor mitochondria were isolated by a method developed in this laboratory. Oxygen uptake was measured with a Clark oxygen electrode (Yellow Springs Instrument Co.) and hydrogen ion movements with a combination glass electrode connected to a Beckman SS-2 pH meter. The concentration of stock solutions of CaCl_2 was checked by atomic absorption. Mitochondrial protein was determined according to Murphy and Ries (S1).

Uptake of 3-hydroxybutyrate and other monocarboxylic acids was determined by accumulation of radioactive label in a mitochondrial pellet (S2) as follows. Mitochondria (2.5 mg of protein) were added to 1 ml of incubation medium (120 mM KCl; 3 mM Hepes; 2.5 mM succinate; 2.5 μM rotenone; 150 μg/ml of bovine serum albumin, pH 7.2, containing [3H]labeled acid (10 μCi/mmol) and [6,6′-3H]sucrose (40 μCi/mmol) in an Eppendorf centrifuge tube. The tubes were incubated aerobically at room temperature (21-23°C) for the times indicated. The incubation was terminated by a 2-min centrifugation in an Eppendorf 3200 centrifuge and a sample of supernatant fluid (500 μl) was added to an equal volume of 0.5 M HClO_4. The remaining supernatant solution was removed with a syringe and liquid adhering to the pellet was carefully removed with a tissue. The pellet was then resuspended in 100 μl of H_2O followed by 300 μl of 0.5 M HClO_4. “Pellet” and “supernatant” tubes then were centrifuged for 4 min to precipitate protein, and samples of each supernatant solution were removed and assayed for [3H] and [3H] by two-channel liquid scintillation counting. The amount of carboxylic acid present in the matrix was calculated by subtracting the amount present in the sucrose-permeable space from the total amount in the pellet. Uptake of 4Ca (200 μCi/mmol) was measured in exactly parallel experiments; the relatively small correction for the sucrose-permeable space was calculated using the mean value for this space obtained from the experiments in which [3H]sucrose was present.

In other parallel experiments the volume of the matrix space was determined using [3H]sucrose and [3H]pyruvate; this was found to be 1.0 μl/mg of protein at zero time or in the absence of extensive uptake. When Ca^{2+} and 3-hydroxybutyrate were being accumulated under the conditions of Fig. 2 the matrix volume rose linearly to 4 μl/mg of protein after 10 min, indicating that the ions accumulating were osmotically active.

All determinations were carried out in duplicate; each figure represents the mean of several determinations on different mitochondrial preparations.

3-Hydroxybutyrate and 4-hydroxybutyrate were added as the sodium salts, the other acids as the potassium salts. All chemicals were of analytical grade. Rotenone was added in a minimal volume of medium salts, the other acids as the potassium salts. All chemicals were of analytical grade. Rotenone was added in a minimal volume of medium salts.

**RESULTS**

**Mitochondrial Swelling—** Aerobic rat liver mitochondria with succinate as substrate will swell in an iso-osmotic KCl suspension medium containing Ca^{2+} and the Na+ or K+ salt of a permeant acid such as phosphoric or 3-hydroxybutyric (e.g. Ref. 24) owing to entry of osmotically active ions and subsequent water movements. Swelling was therefore used to demonstrate the energy-dependence of uptake of Ca^{2+} and 3-hydroxybutyrate. Fig. 1 shows that mitochondria were able to swell in a medium containing Ca^{2+} and 3-hydroxybutyrate in the presence of rotenone only when succinate was added as a source of reducing equivalents (Fig. 1B). No swelling was observed in the absence of 3-hydroxybutyrate (Fig. 1C) or Ca^{2+} (Fig. 1A). Oxidation of 3-hydroxybutyrate was able to support swelling; however, this was abolished by subsequent addition of rotenone (Fig. 1D). Swelling was then restimulated by succinate, and once again prevented by antimycin A, showing the energy-dependent nature of swelling in the presence of Ca^{2+} and 3-hydroxybutyrate.

