INITIATION OF SUCCINATE OXIDATION IN AGED PIGEON HEART MITOCHONDRIA[1]

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In studies of the activation of respiration by succinate in rat liver mitochondria, it was observed some time ago that pretreatment of the mitochondria with ADP for increasing intervals caused an increasing inhibition of respiration (B. Chance and G. R. Williams, unpublished observations). It was also observed that the reduction of pyridine nucleotide by succinate was delayed. More recently, Williams (1960) has shown this lag in mitochondria incubated with high K^+, dinitrophenol, and in the absence of phosphate.

In a recent investigation of the stability of mitochondrial preparations against aging, under aerobic conditions, without added substrate, a number of pigeon heart mitochondria have been examined in detail some days after preparation. Although these aged mitochondria showed good respiratory activity and respiratory control, it was observed that a minute or so elapsed before maximal respiration with succinate was achieved. This problem is also being investigated by other workers (M. Klingenberg and L. Ernster, personal communication). Since succinate is a substrate which elicits a high respiratory activity within a second after its addition to non-phosphorylating Keilin and Hartree heart muscle particles, experiments have been carried out to determine the site at which the respiratory inhibition occurs.

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Materials and Methods. Respiration was measured by the vibrating platinum microelectrode (Chance and Williams, 1955). Pyridine nucleotide reduction was measured by the double-beam spectrophotometer (Chance, 1954) with 340 and 374 mμ as the measuring and reference wavelengths, respectively, and also fluorometrically (Chance, Conrad, and Legallais, 1958) with 365 mμ excitation and 450 mμ measurement. Flavoprotein was measured spectrophotometrically with 460 and 510 mμ as the measuring and reference wavelengths, respectively.

Pigeon heart mitochondria were prepared by a new procedure developed in this laboratory (B. Hagihara, in preparation) involving the use of Bacillus subtilus proteinase. When freshly prepared, these mitochondria have respiratory control ratios with succinate of 6 and a P:O value of 1.8. With one or two days' aging, the respiratory control ratios diminish to about 2 and the phosphorylation efficiency to about 1.5. These activities were preserved to this extent by aeration of the mitochondria in 0.23 M mannitol, 0.07 M sucrose, 1.0 mM "tris," and 0.05 mM versene at 0° C. The added mitochondria were depleted of endogenous substrate and high-energy intermediates and contained oxidized carriers. The reaction medium was similar except that versene was omitted and the buffer concentration was 0.02 M phosphate or "tris." Other details of the experiments are included on the figures themselves and in the figure legend. In the preparation used here, the cytochrome c concentration was 0.95 μM, its turnover number being 5.5 $sec.^{-1}$ (succinate, state 3).

Experimental Results. Fig. 1 shows the time course of the activation of respiration in pigeon heart mitochondria diluted in the aerobic reaction medium. The mitochondria are initially in state 1 (phosphate, but no added ADP present). Addition of 5 mM succinate produces no measurable increment of the slope of the platinum microelectrode trace (0.2 μM O_2/sec.) for half a minute. At the end of a minute, the rate has increased to 0.6 μM O_2/sec.

Since such changes in respiration should result from changes in the steady-state levels of the carriers, the oxidation-reduction level of flavoprotein was considered of most significance. The lowest trace shows the steady-state level of flavoprotein, an upward deflection corresponding to a

