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Dependence of the efficiency of uncouplers on the respiratory rate

It was observed by several investigators[1–4] that the phosphorylating efficiency of cytochrome *c*-deficient mitochondria is increased by the addition of cytochrome *c*. The increase in rate of oxygen uptake upon addition of cytochrome *c* is accompanied by a relatively greater increase in phosphate esterification and, therefore, an increased P:O ratio. It occurred to us that these results could be explained simply if the reactions leading to ATP synthesis and the reactions leading to a dissipation of the respiratory energy compete with different affinities for a common intermediate.

To test this hypothesis, we measured the rate of phosphorylation in intact mitochondria at different respiratory rates, in the presence and absence of low concentrations of uncouplers. As can be seen in Fig. 1, when the rate of succinate oxidation is varied by adding different concentrations of malonate, in the absence of an uncoupler, a straight-line relationship between the rates of phosphorylation and respiration is found, *i.e.* the P:O ratio is independent of the respiratory rate (the slope of the line is 1.85). In the presence of a low concentration of 2,4-dinitrophenol, the phosphorylation rate is depressed at all rates of respiration, but the relative effect is greater at the lower rates of oxygen uptake. This results in a P:O ratio that is dependent on the rate of respiration: the higher the rate of respiration the higher the P:O ratio.

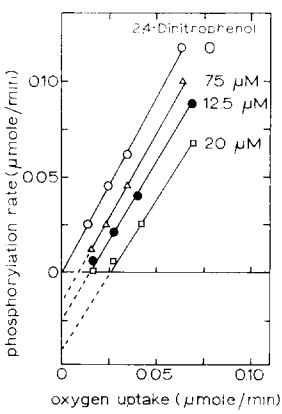

Fig. 1. Relationship between rate of phosphorylation and rate of oxygen uptake by rat-liver mitochondria[5] with succinate as the substrate at different 2,4-dinitrophenol concentrations. Oxygen uptake and phosphorylation were determined in a medium containing 60 mM glucose, 75 mM KCl, 10 mM radioactive phosphate buffer (pH 7.4), 10 mM Tris chloride (pH 7.4), 7.5 mM $MgCl_2$, 1 mM EDTA, 1 mM ADP, 3.3 units (μmoles/min) per ml hexokinase, 20 mM succinate and 1 μg/ml rotenone. The oxygen uptake was varied by the addition of malonate and was monitored with a Clark oxygen electrode (Yellow Springs Instruments). The protein concentration was 1.2 mg/ml and the phosphate had a specific activity of 16 counts/min per nmole. When the suspension became anaerobic, the reaction was stopped with an equal volume of 10% trichloroacetic acid and the inorganic phosphate extracted according to the method of ERNSTER, ZETTERSTRÖM AND LINDBERG[6]. The radioactivity was plated, dried and counted with a Geiger–Müller counter. The amount of radioactivity incorporated in organic phosphates was corrected for the amount incorporated in the presence of 1 μg/ml antimycin.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TTFB, tetrachlorotrifluorobenzimidazole.

It may be noted that at the higher respiration rates, the curve in the presence of uncoupler runs almost parallel to that in its absence. This is to be expected if the uncoupler has a higher affinity than the ATP-synthesizing system for the energy generated during respiration. Once the uncoupler-stimulated reaction is "saturated" the rest of the respiratory energy will be conserved in the form of ATP with the same efficiency as in the absence of the uncoupler.

With increasing uncoupler concentration, the curves at the highest respiration rates form a series of parallel lines extrapolating to successively more negative points on the ordinate (Fig. 1). The value of the intercept with the ordinate represents the maximal rate of energy dissipation by the uncoupler at the concentration used. The magnitude of this intercept is exactly proportional to the uncoupler concentration over the range tested. At relatively high uncoupler concentrations it is no longer possible to observe the straight-line portion of the curve, because of the inability of the respiratory chain to generate high-energy compounds fast enough to saturate the uncoupler.