**Uptake of Ca^{2+} and 3-Hydroxybutyrate—** Both Ca^{2+} and 3-hydroxybutyrate were accumulated by the mitochondria under these conditions; this is shown in Fig. 2. Succinate was present as a source of reducing equivalents while oxidation and subsequent metabolism of the radiolabeled 3-hydroxybutyrate were prevented by rotenone. The reaction was initiated by addition of mitochondria from a stock suspension at 50 mg of protein/ml of sucrose and terminated by centrifugation to prevent ion movements. This took about 20 s; the time scale in the figures has been adjusted accordingly. Initiation of the reaction by Ca^{2+} addition did not alter the effects observed. Uptake was measured by accumulation of radioactive label. It was corrected for the extra-matrix material in the pellet by determination of the sucrose-accessible space as described under “Experimental Procedure.”

In the absence of Ca^{2+} only a small amount (<50 nmol/mg of protein) of 3-hydroxybutyrate was taken up by the mitochondria. Similarly, Ca^{2+} uptake in the absence of 3 hydroxybutyr
3-hydroxybutyric acid) and (b) the addition of a permeant acid from the data in Fig. 3, indicating that for each Ca$^{2+}$ ion taken decrease in the presence of a large excess of the competing assumptions. It also allowed calculation of a best line through the points by orthogonal regression, with a slope equal to the stoichiometry of the mitochondrial matrix. Uptake of $[2, ^{3}C]$succinate was measured under these conditions and was found to be less than 27 nmol/mg of protein; the extra succinate uptake in the presence of Ca$^{2+}$ was less than 10 nmol/mg. These amounts were considered to be negligible.

**Stoichiometry of Uptake: the 3-Hydroxybutyrate/Ca$^{2+}$ Ratio**

It is apparent from Fig. 2 that the time course of uptake was similar for Ca$^{2+}$ and 3-hydroxybutyrate and that the ratio of acid to Ca$^{2+}$ accumulated was about 2. In order to establish this relationship more exactly, the amount of 3-hydroxybutyrate taken up was plotted as a function of Ca$^{2+}$ uptake for time intervals varying from 0.3 to 10 min; this is presented in Fig. 3. This method of calculating the stoichiometry was chosen as it allowed correction for early, nonenergized binding of ions while avoiding a number of complicating assumptions. It also allowed calculation of a best line through the points by orthogonal regression, with a slope equal to the ratio of nanomoles of 3-hydroxybutyrate accumulated to nanomoles of Ca$^{2+}$ accumulated.

Extra 3-hydroxybutyrate uptake was calculated by subtracting the small amount bound in the absence of Ca$^{2+}$ from that bound in the presence of Ca$^{2+}$ (Fig. 2, Line A minus Line D). Uptake of Ca$^{2+}$ was not corrected in a similar way as: (a) Ca$^{2+}$ uptake in the absence of 3-hydroxybutyrate was partially due to endogenous phosphate (such uptake would be expected to decrease in the presence of a large excess of the competing 3-hydroxybutyric acid) and (b) the addition of a permeant acid may allow membrane-bound Ca$^{2+}$ to enter the mitochondrial matrix (15, 20, 32-34) and invalidate the correction.

A slope of 2.04 with standard deviation 0.13 was calculated from the data in Fig. 3, indicating that for each Ca$^{2+}$ ion taken up 2 molecules of 3-hydroxybutyrate were accumulated under these conditions. The intercept on the "Ca$^{2+}$ bound" axis was 50 nmol/mg of protein; small variations in this value between mitochondrial preparations caused some of the scatter of points seen in Fig. 3.

