THE RELATIONSHIP OF THE CA^{++} EFFLUX RATE TO THE "SUPER-STOICHIOMETRY" OF RESPIRATION-LINKED CA^{++} ACCUMULATION BY MITOCHONDRIA

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In an earlier communication (Carafoli, Gamble and Lehninger, 1965 a) it was shown that the number of molecules of Ca^{++} accumulated by rat liver mitochondria per pair of electrons passing from succinate to oxygen following a respiratory jump induced by Ca^{++}, i.e. the Ca^{++}:O accumulation ratio, earlier regarded as constant at about 3.6 - 4.0 (Rossi and Lehninger, 1964; Chance, 1963, 1965; Chappell, Cohn and Greville, 1963), may increase to values of 8 - 10, or even higher, under two sets of conditions: (a) when the concentration of certain neutral salts in the medium, such as NaCl, is increased and (b) when the pH of the medium is increased. These two conditions also produce an increase in the molar ratio H$^+$ ejected:oxygen taken up, from the usually observed value of about 4.0 for succinate to 8.0 or higher (Carafoli, Gamble and Lehninger, 1965). The common denominator in these two sets of conditions producing such "super-stoichiometry" is the increase in the concentration of anions that are impermeant through the mitochondrial membrane: Cl$^-$ and OH$^-$ respectively. In the presence of permeant anions such as phosphate or acetate, the Ca^{++}:O ratio remains constant at the usual value of about 4.0 (succinate), regardless of NaCl concentration or pH.

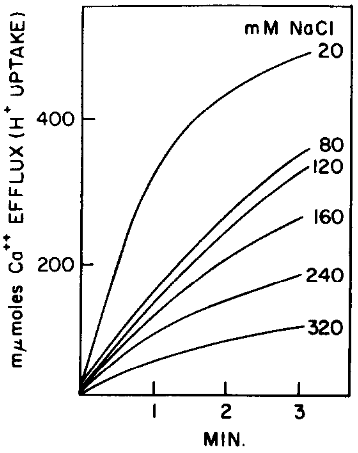
At least two types of explanation for the "super-stoichiometry" of Ca^{++} accumulation and H$^+$ ejection are possible. The first is that the Ca^{++}:O ratio is normally a fixed integral stoichiometric quantity under all conditions, but one having an unexpectedly high value that may exceed 8.0 for succinate, and that the variability in the observed Ca^{++}:O ratios with Cl^- concentration or pH is due to variations produced in the rates of leakage of Ca^{++} from the mitochondria before or during the steady-state following the respiratory jump (cf. Drahota, Carafoli, Rossi, Gamble and Lehninger, 1965). In this steady-state there is usually no net movement of the accumulated Ca^{++}; however, the Ca^{++} is not irreversibly sequestered, but undergoes constant efflux to the medium counterbalanced by active uptake coupled to the "resting" respiration (Carafoli, Rossi and Lehninger, 1965).

The second alternative is that the Ca^{++}:O accumulation ratio is non-stoichiometric, i.e., a "flexible" quantity that may vary widely, and that the Ca^{++} efflux rate is essentially invariant. It is also possible that both the intrinsic Ca^{++}:O accumulation ratio and the Ca^{++} efflux rate may undergo changes with salt concentration and pH of the medium. These alternatives are immediately relevant to the mechanism of mitochondrial ion transport processes, as well as to the concept of chemi-osmotic energy coupling (cf. Mitchell, 1961, 1965).

This communication reports experiments demonstrating that the rate of efflux of accumulated Ca^{++} from liver mitochondria is greatly diminished by an increase in Cl^- concentration and by increasing pH. These changes in the efflux rate can in large part account for the effects of Cl^- concentration and pH on the observed Ca^{++}:O accumulation ratios.

Experimental. Rat liver mitochondria were loaded with $^{45}Ca^{++}$ in media containing varying concentrations of KCl or NaCl, 10 mM Tris chloride adjusted to the desired pH, and 10 mM sodium succinate, by addition of 80 mμmoles $^{45}CaCl_2$ per mg protein to initiate the respiratory jump. Thirty seconds after the end of the jump, antimycin A was added in an amount just sufficient to block respiration, allowing the efflux of Ca^{++} to be measured unopposed by respiration-linked accumulation. Appearance of $^{45}Ca^{++}$ in the suspending medium was followed by the Millipore filtration method, or by following the uptake of H^+ from the medium by means of a recording glass electrode system with scale expansion, since the Ca^{++} and H^+ movements were found to be exactly reciprocal under these conditions (Carafoli, Gamble, and Lehninger, 1965a, b, 1966). Alternatively, Ca^{++} efflux was measured following harvesting of Ca^{++}-loaded mitochondria and resuspension in a fresh medium not containing antimycin A or substrate, in which KCl and pH were varied.

