ON THE MAXIMUM STOICHIOMETRY OF ENERGY-LINKED CA^{++} ACCUMULATION DURING ELECTRON TRANSPORT IN RAT LIVER MITOCHONDRIA

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Several recent investigations have shown that approximately 1.7 - 2.0 molecules of divalent cation (Ca^{++}, Sr^{++}, or Mn^{++}) may be accumulated by isolated mitochondria coupled to the passage of a pair of electrons through each energy-conserving site of the respiratory chain (Rossi and Lehninger, 1963, 1964; Chappell, Cohn, and Greville, 1963; Carafoli, Weiland, and Lehninger, 1965; Carafoli, 1965; Chance, 1963, 1965). Most reported measurements of the $Ca^{++}:\sim$ stoichiometry have been carried out with inorganic phosphate in the medium; under such conditions phosphate is also accumulated. However, limited amounts of divalent cations may also be accumulated in the absence of phosphate, with apparently similar stoichiometry with oxygen uptake (Rossi and Lehninger, 1964; Chappell <u>et al</u>., 1963; Carafoli, 1965).

This communication describes two sets of conditions under which $Ca^{++}:\sim$ accumulation ratios over twice as high as those previously reported may be observed. When the concentration of certain neutral salts in the medium, such as NaCl or KCl, is increased to 240 mM or higher, with pH held constant at 7.4, the $Ca^{++}:\sim$ ratio increases to values exceeding 4.0. When the pH is raised from 7.0 to 8.0, with the NaCl concentration held constant at 80 mM, the $Ca^{++}:\sim$ ratio increases to as high as 7.0. In the presence of phosphate, however, the $Ca^{++}:\sim$ ratio remains at 2.0, regardless of pH or salt concentration.

<u>Experimental</u>. Oxygen uptake was followed with the Clark oxygen polarograph,

uptake of $^{45}Ca^{++}$ by methods described earlier (Rossi and Lehninger, 1964), and H^+ ejection by means of a glass electrode linked to a recording pH meter with scale expansion. Responses of the electrodes were calibrated by addition of known amounts of HCl or dithionite.

Results. When Ca^{++} is added to activate respiration in a system containing 10 mM sodium succinate, 10 mM Tris Cl buffer pH 7.4, NaCl, and rat liver mitochondria, the Ca^{++}:~ accumulation ratio may vary considerably depending on the concentration of NaCl in the medium (Fig. 1). The rates of the initial State 4 respiration and the respiration activated by addition of Ca^{++} were not significantly altered by increasing the NaCl concentration from 40 to 320 mM. However, as salt concentration was increased, the extra oxygen uptake evoked by addition of a fixed amount of Ca^{++} decreased, with a consequent increase in the Ca^{++}:~ ratio calculated from the extra oxygen uptake, from values of about 2.0 at 40 mM NaCl to a value of 4.8 at 320 mM NaCl. The increase in the Ca^{++}:~ ratio beyond the previously reported values of about 2.0 is also evident when the ratio is calculated on the basis of the total oxygen uptake during the respiratory jump evoked by Ca^{++} (cf. Carafoli, Rossi, and Lehninger, 1965). The uptake of the added Ca^{++} was found to be 99% complete within 30 sec following the end of the jump. All of the Ca^{++} uptake during respiratory activation at high NaCl concentrations was inhibited by cyanide.

No increase in the Ca^{++}:~ ratio with increasing concentrations of NaCl was observed when inorganic phosphate was present in the medium (Figs. 1 and 2); the Ca^{++}:~ accumulation ratio remained constant at about 1.8 - 2.0, in agreement with previously observed values (Rossi and Lehninger, 1964), regardless of the NaCl concentration. Under these conditions phosphate is known to be accumulated in the ratio of about 1.0 molecule per pair of electrons per energy-conserving site. Increase in the NaCl concentration from 20 to 240 mM caused no significant change in the efficiency of oxidative phosphorylation as reflected by ADP:0 ratio measurements made