**Ca$^{2+}$/- and 3-Hydroxybutyrate/- Ratios—During uptake of Ca$^{2+}$ in the presence of phosphate, Ca$^{2+}$/- ratios of 1.7 to 2.0 have been reported (1-5). In order to determine whether such a relationship also held during Ca$^{2+}$ uptake supported by 3-hydroxybutyrate we measured oxygen uptake in parallel experiments to those reported above, except that the reaction was initiated with Ca$^{2+}$ added to the system 30 s after the mitochondria. Under these conditions of excess Ca$^{2+}$, oxygen utilization was stimulated and proceeded at a rapid rate until all the oxygen in the vessel was exhausted. The oxygen consumed was determined at each time point for which Ca$^{2+}$ and 3-hydroxybutyrate were measured by subtracting the amount of oxygen consumed in a parallel experiment with no added Ca$^{2+}$, giving a value of "extra oxygen consumed." "Extra 3-hydroxybutyrate uptake" was calculated as described above; "extra Ca$^{2+}$ uptake" was calculated by subtracting the value of 50 nmol/mg of protein obtained from Fig. 3 from each value of Ca$^{2+}$ uptake. This value may not be very precise, due to variations between different mitochondrial preparations and to the small contribution of oxygen-utilizing Ca$^{2+}$ uptake dependent on endogenous phosphate movements (see below). However, its magnitude was not critical since variation by 20% in either direction would alter the calculated Ca$^{2+}$/- ratio only by about 0.2.

The extra uptake of Ca$^{2+}$ and 3-hydroxybutyrate plotted against extra uptake of oxygen is presented in Fig. 4. The slopes of the lines plotted by the method of least squares and forced through the origin were calculated to be 3.8 for Ca$^{2+}$ and 8.2 for 3-hydroxybutyrate. Since succinate was the source of reducing equivalents, these values correspond to a Ca$^{2+}$/- ratio of 1.9 and an associated 3-hydroxybutyrate/- ratio of 4.1. The extra oxygen uptake was also determined in the presence of 3-hydroxybutyrate and limiting amounts of Ca$^{2+}$, in which case oxygen consumption returned to the basal rate before it.
was exhausted. Calculation of $\frac{Ca^{2+}/\sim}{3$-hydroxybutyrate} ratios under these conditions also yielded a value close to 2.0.

**Concentration Dependence of 3-Hydroxybutyrate/Ca$^{2+}$ Ratio**—In order to assess whether 3-hydroxybutyrate could support loads of $Ca^{2+}$ approaching those seen in the presence of phosphate and whether the stoichiometry of acid to $Ca^{2+}$ was an invariant feature of extensive uptake of $Ca^{2+}$ in the presence of 3-hydroxybutyrate, we investigated the dependence of the uptake on $Ca^{2+}$ and acid concentration. Fig. 5 shows that, with a 10-min incubation, optimum uptake of both $Ca^{2+}$ and 3-hydroxybutyrate was seen at about 300 to 400 nmol of $Ca^{2+}$/mg of protein; virtually all the $Ca^{2+}$ was accumulated at $Ca^{2+}$ concentrations of less than 200 nmol/mg of protein.

Fig. 6 shows a similar experiment in which 3-hydroxybutyrate concentration was varied; greatest uptake was observed between 15 and 30 mM acid. Also shown in Fig. 6 is the dependence of 3-hydroxybutyrate uptake on concentration in the absence of $Ca^{2+}$; the fit of the points to the calculated line, assuming 3-hydroxybutyrate diffuses into and equilibrates with a matrix volume of 1 μl/mg of protein (see “Experimental Procedure”), is fairly good. Unlike the $Ca^{2+}$ case, the uptake of 3-hydroxybutyrate did not approach completion at limiting acid concentrations. Thus at 5 mM 3-hydroxybutyrate (2000 nmol/mg of protein), only 100 nmol of 3-hydroxybutyrate/mg of protein were accumulated. Assuming a matrix volume of 1 μl/mg of protein, this corresponded to a 3-hydroxybutyrate gradient of 20:1 with the higher concentration within the mitochondria. A similar gradient was calculated for each point at which 3-hydroxybutyrate was limiting, and may be explained by the limitation on the magnitude of the proton gradient which supported the 3-hydroxybutyrate uptake. Both Figs. 5 and 6 also show that accumulation of $Ca^{2+}$ and 3-hydroxybutyrate had a similar dependence on concentration; the decrease in steady state uptake at higher concentrations was attributed to membrane damage as discussed above.