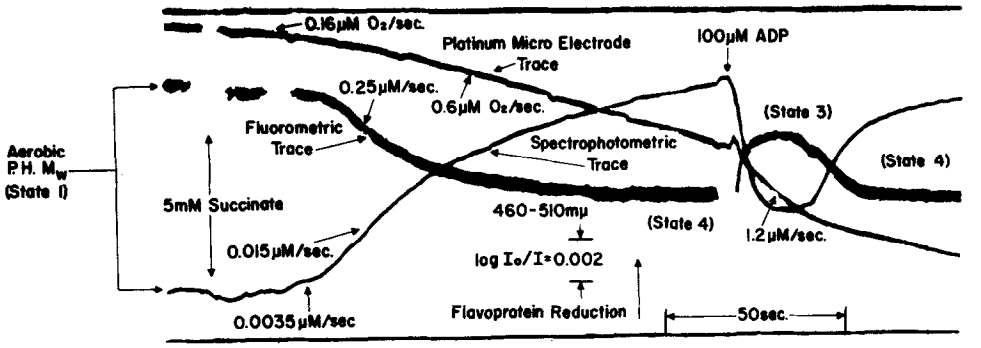

Fig. 1. A study of the delayed activation of succinate respiration in one-day aged pigeon heart mitochondria (P. H. M_w) suspended in the aerobic medium described in the text. The top trace represents respiratory activity measured by the vibrating platinum microelectrode; the middle trace pyridine nucleotide reduction recorded fluorometrically; and the bottom trace the kinetics of flavoprotein reduction recorded spectrophotometrically. The rates of reduction of flavoprotein are directly calculated using $\Delta\varepsilon$ = 11 cm^{-1} mM^{-1}. The rate of pyridine nucleotide reduction measured fluorometrically is calculated from independent spectrophotometric calibrations. 0.95 μM cytochrome c. Optical path, 1 cm. 26° C. (Expt. 101-54).

diminution of absorption at 460 mμ and to flavoprotein reduction. No detectable reduction occurs immediately following substrate addition, and several seconds elapse before an upward deflection of the curve is observable. At half a minute, there is a sharp break in the trace which moves upward at an accelerated rate until the steady state is reached, approximately 140 sec. after treatment of the mitochondria with succinate. The actual rates with which flavoprotein reaches the steady state are very slow compared to the rate with which oxygen is being reduced. In the initial 30 sec., the rate is 3.5×10^{-3} μM fp/sec., and the accelerated rate is 15×10^{-3} μM fp/sec. The inhibited phase of respiratory activity is characterized by a scarcely measurable reduction of flavoprotein.

It has been noted that succinate increases the reduction of pyridine

nucleotide in mitochondria to a greater extent than any other substrate (Chance and Hollunger, 1960). It is therefore of interest to determine at what point in the time course of respiratory activation pyridine nucleotide is reduced. Therefore a simultaneous fluorometric recording of pyridine nucleotide reduction is included. In this case, an increased fluorescence corresponding to pyridine nucleotide reduction is indicated by a downward deflection of the trace. Although the fluorometric recording does not indicate quantitatively the amount of pyridine nucleotide present, independent spectrophotometric calibrations indicate that the amount finally reduced in this recording corresponds to 8 times the amount of cytochrome c present or 7.8 μM DPNH.

The reduction of pyridine nucleotide following the addition of succinate does not proceed at a measurable rate for an interval of half a minute. Thereafter an abrupt reduction at a rate of 0.25 μM/sec. is recorded. It should be noted that by the time pyridine nucleotide reduction has reached its maximal rate, the respiratory activity is increasing significantly and that the pyridine nucleotide reaches its steady state reduction prior to flavoprotein. In order to indicate that a high degree of respiratory activity has been achieved in this incubation period, ADP is added and the typical cycles of oxidation of flavoprotein and pyridine nucleotide accompany the interval of activated respiration.

Discussion. The conditions that lead to an inactivation of succinate oxidation are associated with an oxidized state of the respiratory carriers (state 2) in which the mitochondria are depleted of high-energy intermediates. Thus we may conclude that some essential component has become oxidized. The site of inhibition of succinate oxidation is at the succinic dehydrogenase end of the chain since it is observed that flavoprotein is not reduced on adding succinate.

Respiratory activity increased as flavoprotein and pyridine nucleotide became reduced. It would appear that flavoprotein reduction started before that of pyridine nucleotide. An explanation of the

results is afforded by subsequent experiments showing that pyridine nucleotide reduction and respiration can be reactivated in such preparations by the addition of ATP.

A single explanation for the activation of respiration and pyridine nucleotide reduction is that high energy intermediates are essential for optimal activity in both reactions, succinate itself requiring activation and pyridine nucleotide requiring energy for its reduction. It is more likely, however, that the activation of succinate oxidation requires only the reduction of some oxidizable component of the system, and that this reduction is mediated by high energy intermediates. The latter explanation is consistent with the observations that succinate can be rapidly oxidized in the uncoupled mitochondria or in mitochondria pretreated with amytal (Williams, 1960), whereas the former explanation is not. However, the reduction of pyridine nucleotide in the presence of succinate clearly requires ATP or endogenous high energy intermediates.

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