K^+-DEPENDENT REBOUNDS AND OSCILLATIONS IN RESPIRATION-LINKED MOVEMENTS OF CA^{++} AND H^+ IN RAT LIVER MITOCHONDRIA

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This communication reports conditions in which respiration-linked uptake of Ca^{++} by rat liver mitochondria (cf. Rossi and Lehninger, 1964; Chance, 1965) and the accompanying ejection of H^+ (Saris, 1963; Drahota et al., 1965; Chappell and Crofts, 1965) undergo striking K^+-dependent rebound and oscillatory phenomena, without concomitant fluctuations in oxygen uptake. The findings provide further evidence that the stoichiometry between Ca^{++} uptake and electron transport may vary considerably under different conditions (Carafoli, Gamble, and Lehninger, 1965) and also suggest the existence of a feed-back relationship between active Ca^{++} uptake and the Ca^{++} efflux rate.

Experimental. Mitochondria were isolated from the livers of albino rats and washed 3 times with 0.25 M sucrose. Oxygen uptake was measured with a Clark electrode linked to a 10 mV recorder. H^+ movements were followed with a glass electrode, an expanded scale pH meter, and a 5 - 12.5 mV recorder. The response of the electrode in each reaction medium was calibrated by the addition of known amounts of HCl. Ca^{++} movements were determined by measuring the $^{45}Ca^{++}$ in Millipore filtrates (AAWP, pore size 0.8 μ) of the mitochondrial suspensions.

Results. The rebound and incipient oscillations of the Ca^{++} and H^+ movements, which are reciprocal in nature, are most easily followed with a recording pH meter. The glass electrode trace in Fig. 1 A shows the ejection of H^+ following addition of Ca^{++} to a "normal" system containing rat liver mitochondria, Tris buffer, phosphate, 80 mM NaCl, and succinate (Rossi and Lehninger, 1964). The rapid phase of H^+ ejection was followed by a slower

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approach to a steady-state. However, when phosphate was omitted, and the 80 mM NaCl was replaced with 10 mM KCl (Fig. 1 B), the rapid phase of H^+ ejection was followed by a fast rebound, during which reabsorption of 50-70% of the previously ejected H^+ took place; a slower re-ejection of H^+ led to the final steady-state. Such a "bounce" effect was not seen in the presence of phosphate and was maximal in an all-K^+ medium in which the total K^+ is about 20 - 40 mM. The relatively small "bounce" seen when KCl was replaced by NaCl (Fig. 1 C) is probably due to K^+ arising by ejection from the mitochondria and to leakage of some KCl from the salt bridge. Sr^{++} addition produces even more striking rebounds (Fig. 1 D) in which nearly all of the ejected H^+ was reabsorbed; however, re-ejection of H^+ was incomplete and usually failed to attain the extent observed at the