The stoichiometry of the accumulation under conditions of limiting amounts of $Ca^{2+}$ and 3-hydroxybutyrate is shown in Fig. 7, A and B respectively. In both cases a slope of 1.7 was found. This is slightly lower than the value of 2.0 found in Fig. 3 and may be explained for $Ca^{2+}$ by the decrease in nonenergized binding at low $Ca^{2+}$ concentrations, with a corresponding tendency for the intercept to decrease when $Ca^{2+}$ is limiting.

Brierley et al. (35) have shown that under some conditions respiration-dependent uptake of acetate and $K^+$ may occur in the absence of other cation movements. Had a similar effect existed with 3-hydroxybutyrate in the present work the 3-hydroxybutyrate/$Ca^{2+}$ ratio would have been overestimated due to the replacement of $Ca^{2+}$ by $K^+$. Reducing the $Ca^{2+}$ concentration should therefore have increased the proportion of the acid uptake dependent on $K^+$ and raised the 3-hydroxybutyrate/$Ca^{2+}$ ratio. Fig. 7 shows that this did not occur; $K^+$-dependent movements were therefore unimportant under our experimental conditions.

**Movement of Endogenous Acids**—The cause of the $Ca^{2+}$ uptake not associated with accumulation of 3-hydroxybutyrate seen as the intercept in Fig. 3 and Fig. 7 was investigated by preventing movement of endogenous phosphate by a 2-min preincubation of the mitochondria with N-ethylmaleimide at 20 nmol/mg of protein. The experimental conditions were identical with those described in Fig. 2 except that 3-hydroxybutyrate was not added. $Ca^{2+}$ uptake at zero time of
incubation (i.e., after about 20 s, before centrifugation prevented further uptake) was 80.7 ± 11.7 (S.D.) nmol/mg of protein in the control, whereas with preincubation with N-ethylmaleimide this was reduced by over 50% to 37.0 ± 4.2 (S.D.) nmol/mg of protein. Endogenous phosphate was therefore present at high enough activity to cause significant Ca²⁺ uptake in the absence of 3-hydroxybutyrate. Although the uptake of phosphate in the presence of 20 mM 3-hydroxybutyrate would be greatly reduced it may nonetheless have made some contribution to the 3-hydroxybutyrate-independent uptake. Other factors involved may have been nonspecific binding of Ca²⁺ to the mitochondrial membranes and also the presence of dissolved CO₂ acting as a permeant acid (26).

**Ca²⁺ Uptake Supported by Other Monocarboxylic Acids**

Since 3-hydroxybutyrate is oxidized in the absence of rotenone by rat liver mitochondria, the possibility remained that the effects observed were due to some specific interaction of 3-hydroxybutyrate with the mitochondria and not merely to its property of being a weakly acidic particle. For this reason a number of other monocarboxylic acids were tested for their ability to support Ca²⁺ accumulation, and the stoichiometry of the uptake was measured in the same way as before. The results of such experiments are presented in Table 1.

**Acetate, Propionate, and Butyrate mimicked the effects of 3-hydroxybutyrate with respect to the time course, extent, and stoichiometry of accumulation.** 4-Hydroxybutyrate supported a much slower rate of uptake, which was incomplete at 8 min, and yielded a lower acid/Ca²⁺ ratio, presumably because endogenous ions were able to compete more effectively at lower rates of uptake. Glycolate was not taken up and did not support uptake of Ca²⁺, presumably because it is highly polar and does not pass the membrane. Phenylacetate supported only low levels of Ca²⁺ accumulation, although uptake was maximal at the first time point (i.e., after 20 s).

The extent of proton ejection caused by addition of Ca²⁺ to the medium 30 s after addition of mitochondria was measured and found to be small compared to the uptake of Ca²⁺ and the various acids, being about 80 ng ions of H⁺/mg of protein in the presence of 3-hydroxybutyrate compared to about 120 ng ions/mg of protein in the absence of a permeant acid. The table shows the ability of the acids tested to eliminate net accumulation of protons in the medium by transporting them into the matrix.