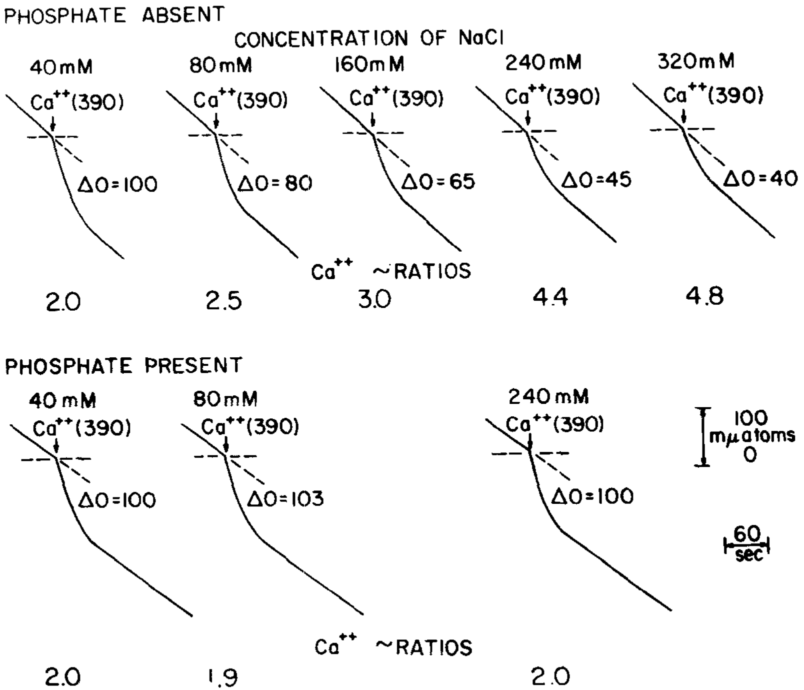
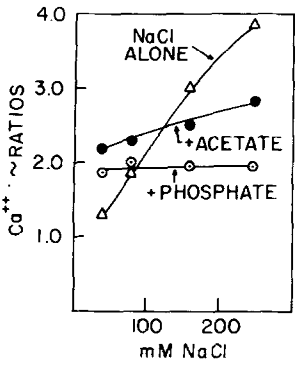
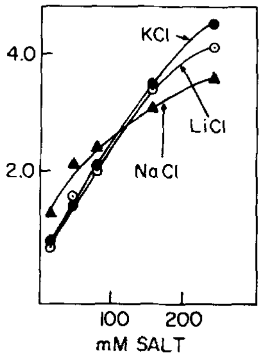


Fig. 1 Effect of NaCl on Ca^{++}:∼ ratio. All systems contained 10 mM Na succinate, 10 mM Tris Cl pH 7.4, 5.0 mg mitochondrial protein and NaCl as shown in volume of 1.9 ml. At times shown, 390 mμmoles $CaCl_2$ was added; T=25°. Lower traces were from systems also containing 1.0 mM phosphate and 5.0 μg oligomycin.

by the respiratory jump method. It is therefore clear that increasing the NaCl concentration produces a large increase in the Ca^{++}:ADP ratio, which Chance (1965) has proposed as the most valid measure of the stoichiometry of ion uptake.

Fig. 3 shows that replacement of NaCl with KCl or LiCl produced similar increases in the Ca^{++}:∼ accumulation ratio. Furthermore, such increases were also given by NaBr, NaCNS, and NaI. On the other hand, when 10 mM sodium acetate was present in the system, the Ca^{++}:∼ ratio increased only slightly with increasing NaCl concentration; the action of acetate thus resembles that of phosphate, also shown in Fig. 3. The high Ca^{++}:∼ratios observed in the presence of high concentrations of some neutral salts may possibly be associated with the relative impermeability of the mitochondrial membranes to their anions. The anions Cl^-, Br^-, and I^- are believed to be relatively non-permeant, whereas the anions phosphate and





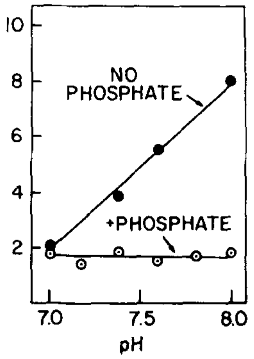


Fig. 2 Effect of NaCl concentration. System contained in 1.9 ml 5.0 mg mitochondrial protein, 10 mM sodium succinate, 10 mM Tris Cl pH 7.4, and, where shown, 1.0 mM phosphate + 5.0 μg oligomycin, or 4.0 mM sodium acetate. NaCl was varied as shown. T=25°. $CaCl_2$ (390 mμmoles) added to activate respiration.

Fig. 3 Effect of different salts on Ca^{++}:$\sim$ ratio. Details as in Fig. 1; no Pi or acetate present.