As is seen in Fig. 1, increasing the concentration of KCl in the medium from 20 mM to 320 mM caused a progressive decrease in the rate of Ca^{++} efflux from previously loaded mitochondria, so that at 240 mM the initial rate of efflux was less than half of that observed at 80 mM KCl. Since the observed Ca^{++}:O ratio (succinate) at 80 mM salt is about 4.0 (Rossi and Lehninger, 1965) and at 240 mM about 8 - 10 (Carafoli, Gamble, and Lehninger, 1965), i.e., a 2-fold increase, the 50% inhibition of Ca^{++} efflux in the 240 mM KCl medium could approximately account for the increase in observed net Ca^{++}:O ratio with KCl concentration, if it is assumed that the intrinsic Ca^{++}:O accumulation stoichiometry is constant at about 8.0 at all salt concentrations.



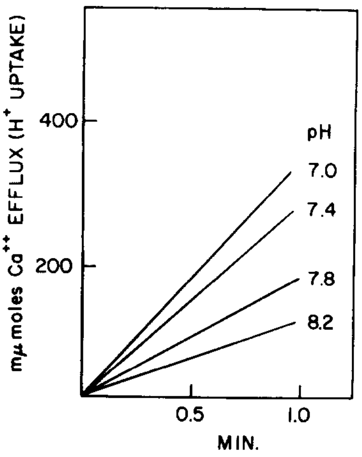


Fig. 1. (Left) To 2.0 ml system containing rat liver mitochondria (5.0 mg), 10 mM sodium succinate, 10 mM tris HCl (pH 7.4), and KCl at concentrations shown, 500 mμmoles $CaCl_2$ were added. Thirty seconds after end of respiratory jump, 1.25 μg antimycin A was added and H^+ uptake corresponding to Ca^{++} efflux measured with recording expanded scale pH meter. T = 25°.

Fig. 2 (Right) Details as in Fig. 1, but with KCl fixed at 80 mM and pH varied as shown.

As is seen in Fig. 2, increasing the pH of the medium over the range from 7.0 to 8.2, with KCl held constant at 80 mM, decreased the efflux rate to about 30% of the maximum. This effect may be compared with the earlier finding that the observed Ca^{++}:O ratio (succinate) increased from a value of about 4.0 at pH 7.4 to a value of over 10 at pH 8.5 (Carafoli, Gamble, and Lehninger, 1965).

Discussion. The finding that increasing salt concentration and increasing pH of the medium decrease the rate of efflux of Ca^{++} from loaded mitochondria more clearly defines the dynamic aspects of the "super-stoichiometry" of respiration-linked Ca^{++} accumulation. Taken at face value this finding is consistent with the view that the intrinsic Ca^{++}:O accumulation and H^{+}:O ejection ratios have fixed stoichiometry, but with unexpectedly high values of 8.0 or higher (succinate). The usually observed values of about 4.0 at 80 mM KCl and pH 7.4 could be the resultant of an intrinsic accumulation ratio of 8.0, but a significantly high efflux rate to yield a net value of 4.0. Any "flexibility" in observed Ca^{++}:O and H^{+}:O ratios could then be a reflection of alterations of the Ca^{++} efflux rate.

Although this finding clarifies the dynamics of the "super-stoichiometry" observed in the presence of high salt concentration or high pH, it still does not rationalize satisfactorily the uncomfortably high Ca^{++}:O accumulation and the H^{+}:O ejection ratios observed in these conditions, either on the basis of the traditional chemical coupling hypothesis (cf. Rossi and Lehninger, 1963, 1964; Chance, 1965) or on the basis of the chemi-osmotic coupling hypothesis (Mitchell 1961, 1965). A stoichiometric interaction of 4 or more molecules of Ca^{++} with a single high-energy bond seems unlikely, in the case of chemical coupling hypothesis. In the case of chemi-osmotic coupling, it must be assumed that as many as 4 - 5 H^{+} could be ejected per site, or up to 15 H^{+} as each pair of electrons travels from NADH to oxygen, a stoichiometry that existing knowledge of the respiratory chain does not appear to allow. It appears possible that some of the Ca^{++} accumulated and H^{+} ejected under these conditions occurs without concomitant electron transport. High NaCl and high pH might favor exchange of added Ca^{++} with preexisting protons bound to fixed charged groups in the mitochondrion, such as those of phospholipids or of structure protein. Against this is the fact that respiratory inhibitors such as cyanide or antimycin A inhibit all Ca^{++} accumulation and H^{+} ejection in systems showing "super-stoichiometry" (Carafoli, Gamble, and Lehninger, 1965). The kinetics of Ca^{++} efflux (Fig. 1 and 2) suggests that some of the accumulated Ca^{++} may undergo a transition in the presence of high KCl or high pH to a stabler form that may not leave the mitochondria readily. These facts, plus the effect of permeant anions such as phosphate or acetate in fixing the Ca^{++}:O stoichiometry at 4.0 (succinate) (Carafoli, Gamble, and Lehninger, 1965), must represent the most pertinent keys to establishing the true intrinsic stoichiometry between Ca^{++} accumulation, H^+ ejection, and electron transport in mitochondria. Details of these and related experiments will be published elsewhere.

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