The same picture as in Fig. 1 has been obtained with other uncouplers including dicoumarol, FCCP and TTFB. Changing the rate of succinate oxidation by another means, *viz.* with antimycin, led to the same results.

With β-hydroxybutyrate as substrate, similar results were obtained with the difference that the slope of the straight-line portions was steeper, as was to be expected. The effect of 10^{-7} M FCCP on the rate of phosphorylation with succinate or β-hydroxybutyrate is compared in Fig. 2. The intercept on the ordinate, obtained by extrapolation of the straight-line portions, is the same in both cases. This means that the maximal decrease in phosphorylation rate induced by the uncoupler is the same

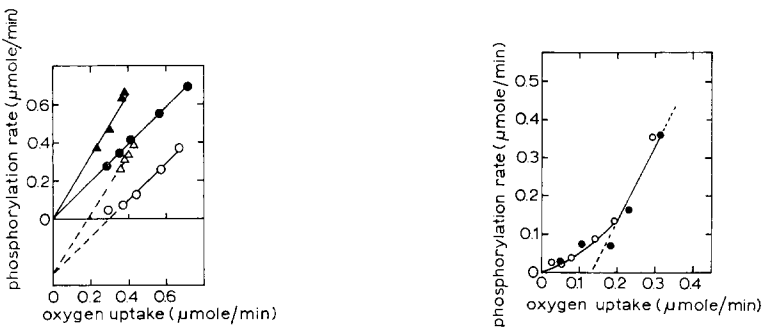

Fig. 2. Effect of substrate on the uncoupling efficiency. Oxygen uptake and phosphorylation were measured as described under Fig. 1. The substrates were 20 mM succinate *plus* 1 μg/ml rotenone (○—○ and ●—●) or 20 mM β-hydroxybutyrate (△—△ and ▲—▲). In both cases the rate of oxygen uptake was varied by the addition of malonate. The closed symbols refer to the system in the absence of FCCP, the open symbols are in the presence of 10^{-7} M FCCP. The protein concentration was 1.7 mg/ml, and the phosphate had a specific activity of 58 counts/min per nmole.

Fig. 3. Relationship between the rate of phosphorylation and the rate of oxygen uptake in cytochrome *c*-deficient mitochondria. Oxygen uptake and phosphorylation were measured as described under Fig. 1. The substrate was 4 mM (○—○) or 40 mM (●—●) succinate *plus* 1 μg/ml rotenone. The rate of oxygen uptake was varied by the addition of cytochrome *c* up to 1.2 μM. The deficient mitochondria (prepared according to the procedure of JACOBS AND SANADI[2]) were preincubated with the cytochrome *c* for 3 min before addition of substrate. The protein concentration was 2.6 mg/ml, and the phosphate had a specific activity of 57 counts/min per nmole.

for both substrates or, in other words, that the uncoupler was saturated by the same rate of generation of high-energy compounds.

Cytochrome *c*-deficient mitochondria resemble intact mitochondria in the presence of a low concentration of uncoupler. A plot of the rate of phosphorylation *versus* the rate of oxidation of succinate (in this case varied by adding different amounts of cytochrome *c*) gives a curve bending upwards at the higher respiration rates (Fig. 3). The last portion of this curve approaches a straight line with a slope of 2. Recalculating the data from the paper of SLATER[1], one finds a P:O ratio of 4 for α-oxoglutarate oxidation at the highest respiration rate induced by cytochrome *c* in cytochrome *c*-deficient mitochondria.

We conclude, then, that the experimental results support the idea that there is a true competition between uncoupling and ATP synthesis for a common high-energy intermediate or state with a higher affinity of the former process and that it is possible to saturate the uncoupling reactions. The nature of the "endogenous" uncoupler present in the cytochrome *c*-deficient mitochondria is unknown, but it could very well be fatty acid.

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