THE EFFECT OF SALT CONCENTRATION AND pH ON THE RELATIONSHIP BETWEEN H^+ EJECTION AND ELECTRON TRANSPORT IN ISOLATED MITOCHONDRIA

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In an earlier communication we reported that increasing the concentration of certain salts such as NaCl or increasing the pH of the suspending medium increased the Ca^{++}:O accumulation ratio during Ca^{++}-induced respiratory jumps in rat liver mitochondria, from the normal value of about 4.0 for succinate (Rossi and Lehninger, 1964) to values exceeding 8-10 (Carafoli, Gamble, and Lehninger, 1965). These findings raise some fundamental questions as to whether the relationship between ion accumulation and electron transport is fixed and stoichiometric, as usually thought, or whether it is dependent, at least in part, on the magnitude of ion gradients across the mitochondrial membrane.

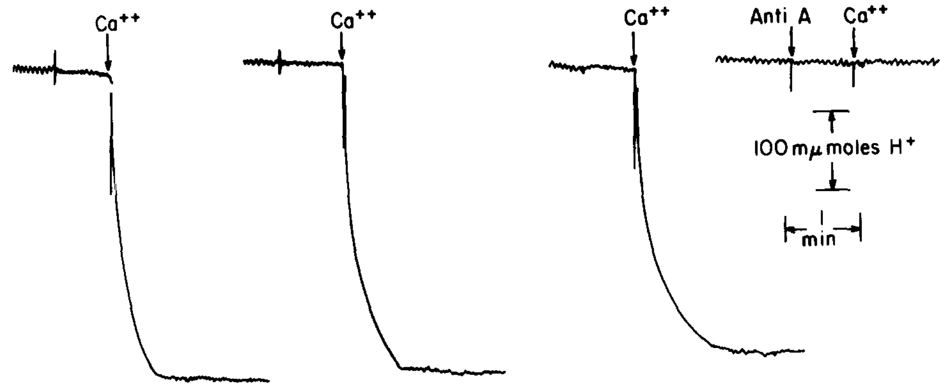
This communication reports on the effect of salt concentration and pH of the medium on the quantitative relationships between H^+ ion ejection and electron transport during Ca^{++}-induced respiratory jumps. Several investigators have reported that approximately 1.0 H^+ ion is ejected for each Ca^{++} ion accumulated in such jump experiments, thus yielding under "normal" conditions, the ejection (per pair of electrons) of 2 H^+ per "site" or 6 H^+ for the full length of the respiratory chain (cf. Saris, 1963; Engstrom and DeLuca, 1964; Chappell and Crofts, 1965a, b; Chance, 1965).

We wish to report that increasing the NaCl concentration in the suspending medium over the range 80-240 mM or increasing its pH from 7.0 to 8.5 causes no change in the molar ratio H^+ ejected : Ca^{++}

accumulated, but does cause a large increase in the H^+:O ejection ratio from "normal" values of about 2.0 per "site" to values of 5 per "site" or sometimes even higher.

Experimental details. Unaccumulated $^{45}Ca^{++}$ remaining in the suspending medium was measured following rapid filtration of the mitochondrial suspensions through a Millipore filter (AAW, 0.8 μ) or rapid centrifugation in a Beckman-Spinco Microfuge (Carafoli, Gamble, and Lehninger, 1966). Ejection of H^+ was followed with a glass electrode and Beckman Expandomatic pH meter equipped with a Sargent SR recorder; the absolute amounts ejected were determined by calibration with internal standards of HCl. Oxygen uptake was determined with the Clark electrode.

Results. Addition of $^{45}CaCl_2$ to a system containing succinate, Tris chloride buffer pH 7.4, rat liver mitochondria, and concentrations of NaCl increasing from 80 to 240 mM produced stimulation of respiration, accumulation of Ca^{++}, and ejection of H^+. Ca^{++} accumulation was measured 30-60 sec after return of the respiration to the original State 4 or resting rate (Table 1). Under these conditions increasing the salt concentration caused progressive decreases in the extra oxygen uptake evoked by addition of a fixed amount of Ca^{++}, with a resulting increase in the actual Ca^{++}: extra O accumulation ratio as reported earlier (Carafoli, Gamble, and Lehninger, 1965). As is seen in Fig. 1, increasing the NaCl concentration in the medium caused no significant change in the molar ratio H^+ ejected : Ca^{++} accumulated, which remained fairly constant at about 1.10 from 80 to 240 mM NaCl. However, the data in Fig. 1 and Table 1 show that the molar ratio H^+ ejected : extra oxygen consumed rose from 4.0 to 9.25 (succinate) as NaCl was increased from 80 to 240 mM.



mM NaCl :	80	160	240	240
$H^+:Ca^{++}$:	1.08	1.10	1.09	-
$H^+:O^{--}$:	4.00	5.70	9.25	-
$Ca^{++}:O^{--}$:	3.70	5.08	8.50	-

Fig. 1. H^+ ejection curves for Exp. 1, Table 1. Antimycin A was added at 0.25 μg per mg protein where shown in trace at right.

