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A New Procedure for the Separation of Mitochondrial Membrane Proteins

By P. J. Curtis. (*Beatson Institute for Cancer Research, Royal Beatson Memorial Hospital, Glasgow C.3, U.K.*)

Studies on mitochondrial membrane proteins so far have been in aqueous solutions. The hydrophobic nature of the membrane protein suggests that a large part of the protein may exist in the non-polar section of the membrane. Therefore organic solvents might be more suitable and there have been reports of membrane protein dissolved in such solvents (Lowden, Moscarello & Morecki, 1966; Zahler & Wallach, 1967; Green, Haard, Lenaz & Silman, 1968). Preliminary work by Curtis (1969) showed that rat liver mitochondrial membrane protein dissolved completely in acidic chloroform–methanol (2:1, v/v) in the absence of phospholipids. Further use of chloroform–methanol as a solvent for membrane proteins has been investigated.

Exhaustive methylation of Sephadex G-75, G-100 and G-200 (Nyström & Sjövall, 1965) gave products that swelled in chloroform–methanol (2:1, v/v) approximately as much as in water. Chromatography of polystyrene fractions of narrow molecular-weight range in chloroform–methanol (3:1, v/v) showed that separation was according to size. Gel filtration of the mitochondrial membrane protein dissolved in chloroform–methanol (2:1, v/v) was achieved most successfully on methylated Sephadex G-100 equilibrated with chloroform–methanol (2:1, v/v) containing acetic acid (10mM). Polyacrylamide-gel electrophoresis (Neville, 1967) at pH 2.7 in 8M-urea showed that the major component of membrane protein was largely separated from the other components, and further purification was possible by rechromatography on methylated Sephadex G-100.

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Presence and Spatial Localization of Carbonic Anhydrase in Rat Liver Mitochondria

By F. A. Holton. (*Department of Physiology, Royal Veterinary College, London N.W.1, U.K.*)

About 2–10% of the carbonic anhydrase activity in homogenates of rat liver, kidney and cerebral cortex is recovered in the mitochondrial fraction (Datta & Shepard, 1959; Karler & Woodbury, 1960). Datta & Shepard (1959) believed these low recoveries indicated that the enzyme was confined to the cytoplasm, but Karler & Woodbury (1960) concluded that carbonic anhydrase was present in mitochondria and was tightly bound to membranes.

We recently reported that choline oxidation in liver mitochondria was accelerated under conditions enabling carbon dioxide to pass into the mitochondria and suggested that carbonic anhydrase in the matrix space accelerated the production of internal H^+ ions, which then facilitated choline entry by ionic exchange (Holton & Tyler, 1969). This hypothesis has now been tested by making electrometric measurements of carbonic anhydrase activity in mitochondria. Reaction mixtures containing carbon dioxide were rapidly stirred in a closed cell at 0.5–2°C. Changes of pH were measured by recording the e.m.f. of a glass electrode–calomel electrode system, with a strip-chart recorder running at 4in/min. Rapid addition of acid or alkali yielded biphasic traces, which were analysed to yield a first-order velocity constant for the rate of approach to pH equilibrium. Between pH 6 and pH 7 mitochondria in distilled water increased the rate constant for the approach to equilibrium from the alkaline side (CO_2 hydration predominating). At pH 6.4 the rate constant was doubled by 1.2mg of mitochondrial protein/ml. In contrast, mitochondria had no effect on the rate constant for approach to equilibrium from the acid side (HCO_3^- and H_2CO_3 dehydration predominating).

These properties suggest the presence of a membrane-bound enzyme accessible to substrate by a diffusion path traversed more rapidly by CO_2 than by HCO_3^- ion. They are interpreted as showing that liver mitochondria contain carbonic anhydrase located on the matrix side of the inner membrane, which is known to be permeable to CO_2 but impermeable to

HCO_3^- ions (Chappell & Crofts, 1966). The physiological role of the enzyme may be to provide a rapidly reacting buffer system in the matrix space and thus to moderate pH changes resulting from variations in the rate of secretion of H^+ ions, which is thought to be a continuous process in respiring mitochondria (Mitchell, 1966).

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Spatial Arrangement of Respiratory-Chain Components in Mitochondrial Membranes

By D. D. Tyler. (*Department of Physiology, Royal Veterinary College, University of London, London N.W.1, U.K.*)

Studies of the swelling behaviour of rat liver mitochondria and cytochrome *c*-deficient mitochondria indicate that the cristae membrane is impermeable to ferricyanide ions (cf. Mitchell & Moyle, 1969). Ferricyanide should therefore be a useful electron-accepting reagent to test whether respiratory-chain components exhibit a sidedness in their reactivity, as predicted by the chemiosmotic theory (Mitchell, 1966).

The reduced cytochromes *c* (+ c_1) and *a* of mitochondria treated with cyanide, succinate and malonate were oxidized by ferricyanide in an antimycin-insensitive reaction, whereas the oxidation of cytochrome *b* was blocked by antimycin, in agreement with Estabrook (1961). Similar studies with cytochrome *c*-deficient mitochondria showed that cytochrome c_1 oxidation is insensitive to antimycin, and revealed that cytochrome *a* oxidation proceeds slowly in the absence of cytochrome *c*. These results suggest that in mitochondria both cytochromes *c* and c_1 are situated, and react with ferricyanide, at the outer cristae surface, whereas neither cytochrome *b* nor cytochrome *a* is directly accessible to ferricyanide. The conclusion that the cytochrome *c*-binding site is situated at the outer cristae surface (Lenaz & MacLennan, 1966; Lee & Carlson, 1968; Muscatello & Carafoli, 1969) was confirmed by studies of the succinate oxidation system in submitochondrial digitonin-particles and sonic-particles. Most of the digitonin-particles present fragments of the outer cristae surface to the suspending medium, but most of the sonic-particles present the inner surface (Lee & Ernster, 1966; Malviya, Parsa, Yodaiken & Elliott, 1968). Succinate oxidation by cytochrome *c*-deficient digitonin-particles was restored by exogenous cytochrome *c*, whereas succinate oxidation by cytochrome *c*-deficient sonic-particles required both exogenous cytochrome *c* and membrane-lytic concentrations of Triton X-100.

With sonic-particles, reduced cytochromes *c* (+ c_1), *a* and *b* and the modified cytochrome *b* component reduced by succinate in the presence of antimycin (Chance, 1958) were all oxidized by ferricyanide in an antimycin-insensitive and thenoyltrifluoroacetone (1,1,1-trifluoro-3-then-2′-oylacetone)-insensitive reaction. The kinetics of cytochrome *a* oxidation by ferricyanide were similar in sonic-particles and in cytochrome *c*-deficient sonic-particles. Sonic-particles also catalyse thenoyltrifluoroacetone-insensitive succinate–ferricyanide reductase and succinate dehydrogenase activities (Tyler, Gonze, & Estabrook, 1966). The results with sonic-particles suggest that ferricyanide reacts with succinate dehydrogenase, cytochrome *b* and cytochrome *a*, and that in whole mitochondria all these components are situated at the inner cristae surface. Independent evidence that cytochromes *a* and a_3 are situated at the inner surface has been presented (Palmieri & Klingenberg, 1967; Mitchell & Moyle, 1967).

The present results are consistent with the view (Mitchell, 1966) that the respiratory chain contains components organized in a sided manner to provide a series of oxidoreduction loops, and suggest that cytochrome *b* may be the electron-carrying arm of loop 2.

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