ENERGY-COUPLING IN MITOCHONDRIA DURING RESTING OR STATE 4 RESPIRATION

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The low rate of respiration of mitochondria in the absence of phosphate acceptors (i.e. State 4) and the high rate of respiration stimulated by ADP (i.e. State 3) are fundamental elements not only in intracellular control of respiration and ATP formation, but also in determination of the stoichiometry of oxidative phosphorylation with the oxygen electrode (cf. Lardy and Wellman, 1952; Chance and Williams, 1956). Recent work shows that respiratory stimulation also accompanies ion transport: the addition of Ca^{++}, Sr^{++}, or Mn^{++} to acceptor-less mitochondria causes a stoichiometric "jump" in oxygen uptake, which is accompanied by quantitative accumulation of the metal ion (cf. Chance, 1963; Chappell, Cohn, and Greville, 1963; Rossi and Lehninger, 1964; Carafoli, 1965).

Little evidence exists as to whether resting respiration is capable of energy-coupling, yet this question has a bearing not only on the mechanism and control of oxidative phosphorylation and active ion accumulation, but also on the calculation of ADP:O or Ca^{++}:O ratios from "respiratory jump" data (cf. Chance and Williams, 1956; Chappell et al, 1963; Ernster and Lee, 1964; Rossi and Lehninger, 1964). We have recently reported that during the "resting" respiration which ensues after a Ca^{++} jump, the accumulated Ca^{++} is retained in a steady-state in which passive efflux of Ca^{++} is counterbalanced by active accumulation of Ca^{++} linked

to the resting respiration (Drahota, Carafoli, Rossi, Gamble, and Lehninger, 1965). This demonstration of energy coupling during resting or State 4 respiration has led to an experimental method for measurement of its efficiency.

Methods. Uptake of oxygen and accumulation of $^{45}Ca^{++}$, and $^{85}Sr^{++}$ were measured as described earlier (Rossi and Lehninger, 1964; Carafoli, 1965); ATP was determined by the hexokinase-Zwischenferment method.

Approach and results. Earlier work has shown that the concentration of Ca^{++} required for half-maximal rates of stimulated oxygen uptake in Ca^{++} jump experiments with rat liver mitochondria (2 mg protein per ml) is about 20 μM; however, no detectable extra O_2 uptake ensues if Ca^{++} is added at less than 10 μM (Rossi and Lehninger, 1964). Nevertheless, Ca^{++} accumulation evidently can still occur at concentrations less than 10 μM since in the steady state after a Ca^{++}-jump, in which the concentration of Ca^{++} left in the medium is but 1-2 μM, active pumping of Ca^{++} still occurs (Drahota, et al., 1965). It therefore appeared possible to obtain measurements of net Ca^{++} uptake during unstimulated State 4 respiration if Ca^{++} could be added to the mitochondrial suspension in such a way that its concentration is prevented from exceeding $\sim$10 μM. Such measurements were achieved by continuous infusion of Ca^{++} into the polarograph cuvette from a syringe microburette; the micrometer drive of the latter was driven at a constant controlled rate by a geared-down motor. In the following experiments, known amounts of Ca^{++}, Sr^{++}, or ADP were infused at different rates into suspensions of rat liver mitochondria with simultaneous monitoring of respiratory rate with the oxygen electrode; the accumulation of Ca^{++} or Sr^{++} or the ATP formed was determined at the end of the infusion period. The volume of the infused addition was kept below 0.01 ml; the total volume of the test system was 2.0 ml.

In Fig.1 are shown a series of such experiments in which 240 mμmoles $^{45}Ca^{++}$ were added to State 4 mitochondria respiring with succinate as

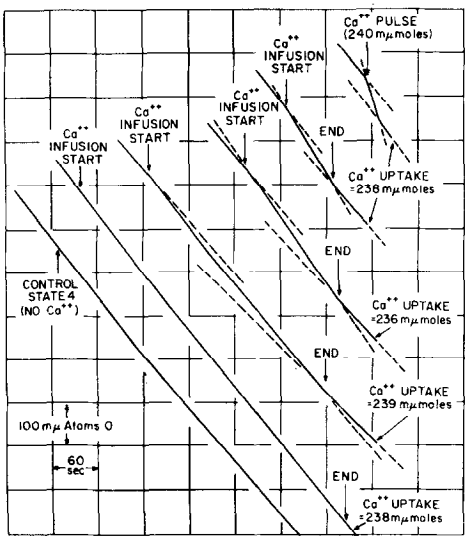

Fig. 1

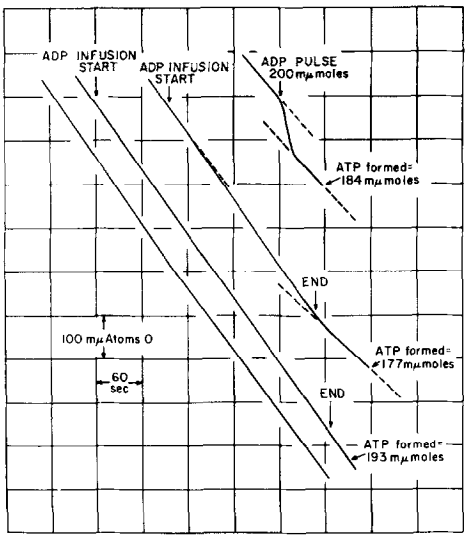

Fig. 2

Ca++ uptake (Fig.1) and ADP phosphorylation (Fig.2) during State 4 respiration. Reaction media contained 10 mM Tris.HCl, pH 7.3, 80 mM NaCl, 10 mM succinate, and 5 mg mitochondrial protein in a final volume of 1.9 ml (Fig.1). Inorganic phosphate (5 mM) was added when ADP phosphorylation was determined (Fig. 2). To these media were added 240 mμmoles $^{45}CaCl_2$, or 200 mμmoles ADP, either in a single pulse or infused at the rates shown. Ca++ uptake and ATP formation were determined 60 sec after the additions were completed, as described in the text. T=25°.