Fig. 4 Effect of pH. System contained 10 mM Na succinate, 80 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris Cl buffer. $CaCl_2$(350 mμmoles) added to activate respiration.

acetate penetrate readily (cf. Rasmussen, Chance, and Ogata, 1965). Sucrose cannot replace NaCl in producing increases in the Ca^{++}:$\sim$ ratio.

Another condition causing elevation of the Ca^{++}:$\sim$ ratio to values higher than reported earlier is increase of pH of the suspending medium, with NaCl held constant at 80 mM (Fig. 4). The Ca^{++}:$\sim$ ratio increased from the normal value of about 2.0 at pH 7.0 to a value of about 8 at pH 8.0. This effect did not occur in the presence of phosphate; as is shown in Fig. 4, the Ca^{++}:$\sim$ activation ratio remained constant at about 2.0. This finding not only demonstrates that high Ca:$\sim$ ratios are not an artifact of high salt concentration, but also suggests that gradients of H^+ or OH^- across the mitochondrial membrane may also determine the efficiency of net accumulation of Ca^{++}.

Discussion. From the findings reported here, earlier observations that the Ca^{++}:$\sim$ ratios are approximately identical in the absence and presence of phosphate must be attributed to fortuitous choice of salt concentrations and pH for the reaction media.

We have recently reported that the accumulated Ca^{++} present in mitochondria following a Ca^{++}-induced respiratory jump is not irreversibly sequestered, but exists in a dynamic steady-state, in which constant Ca^{++} efflux is counterbalanced by Ca^{++} uptake coupled to the resting respiration (Drahota, Carafoli, Rossi, Gamble, and Lehninger, 1965; Carafoli, Rossi, and Lenninger, 1965). The observed increase in the <u>net</u> Ca^{++}:$\sim$ accumulation ratio that is evoked by increasing the NaCl concentration or by increasing the pH can therefore be accounted for in two different ways, either by an increase in the efficiency in Ca^{++} uptake per pair of electrons, with no change in efflux rate, or by a condition in which there is a constant stoichiometry of Ca^{++} uptake with electron transport, but a decrease in the efflux rate of Ca^{++} as NaCl concentration or pH is increased. Experiments now under way on the effect of NaCl concentration and pH on the rate of Ca^{++} efflux should permit a decision as to these alternatives. Whatever the mechanism, it is obvious that the maximum value for the stoichiometric ratio Ca^{++}:$\sim$ must be at least 4.0 - 5.0, or more than twice as high as the previously accepted value of 2.0. Such a maximum efficiency of 8 - 10 or more positive charges moved per pair of electrons per energy-conserving site as described here for Ca^{++} uptake is approximately equivalent electrochemically to the extrapolated uptake of about 10 K^+ per pair of electrons per site observed by Pressman (1965) in valinomycin-treated mitochondria.

Full details of this and related studies on the coupling of ion movements to electron transport will be submitted for publication elsewhere. The authors thank Drs. Carlo S. Rossi and Jozef Bielawski for confirmation of some of the experiments described, and Brenda Cimino for technical assistance. This investigation was supported by the National Institutes of Health and the National Science Foundation.

References

Carafoli, E. (1965), Biochim. Biophys. Acta, 97, 107.
Carafoli, E., Rossi, C. S. and Lehninger, A. L. (1965), Biochem. Biophys. Research Commun., 19, 609.
Carafoli, E., Weiland, S., and Lehninger, A. L. (1965), Biochim. Biophys. Acta, 97, 88.
Chance, B. (1963), in B. Chance (ed), Energy-linked functions of mitochondria, Academic Press, Inc., New York, p. 253.
Chance, B. (1965), J. Biol. Chem., 240, 2729.
Chappell, J. B., Cohn, M., and Greville, G. D. (1963), in B. Chance (ed) Energy-linked functions of mitochondria, Academic Press, Inc., New York, p.219
Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L., and Lehninger, A. L., (1965), J. Biol. Chem., 240, 2712.
Pressman, B. C. (1965), Proc. Nat. Acad. Sci. (U.S.), 53, 1076.
Rasmussen, H., Chance, B., and Ogata, E. (1965), Proc. Nat. Acad. Sci. (U.S.), 53, 1069.
Rossi, C. S. and Lehninger, A. L. (1963), Biochem. Zeit., 338, 698.
Rossi, C. S. and Lehninger, A. L. (1964), J. Biol. Chem., 239, 3971.