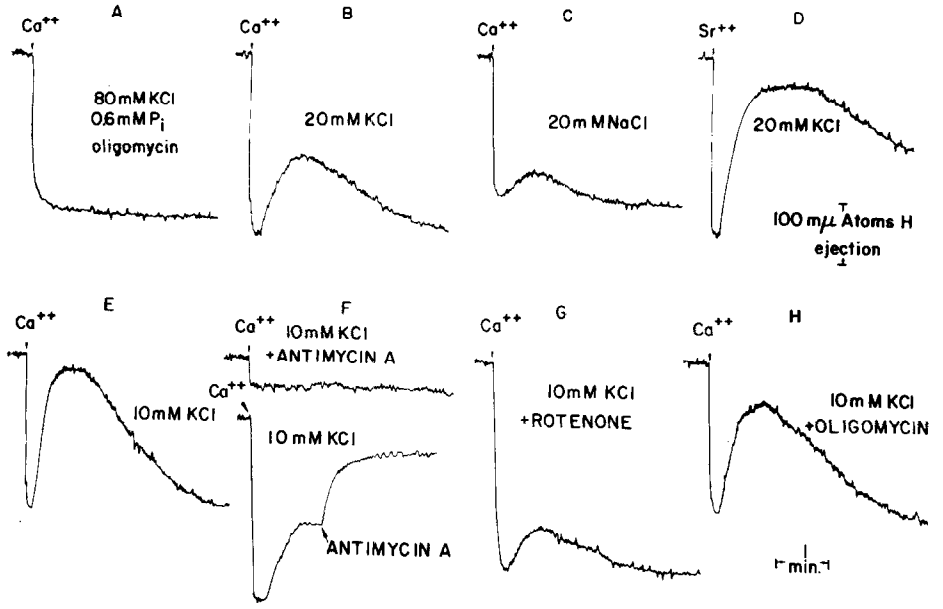

Fig. 1 Rebound and oscillation in H^+ ejection. All media (2.0 ml) contained 5.0 mg mitochondrial protein and 10 mM Tris chloride (pH 7.4). Other components of the media follow; A. 80 mM NaCl, 6.0 mM Pi, 2 μg oligomycin, 10 mM K-succinate; B. 20 mM KCl, 10 mM K-succinate; C. 20 mM NaCl, 10 mM Na-succinate; D. same as B. The media for E-H contained 10 mM K-succinate and 10 mM KCl. Concentrations of the inhibitors were: 1 μM rotenone, 1 mM cyanide, 10 μg Antimycin A. At the arrows 390 mμmoles Ca^{++} or Sr^{++} was added. Temperature 25°.

peak of the first ejection. Mn^{++} addition gave a slow H^+ ejection but no rebound. The "bounce" effects do not occur in media containing sucrose at 100 - 250 mM.

The series of traces in Fig. 1 E - H show that the respiratory inhibitor antimycin A (added before Ca^{++}) blocked the ejection of H^+. Similar results were given by cyanide. Furthermore, the addition of antimycin A at the peak of the rebound prevented the second ejection of H^+, which must therefore also be dependent on electron transport. Rotenone, which does not inhibit succinate oxidation, was found to dampen the "bounce" considerably. When succinate was replaced by β-hydroxybutyrate as the respiratory substrate, the rebound still occurred but there was no re-ejection of H^+. These findings suggest that reversal of electron transport to NAD may be involved in the oscillatory response. Oligomycin has no effect on the "bounce," but ATP, phosphate, and Mg^{++} tested singly inhibited the "bounce" completely. The "bounce" effect also was inhibited when 10 mM KCl was replaced by 10 mM K acetate.

The curves in Fig. 2 A show that in the normal "non-bouncing" system, Ca^{++} uptake and H^+ ejection proceeded together following addition of Ca^{++}. H^+ ejection was complete when the stimulated oxygen uptake had just returned to the original "resting" level. Over 85% of the added Ca^{++} was rapidly taken up during H^+ ejection and the remainder was accumulated somewhat more slowly. In the ensuing phase of resting respiration following the Ca^{++}-jump, the accumulated Ca^{++} normally remains in the mitochondria in a steady-state, in which accumulation of Ca^{++} linked to resting respiration (Carafoli, Rossi, and Lehninger, 1965) is counterbalanced by passive efflux (Drahota et al., 1965).

The curves in Fig. 2 B show that in the "bouncing" system the activation of respiration on adding Ca^{++} is also accompanied by the rapid uptake of $^{45}Ca^{++}$ and ejection of H^+. Following the completion of the respiratory jump and return of respiration to the resting rate, there was a large release of some of the newly accumulated $^{45}Ca^{++}$ back into the medium, accompanied by reabsorption of H^+. In the third phase, Ca^{++} was re-accumulated from the medium again, accompanied by H^+ ejection, without any change in the rate of resting respiration.