substrate, in a single pulse (top), or infused over increasing time intervals (lower curves). After the pulsed addition, the usual stoichiometric extra oxygen uptake and Ca++ uptake occurred (cf. Table 1). However, on infusing 240 mμmoles Ca++ at a continuous rate over time intervals increasing from 1.0 to 4.0 minutes, the stimulation of oxygen uptake declined with the decrease in rate of infusion; in each case essentially all the Ca++ was accumulated. When 240 mμmoles of Ca++ was infused over a period of 6 min. (0.67 mμmoles Ca++ sec^{-1}), no detectable respiratory stimulation occurred in comparison with a control curve of State 4 respiration in the absence of Ca++ additions, yet essentially all the Ca++ infused was accumulated by the mitochondria. In Table I it is seen that after the pulsed addition of Ca++, the ratio Ca++:O is about 2.1 per coupling

Table 1. Ca^{++} uptake during State 4 respiration. Reaction medium (T=25°) contained 10 mM Tris.HCl, pH 7.3, 80 mM NaCl, 10 mM succinate, and 5 mg mitochondrial protein in a final volume of 1.9 ml., 250 mμmoles $^{45}CaCl_2$ was added either in a single pulse or infused at rates given. The Ca^{++} uptake was determined 60 sec after Ca^{++} addition was complete.

Rate of infusion mμmoles x sec^{-1}	Ca^{++} uptake mμmoles	Extra O_2 uptake mμatoms	Ca^{++}: (extra oxygen)	Total O_2 uptake mμatoms	Ca^{++}: (total oxygen)
(Pulsed)	238	57	2.1	70	1.70
4.00	238	42	2.9	170	0.70
2.00	236	40	3.0	230	0.52
1.00	239	39	3.1	470	0.26
0.667	238	0	∞	680	0.18

site when calculated on the basis of the extra oxygen uptake and 1.7 per coupling site calculated on the basis of total oxygen uptake. In the continuous infusion experiments, the Ca^{++}: ∼ ratio, calculated on the basis of extra oxygen uptake, rose to 3.0 as the rate of infusion declined and yielded the absurd value of infinity when no extra oxygen uptake was stimulated. However, when calculated on the basis of the total oxygen consumed over the period of infusion, then the Ca^{++}: ∼ accumulation ratio declined from 1.7 to a value of about 0.18 in the case in which no stimulation of respiration occurs.

Essentially similar results were observed in experiments on Sr^{++} accumulation (Carafoli, 1965). When $^{85}Sr^{++}$ was infused at the rate of 0.71 mμmoles x sec^{-1} into systems such as described in Fig.1 and Table 1, there was no detectable increase in the rate of State 4 oxygen uptake. In a typical experiment, the Sr^{++}: ∼ accumulation ratio calculated on the basis of total oxygen uptake was 2.03 for the pulsed addition and 0.43 during unstimulated State 4 respiration.

Oxidative phosphorylation of ADP without concomitant respiratory stimulation can also be observed when ADP is continuously infused in such experiments. The top curve in Fig.2 shows the usual "ADP-jump" following a pulsed addition of 200 mμmoles ADP. The observed ADP: ∼ ratio was 1.00 on the basis of extra O_2 uptake and 0.88 on the basis of total oxygen

uptake. The second curve shows the result of infusing ADP at the rate of 1.11 mμmoles x sec^{-1}; a stimulation of respiration is evident from the distinct points of inflection at the beginning and end of the infusion. The third curve (infusion rate = 0.667 mμmoles x sec^{-1}) shows no points of inflection, as compared with the control State 4 trace; in this experiment 193 mμmoles ADP were phosphorylated to ATP out of 200 mμmoles infused, the ADP: $\sim$ ratio calculated on the basis of total oxygen uptake was 0.12.

These findings show that calculation of ADP:O or Ca^{++}:O ratios from respiratory jump experiments based on extra oxygen uptake are overestimated. The degree of overestimation depends on the acceptor control ratio (or Ca^{++} control ratio); it is relatively small ($<10\%$) in systems showing maximal acceptor control. It is not determined by these experiments whether that part of the total oxygen uptake in fully stimulated State 3 respiration that is represented by the original State 4 rate of respiration has the same efficiency as it does when measured by itself in experiments of the kind described here. Presumably, it is higher, since the higher ADP (or Ca^{++}) concentrations required to evoke maximal rates of respiration can compete more effectively for high-energy intermediates against H_2O or endogenous uncoupling factors (Remmert and Lehninger, 1960).

These findings also have a bearing on the mechanism of loose-coupling (cf. Remmert and Lehninger, 1960) and of reverse acceptor control by ADP (Lehninger and Gregg, 1963; Gregg and Lehninger, 1963; Chance and Hagihara, 1962).

Summary. The resting or State 4 respiration of freshly prepared, intact rat liver mitochondria can support energy-linked accumulation of Ca^{++} or Sr^{++} or the oxidative phosphorylation of ADP. However, under these conditions the coupling efficiency is relatively low; about 10% maximal for Ca^{++}, about 20% for Sr^{++} accumulation, and about 12% maximal for oxidative phosphorylation of ADP.

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