Short Communications

Uptake of Dicoumarol by Rat Liver Mitochondria

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Bivalent cations, being able to traverse the mitochondrial membrane against a concentration gradient, do so at the expense of metabolic energy derived from respiration or from the hydrolysis of ATP. Such transport results in discharge of the high-energy state of mitochondria as seen in stimulation of respiration and decline in ATP synthesis. In other words, under conditions where transport occurs, certain cations are to be termed uncouplers of oxidative phosphorylation (Lehninger, 1948). Other uncoupling compounds, frequently used in studies of energy transfer, include various nitro- and halogen-substituted phenols, dicoumarol (3,3'-methylenebis-4-hydroxycoumarin) and carbonyl cyanide *m*-chlorophenylhydrazone. These compounds are weak acids and exhibit, in common, considerable lipid solubility, but the precise nature of their uncoupling action is not well understood. An approach suggested by van Dam & Slater (1968) is that uncoupling by such anions may be a function of their energy-dependent transport in a fashion similar to that of uncoupling on cation translocation. For example, if energy-linked inward transport be paired with passive but rapid efflux, then low concentrations of uncouplers might capture available energy for finite periods of time.

This view of uncoupling predicts, among other things, demonstrable transport of uncoupler molecules into mitochondria and competition between various uncoupling agents for available energy. This communication reports translocation of [¹⁴C]dicoumarol into rat liver mitochondria and the inhibition of its transport by other uncoupling agents.

Rat liver mitochondria were isolated according to the method of Myers & Slater (1957). In preliminary experiments, where conditions were similar to those described in Table 1, it was found that dicoumarol entered mitochondria rapidly, approaching maximum uptake in about 10 sec. at 0°. Table 1 describes experiments terminated at 10 sec. and shows that the addition of a second uncoupling compound depresses dicoumarol uptake. Addition of 2,4-dinitrophenol produces slight inhibition, whereas its octyl derivative inhibits extensively, presumably assisted by its greater

solubility in the lipid of the mitochondrial membrane (Hemker, 1964). Both carbonyl cyanide *m*-chlorophenylhydrazone and gramicidin produce significant inhibition, the latter undoubtedly through its ability to permit the active translocation of K⁺ ions from the reaction medium (Pressman, 1965). The second part of Table 1 shows that preincubation of the mitochondria with glucose and hexokinase to trap intramitochondrial phosphate leads to a lessened dicoumarol uptake. Addition of phosphate produces about a twofold increase in uptake when compared with the control. This influence of phosphate should be considered in the light of similar stimulation by phosphate of the energy-requiring translocation of a number of anions across the mitochondrial membrane

Table 1. *Uptake of [¹⁴C]dicoumarol into rat liver mitochondria*

Mitochondria were incubated with dicoumarol (ammonium salt) for 10 sec. followed by filtration on a wet 0.65 μ Millipore filter. Filtration was complete in less than 2 sec. The reaction was begun by rapid addition of dicoumarol with a syringe and terminated by pouring the reaction mixture on to the filter, which was then washed with 5 ml. of 0.25 M-sucrose and dried, and the radioactivity was measured with a gas-flow counter. All operations up to drying the filter were carried out at 0°. The reaction medium contained 15 mM-KCl, 2 mM-EDTA, 50 mM-tris-HCl, 5 mM-MgCl₂, 21.5 μ M-dicoumarol and 0.6 mg. of protein in a volume of 2 ml. and at pH 7.4. In this experiment, 1 nmole of dicoumarol gave 1666 counts/min. In the experiment with glucose and hexokinase, the mitochondria were preincubated with the reaction mixture for 5 min. before the addition of dicoumarol.

Addition	[¹⁴ C]Dicoumarol uptake (counts/min.)
None	1659
2,4-Dinitrophenol (250 μ M)	1231
2,6-Dinitro-4-octylphenol (250 μ M)	73
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone (25 μ M)	994
Gramicidin (50 μ g.)	565
Glucose (30 mM) + hexokinase (50 units)	965
Phosphate (2.5 mM)	3354

(Chappell & Haarhoff, 1966). It must be noted that considerable transport occurs even in the absence of phosphate, an observation consistent with the important fact that uncoupling agents do not require phosphate for their stimulation of respiration or for their inhibition of other energy-linked functions (Borst & Slater, 1961).

In conclusion, dicoumarol enters mitochondria by a process similar to the translocation of other anions in being stimulated on the addition of inorganic phosphate. Entry of the uncoupler, dicoumarol, is suppressed by the presence of a second uncoupler, representing the sort of competition which would be predicted on the supposition that uncouplers are actively translocated and, indeed, that the uncoupling effect is a direct consequence of their energy-requiring transport.

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- Borst, P. & Slater, E. C. (1961). *Biochim. biophys. Acta*, **48**, 326.
- Chappell, J. B. & Haarhoff, K. N. (1966). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtezak, L. London: Academic Press (Inc.) Ltd.
- Hemker, H. C. (1964). *Biochim. biophys. Acta*, **81**, 9.
- Lehninger, A. L. (1948). *J. biol. Chem.* **178**, 625.
- Myers, D. K. & Slater, E. C. (1967). *Biochem. J.* **67**, 888.
- Pressman, B. C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1076.
- van Dam, K. & Slater, E. C. (1968). *Proc. nat. Acad. Sci., Wash.*, (in the Press).