These acids which supported Ca²⁺ uptake and were themselves readily taken up by the mitochondria (acetate, propionate, butyrate, 3-hydroxybutyrate) were most efficient at reducing proton production. 4-Hydroxybutyrate only partially eliminated acidification of the medium, consistent with its entry being restricted; glycolate could neither support uptake of Ca²⁺ nor reduce proton ejection, consistent with an inability to penetrate the mitochondrial membrane together with a proton. Phenylacetate reduced proton production but did not support high levels of Ca²⁺ uptake, owing to its uncoupling action at this high concentration. The table also shows the pK' values for the acids; entry and support of Ca²⁺ uptake were not obviously related to pK'; other factors, particularly lipid solubility, presumably determine the differences between isomers such as 3- and 4-hydroxybutyrate. It should be noted that the acid/Ca²⁺ ratio was the same for both the sodium salt (3-hydroxybutyrate) and the potassium salts (other acids), showing that the effects observed were independent of the accompanying monovalent cation.

**Mitochondria from Malignant Cells**—Mitochondria isolated from some malignant cells are able to accumulate Ca²⁺ and phosphate with particularly pronounced capacity to retain high loads (36-38). Uptake of Ca²⁺ and 3-hydroxybutyrate by mitochondria isolated from AS-30D ascites cells was investigated in experiments similar to those of Fig. 2 and Fig. 3. Ca²⁺ and 3-hydroxybutyrate uptake reached levels similar to those seen above, and had a similar stoichiometry and time course.

**DISCUSSION**

Lipophiplic monocarboxylic acids such as acetate acid enter mitochondria readily by simple diffusion as the undisassociated
acid (13, 24, 28-30, 39, 40). Accumulation of the anion may therefore be used as a quantitative measure of proton entry in the absence of other agents causing proton uptake since, for each acetate anion translocated into the mitochondria, 1 proton must also move inward. The advantages of using permeant monocarboxylic acid uptake as a measure of \( H^+ \)-entry (and thus of the preceding \( H^+ \) ejection) during \( Ca^{2+} \) accumulation by mitochondria rather than using phosphate or bicarbonate may be summarized as follows.

1. Both \( Ca^{2+} \) and carboxylic acid accumulate to extents approaching those seen with phosphate under conditions of “massive loading” (1), allowing accurate determination of the amounts taken up and diminishing the importance of other events such as nonenergized binding to membranes.

2. Since these acids have only one dissociable group at the \( pH \) used the ambiguity over the ionic species present in solution within the mitochondria is reduced to a minimum.

3. Net penetration by the dissociated anion is probably negligible relative to net penetration by the acid.

4. Unlike calcium phosphate or carbonate, the \( Ca^{2+} \) salts of monocarboxylic acids are highly water-soluble, allowing extensive uptake in the absence of precipitation and concomitant proton release.

5. Metabolism of these acids by mitochondria in the presence of rotenone is negligible.

The results presented here show that \( Ca^{2+} \) and permeant acids were accumulated by mitochondria with an acid/\( Ca^{2+} \) ratio of close to 2.0. This stoichiometry was maintained over a wide range of concentrations of both \( Ca^{2+} \) and 3-hydroxybutyrate and was independent of the permeant acid used. Simultaneous measurements of oxygen uptake showed that the usual \( Ca^{2+}/\text{O}_{2} \) ratio of 2.0 (1-5) held under the conditions employed and that the 3-hydroxybutyrate/\( \text{O}_{2} \) ratio was thus close to 4.0. This is equivalent to an \( H^+/\text{Ca}^{2+} \) ratio of 4.0 during \( Ca^{2+} \)-stimulated electron transport.

The actual \( H^+ /Ca^{2+} \) ratio calculated by dividing the protons which appeared in the medium by the \( Ca^{2+} \) ions accumulated after 10 min was about 0.25, in agreement with earlier results (15, 16, 32). However, the observed \( H^+ \)-production was a rather rapid, early event, nonsynchronous with the uptake of \( Ca^{2+} \) and 3-hydroxybutyrate. Thus, correction of the data used to plot Fig. 3 would have affected the slope only close to the origin and would not have altered the stoichiometry obtained over the rest of the range.

