PRELIMINARY NOTES

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The determination of the rate of uptake of substrates by rat-liver mitochondria

The fact that mitochondria accumulate anions to a concentration greater than that in the surrounding medium is well documented[1–8]. Such an accumulation can be ascribed to the intramitochondrial K^+ which is rather firmly retained[1,6,8]. Furthermore, it was observed that the intramitochondrial concentration of anions was lowered in a competitive manner by the addition of uncouplers, even in the absence of a utilizable source of energy[8]. This led to the proposal that uncouplers are transported into the mitochondrion *via* the same carriers that are responsible for the transport of substrates[9,10]. However, this conclusion could only be tentative so long as only equilibrium states had been measured as was the case in former reports[8,10].

This paper describes a technique with which actual rates of substrate movement can be measured. The results obtained so far confirm the hypothesis that substrate and uncoupler anion compete for a common mechanism of entry into the mitochondrion.

The technique used consists of a modification of that described earlier, in which a rapid separation of mitochondria from the medium is achieved by centrifugation through a layer of silicone[11–13]. The main difference is that the mixing of the mitochondria with the incubation medium occurs during centrifugation (*cf.* ref. 12). To this end small plexiglass caps (Fig. 1) are mounted on top of normal Eppendorf centrifuge tubes (No. 3810) in which from bottom to top are layered: 0.075 ml 15% $HClO_4$ (density 1.09), 0.40 ml silicone oil (Wacker Chemie, Silikonöl AR 100, density 1.05) and 0.45 ml medium containing ^{14}C-labelled substrates and ^3H_2O. The tubes *plus* caps are placed horizontally in an alumina centrifuge head, consisting of a ring with horizontal holes into which the tubes are fitted. After 15-sec centrifugation in the Eppendorf 3200 centrifuge to settle the layers the caps are filled with 0.05 ml mitochondrial suspension.

Incubation is started by a brief pulse of centrifugation to force the mitochondria into the medium and stopped by a second acceleration to maximum speed ($20000 \times g$) which is maintained for 0.5 min. During the second period, the mitochondria migrate through the silicone layer into the acid. It was found that the time required to finish this latter process was considerably shortened by lowering the difference in density between medium and silicone oil by adding dextran (*cf.* ref. 14). All operations with the centrifuge were controlled by an electronic device on which the time interval between starting and stopping procedures could be preset.

After the centrifugation, samples were taken from supernatant and acid layers to determine the radioactivity and, thereby, the amount of substrate contained

within the mitochondria[13]. In parallel runs, the sucrose-permeable space of the mitochondria was determined and matrix concentrations of the substrates calculated as described before[8].

The experiments were carried out at 0° since it was found that the rate of uptake is strongly temperature-dependent (the Q_{10} is approx. 2.5). This tends to favour the view that the uptake is not a simple diffusion-controlled process.

An example of the accumulation of succinate is given in Fig. 2. Under the conditions used the rate of this process can be measured easily. Also the inhibition by 2,4-dinitrophenol of the rate of succinate entry is demonstrated. It may be noted that the inhibition of the initial rate is similar to the inhibition of the final level of accumulation. (The value at 30 sec approximates that at equilibrium as determined in other experiments.)

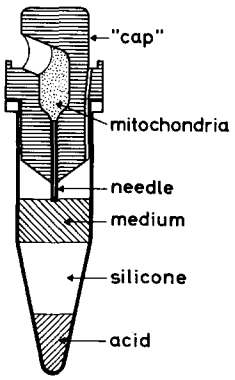

Fig. 1. Cap for mitochondria to be introduced into the medium during centrifugation. The main body is made of plexiglass and the needle of stainless steel.

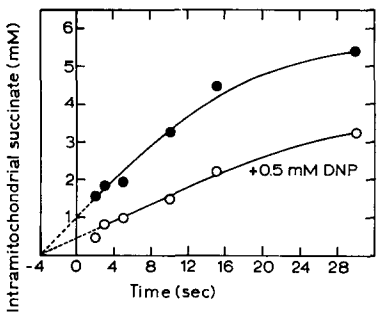

Fig. 2. Kinetics of uptake of succinate by rat-liver mitochondria. The mitochondrial suspension (0.05 ml), containing 1.4 mg protein, was incubated with 0.45 ml medium containing 1 mM [^{14}C]succinate (0.5 μC/μmole), 50 mM Tris chloride, 50 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA and 60 mg/ml dextran (British Drug Houses, A-grade) at a pH of 7.4. The temperature was 0°. The medium further contained 0.5 μC/ml ^3H_2O; 2,4-dinitrophenol (DNP) was added at a concentration of 0.5 mM. After centrifugation as described in the text, samples were taken and radioactivity determined by liquid-scintillation counting[13]. The time indicated is that between the two pulses of centrifugation. The lines are extrapolated to -4 sec, the maximum 'dead time' of the procedure, as determined by separate measurements (see text).

The extrapolated lines converge at a point a few sec before the zero time. This period is presumably the time it takes for the mitochondria to move through the medium into the layer of silicone. The magnitude of this 'dead time' was assessed by using the ATP–P_i exchange reaction as a marker. The incorporation of $^{32}P_i$ into intramitochondrial ATP was determined by the centrifugation method and in parallel incubations carried out in test tubes according to the method of GROOT[15]. From these measurements, it could be calculated that the mitochondria are in contact with the $^{32}P_i$ for a period of 4 ± 0.2 (S.E.) sec more than the preset incubation time. This measurement gives, however, a maximum value for the 'dead time' for anion uptake, since the ATP–P_i exchange reaction is still proceeding while the mitochondria are enclosed in the silicone layer, whereas the net anion uptake stops as soon as the mitochondria leave the incubation medium.

We have also used the technique described to measure uptake of pyruvate and malonate. The K_m for the rate of uptake of malonate, 1.4 mM, was similar to that determined for equilibrium accumulation, 1.3 mM (ref. 8). The uptake of 2,4-dinitrophenol, on the other hand, is too fast to allow measurement of the initial rate. Already at the shortest experimental time the uptake of this uncoupler is more than 50% complete*.

The conclusions drawn from the measurement of equilibrium accumulation values[8,10] are supported by the data obtained from the measurement of the rate of substrate uptake.

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* The rate of penetration of malate measured with a spectrophotometric technique by HASLAM AND GRIFFITHS[16] is two orders of magnitude smaller than that found with our technique.

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