Table 1

Effect of NaCl concentration and pH on Ca^{++} uptake and H^+ ejection

The systems (2.0 ml) contained rat liver mitochondria (5.0 mg protein), 10 mM sodium succinate, 10 mM Tris chloride at pH shown, and sodium chloride at concentration shown. The respiratory jump was initiated by additions of 400 mμmoles $^{45}CaCl_2$. The Ca^{++} accumulation and H^+ ejection data were obtained 60 seconds after completion of the jump. Temperature = 25°.

pH	NaCl mM	Ca^{++} uptake mμmoles	Extra oxygen uptake mμatoms	$Ca^{++}:O$	H^+ ejection mμmoles	$H^+:Ca^{++}$	$H^+:O$
7.4	80	370	100	3.70	400	1.08	4.00
7.4	160	355	70	5.08	400	1.10	5.70
7.4	240	340	40	8.50	370	1.09	9.25
7.0	80	385	110	3.50	380	0.99	3.45
7.4	80	382	100	3.82	360	0.94	3.60
8.2	80	360	72	5.00	425	1.18	5.90
8.6	80	268	36	7.20	350	1.30	9.80

Data in Table 1 also show the effect of keeping NaCl concentration constant at 80 mM and raising the pH from 7.0 to 8.2. It is seen that the ratio H^+ ejected :Ca^{++} accumulated remained in the range 0.94 - 1.3, but the

Ca^{++}:O accumulation ratio and the H^{+}:O ejection ratio rose sharply in a parallel manner, the latter from values of about 3.5 to about 10.0 as pH was increased. The ejection of H^{+} and accumulation of Ca^{++} at high salt concentrations or high pH are completely prevented by uncoupling agents or respiratory inhibitors such as antimycin A (Fig. 1); the "super-stoichiometry" is therefore not a respiration-independent artifact.

The Ca^{++}:O ratios reported here are measured accumulation ratios, not the respiratory activation ratios. At pH 7.4 and 80 mM salt over 99% of added Ca^{++} is accumulated, and the Ca^{++}:O accumulation and activation ratios are essentially identical. This is not always the case at high salt concentrations or high pH; under the latter conditions a significantly smaller fraction of the added Ca^{++} may actually be accumulated. Conclusions on the stoichiometry between Ca^{++} uptake and H^{+} ejection based on activation ratios alone can be in serious error.

Discussion. The data reported here indicate that the H^{+}:2e ejection ratio during electron transport activated by Ca^{++} may be much higher than the usually accepted value of 2.0 per "site" or 6 H^{+} for the entire chain when the salt concentration or pH of the medium is increased. Unambiguous values as high as 15 per chain have been observed. Since present knowledge of the mechanism of electron transport does not appear to allow so large a number of proton exchanges per pair of electrons, some alternative explanations must be considered. One point appears significant: the molar ratio H^{+} ejected :Ca^{++} accumulated did not change appreciably with salt concentration or pH; it remained rather constant at about 1.0 - 1.2. Actually, as long as permeant anions such as acetate or phosphate are not present, the H^{+}:Ca^{++} ratio appears to remain constant at about 1.0 - 1.2 under all circumstances, either during Ca^{++} uptake or efflux (cf. Drahota, Carafoli, Rossi, Gamble, and Lehninger, 1965). Earlier observations showed that when phosphate or other permeant anions such as acetate are added, the Ca^{++}:2e accumulation ratio remains fixed at about 2.0 per site regardless of salt concentration or pH (Carafoli, Gamble, Lehninger, 1965). We have also found that the H^+:2e ejection ratio in the presence of phosphate or acetate also remains at values of 2.0 per "site" or less, regardless of salt concentration or pH. The "super-stoichiometry" of Ca^{++} uptake and of H^+ ejection is thus apparent only in the absence of the permeant anions phosphate or acetate.

The common denominator in the high salt or high pH media that produce the "super-stoichiometry" is the presence of increasing concentrations of relatively impermeant anions, i.e. Cl^-, or OH^-. High gradients of these across the mitochondrial membrane may in some manner poise the membrane electrochemically, possibly by a Donnan effect, so that it gives the super-stoichiometry effect. On the other hand, the presence of permeant anions such as phosphate would not permit such a high gradient of negative ions to develop across the membrane and could be expected to yield a different electrochemical balance.

Whatever the explanation, these findings are immediately relevant to the mechanism of respiration-dependent mitochondrial ion transport and to the question whether this is an exactly stoichiometric process, as would be expected in a strictly chemical coupling mechanism, or a "flexible" process dependent on ion gradients, as could be expected in chemi-osmotic energy coupling (Mitchell, 1961, 1962).

Full details of these and related experiments will be reported elsewhere.

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