A value of 0.8 for the acid/\( Ca^{2+} \) ratio has been reported by Rasmussen and colleagues (15) by simply dividing total acetate accumulated by total \( Ca^{2+} \) accumulated. However, they did not correct their data for extra-matrix acid and \( Ca^{2+} \) in the mitochondrial pellets and for anion bound in the absence of \( Ca^{2+} \). In particular, the method of calculating the stoichiometry from the slope of the plot of anion bound against \( Ca^{2+} \) bound used here allowed correction for early events such as nonspecific \( Ca^{2+} \) binding without requiring assumptions as to their extent.

Our finding that the \( H^+ /Ca^{2+} \) ratio is 2.0 when permeant monocarboxylic acids are the anion source must be regarded as a more valid and reliable measurement than the rather variable data obtained in the presence of phosphate (1, 16, 17) since the approach described here is independent of the uncertainties regarding the actual ionic species of phosphate accumulated with \( Ca^{2+} \) as well as secondary proton exchanges accompanying precipitation of insoluble calcium phosphate salt(s).

The available evidence indicates that \( Ca^{2+} \) enters nonrespiring mitochondria by electrophoretic unipor (24, 41). Thus nonrespiring mitochondria swell rapidly in the absence of uncouplers when suspended in iso-osmotic solutions of the calcium salts of permeant anions such as thiocyanate (41) or nitrate (24); moreover, \( Ca^{2+} \) under some conditions appears to follow the distribution predicted for a two-charge translocation (42). If \( Ca^{2+} \) passes through the membrane of respiring mitochondria by the same electrophoretic unipor mechanism, it follows that the 4 protons ejected/site during \( Ca^{2+} \) accumulation must have originated from an event directly associated with electron transport. This interpretation suggests that the primary \( H^+/\text{Ca}^{2+} \) ratio of electron transport is actually 4.0, rather than the value \( H^+/\text{Ca}^{2+} \approx 2 \) obtained from measurements of \( pH \) changes during oxygen pulse experiments (43, 44) or ATP hydrolysis (45-47), which in some cases are accompanied by \( Ca^{2+} \) movements (48, 49). Recent observations on photosynthetic systems indicate that the number of protons moved per energy-conserving site in chloroplasts definitely exceeds 2 and may be 3 or higher (50-52).

An alternative explanation for the \( H^+/\text{Ca}^{2+} \) ratio of 4.0 is that under the aerobic conditions respiration-energized \( Ca^{2+} \) uptake may proceed by electrogenic \( Ca^{2+} -H^+ \) antipor (53), rather than by the electrogenic unipor of \( Ca^{2+} \) known to take place anaerobically (24, 41). For the electrogenic \( Ca^{2+} -H^+ \) antipor model to accommodate the data reported here it must be assumed that electron transport yields 2.0 \( H^+ \) per energy-conserving site and that 2.0 \( Ca^{2+} \) enter in exchange for 2 \( H^+ \) from some other source, leading to electroneutrality and an overall \( H^+/\text{Ca}^{2+} \) ratio of 4.0. This explanation can only be correct if the mechanism of \( Ca^{2+} \) transport across the membrane is fundamentally different under aerobic and anaerobic conditions. Because of the important implications of these considerations with regard to the chemiosmotic coupling hypothesis, these questions are under further investigation.

Note Added in Proof—Since this paper was submitted the major energy source for superstoichiometric \( Ca^{2+} \) uptake and \( H^+ \) ejection has been identified as intramitochondrial ATP (54). Although such superstoichiometric ATP-dependent ion movements are negligible in comparison with the rather large magnitude of the \( Ca^{2+} \) and anion uptakes described in this paper, other experiments employing oligomycin to eliminate ATP-dependent \( Ca^{2+} \) and \( H^+ \) movements in studies of the \( H^+/\text{Ca}^{2+} \) ratio are described elsewhere (55).

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