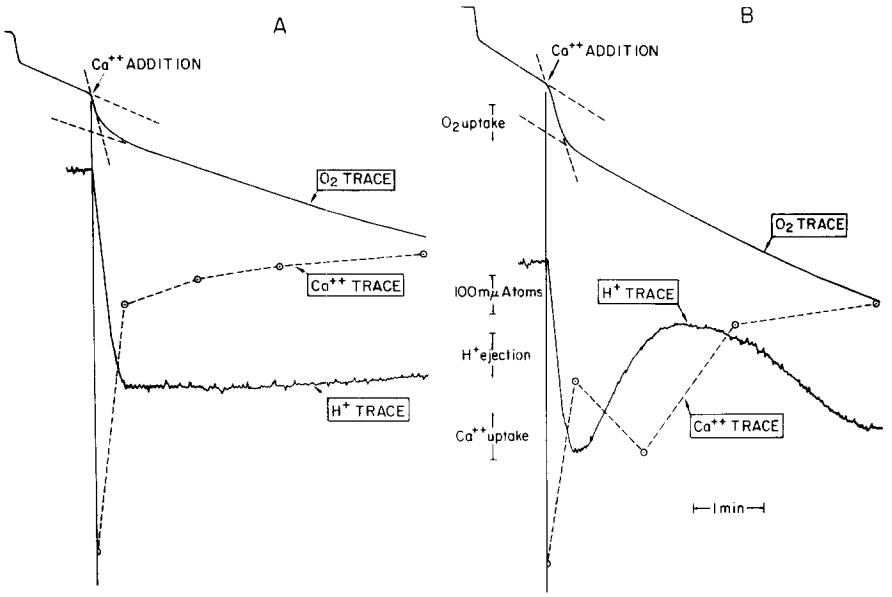

Fig. 2 Relationship between O_2 uptake and H^+ and Ca^{++} movements. O_2 uptake was determined in a medium containing 10 mM Tris Cl, pH 7.6, 10 mM K-succinate, 80 mM KCl (A) or 10 mM KCl (B) and 5 mg mitochondrial protein in a final volume of 1.9 ml. Ca^{++} (390 mμmoles) was added at the arrows. Ca^{++} and H^+ movements were determined in separate but identical vessels. Data are for 1.9 ml system. Temperature, 25°.

The first peaks in the H^+ and Ca^{++} curves at the end of the respiratory jump coincide closely, but the following peak of Ca^{++} release appears in this experiment to precede the peak of H^+ reabsorption. However, because of inability to obtain more frequent samples for Ca^{++} analysis and because of the relatively large timing error of 5 - 8 sec in the sampling and filtration method, it was not possible to determine whether Ca^{++} movements are exactly synchronous (but reciprocal) with H^+ movements, or whether Ca^{++} movements precede or follow H^+ movements. For the same reason, the depression in the Ca^{++} uptake curve which corresponds to the peak of H^+ ejection may in fact be a much deeper one than shown.

The fact that respiration remains in the resting state during the large fluctuations in H^+ and Ca^{++} movements indicates that large changes in the relative rates of Ca^{++} uptake and Ca^{++} efflux may take place in the so-called resting respiration without changes in respiratory rate. Following such a

cycle of events, the addition of another aliquot of Ca^{++} will evoke both respiratory stimulation and H^{+} ejection, indicating that respiratory control has not been damaged. Similar observations have been made following Sr^{++} addition.

Discussion. The incipient oscillatory phenomena described here represent another instance in which a complex enzyme system shows periodic behavior, this one involving ion movements across a membrane system. Our observations may possibly be related to the oscillations of K^{+} movements in mitochondria induced by valinomycin that were briefly reported by Pressman (1965) and to oscillations in K^{+}-stimulated valinomycin-dependent ATPase activity (Lardy and Graven, 1965), but differ in that they do not require the presence of the antibiotic. The apparent specificity of K^{+} in inducing such "bounces" is probably related to our earlier finding that K^{+} is lost from mitochondria as Ca^{++} is accumulated (Carafoli, Rossi, and Lehninger, 1964), and that discharge of Ca^{++} from loaded mitochondria is promoted in an all-K^{+} medium (Drahota and Lehninger, 1965).

These experiments provide further evidence supporting the conclusion reached in a preceding communication (Carafoli, Gamble, and Lehninger, 1965) that net Ca^{++} accumulation and electron transport do not always stand in an exact and constant stoichiometric relationship as has been generally assumed. Actually, Ca^{++}:$\sim$ accumulation ratios as high as 4 - 5 were observed depending on salt concentration and pH. In the experiment of Fig. 2 B, the Ca^{++}:0 accumulation ratio at the end of the period of respiratory stimulation was about 4.5 (succinate), in approximate agreement with the stoichiometry of 1.7 - 2.0 molecules of Ca^{++} uptake per pair of electrons per energy-conserving site measured by Rossi and Lehninger (1964). However, immediately after the jump the Ca^{++} efflux rate is evidently very high in relation to the uptake rate, and Ca^{++} is lost from the mitochondria. After a lag period, during which a feed-back adjustment of either the efflux rate or of the efficiency of Ca^{++} uptake was evidently taking place, the system began a slower return to a steady state in which Ca^{++} efflux and uptake rates came into balance again.

Detailed reports on this and related investigations on maximum stoichiometry of Ca^{++} uptake and on Ca^{++} efflux rates will be presented for publication elsewhere.

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