RELEASE OF β-HYDROXYBUTYRIC APODEHYDROGENASE FROM BEEF HEART MITOCHONDRIA BY THE ACTION OF PHOSPHOLIPASE A.

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Most of the primary dehydrogenases of the mitochondrion are separated from the electron transport enzymes by relatively simple means. Procedures such as freezing and thawing, mild sonication, extraction with dilute salt, etc. are sufficient to dislodge these enzymes from the remainder of the mitochondrial membranes. In contrast, β-hydroxybutyric dehydrogenase is released only after treatment of mitochondrial fragments with cholate and ammonium sulfate (Sekuzu et al., 1963). Such reagents have a profound effect on the binding of phospholipid to protein (Fleischer and Brierley, 1961a; Fleischer et al., 1963).

The involvement of phospholipid in the function of mitochondrial enzymes was first demonstrated by depleting the mitochondrion of phospholipid with aqueous acetone and showing that rebinding of phospholipid led to reactivation of electron transport activity, (Fleischer et al., 1962). The purified β-hydroxybutyric dehydrogenase prepared with the cholate and ammonium sulfate procedure (Sekuzu et al., 1963) had a specific and absolute requirement for added lecithin. More recently, a phospholipid

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requirement for DPNH oxidation and Mg dependant ATP'ase activity was demonstrated after removal of phospholipid by digestion with phospholipase A. The hydrolysis products of phospholipase digestion, i.e. fatty acids and lysophosphatides, were washed out with a solution containing serum albumin (Fleischer et al., 1964; Casu et al., 1966).

In the present communication, we describe a new procedure for releasing β-hydroxybutyric dehydrogenase from the mitochondrion by digestion with phospholipase A. The specific requirement of the enzyme for lecithin could thus be confirmed with a procedure which does not involve treatment with bile acids. The results are compatible with the view that the enzyme exists in the mitochondrion complexed to phospholipid containing lecithin.

MATERIALS AND METHODS

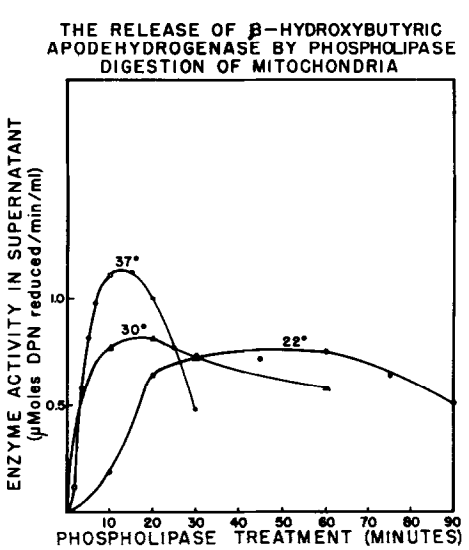
Lecithin was obtained from Sylvana Co., Orange, N. J. Before use it was further purified by chromatography on silicic acid (Fleischer et al., 1961b). Cardiolipin and phosphatidyl ethanolamine were isolated from beef heart mitochondrial phospholipids by chromatography on DEAE cellulose (Rouser et al., 1963). Phosphatidyl inositol, of wheat germ origin, was a gift from Dr. Coulon-Morelec, Institut Pasteur, Paris. Phospholipids were dispersed in aqueous medium prior to their use (Fleischer and Klouwen, 1961c). Crystalline bovine plasma albumin was obtained from Armour Pharmaceutical Co.

Naja naja venom was obtained from Miami Serpentarium Laboratories. Before use, it was boiled 5 min. according to Klibansky and DeVries (1963). "Heavy" beef heart mitochondria (Hatefi & Lester, 1958) were incubated with the venom (Edwards and Ball, 1954). Four micrograms of heated venom were added for each milligram of mitochondrial protein used. β-hydroxybutyric apodehydrogenase activity was measured as described by Jurtshuk et al., (1963).

Other analytical determinations including protein and phosphorus determinations were made as described previously (Fleischer et al., 1962).

RESULTS AND DISCUSSION

Treatment of "heavy" beef heart mitochondria with phospholipase A results in the release of β-hydroxybutyric apodehydrogenase into the medium.



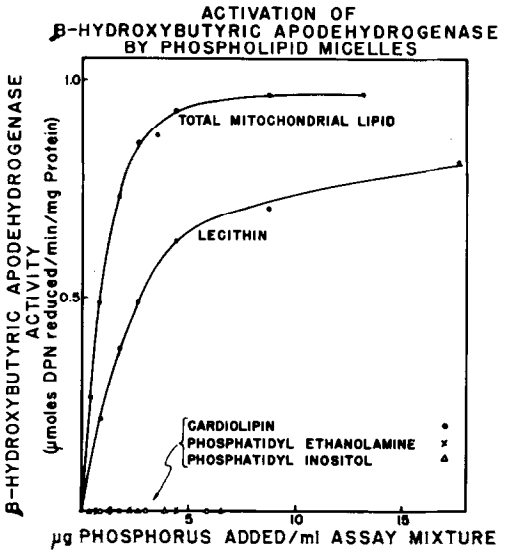


Figure 1. Mitochondria, to a final concentration of 10 mg protein/ml, were incubated with Naja naja venom as described in the text. At the end of the incubation time, samples were removed from the mixture, adjusted to 0.009 M with respect to EDTA, and sedimented at 59,000 x g for 15 minutes. The supernatants were assayed in the presence of 0.2 mg total mitochondrial lipid microdispersed as described in the text.

Figure 2. The enzyme was prepared by the treatment of mitochondria with phospholipase A at 37° C for 12.5 min (as in Fig. 1). The enzyme was partially purified by ammonium sulfate precipitation and reprecipitated twice in the presence of excess crystalline bovine plasma albumin as described in the text.

The release of enzyme as a function of the time and temperature is shown in Fig. 1. At temperatures lower than 37° C, the release of the enzyme is not as efficient. The yield of activity in the supernatant also decreases during prolonged incubation at 37° C whereas the activity recovered in the pellet after the optimum time remains the same. The decrease of activity recovered in the supernatant is thus probably due to inactivation of the enzyme in the supernatant.

The enzyme released by treatment with phospholipase A is inactive in the absence of added lipid (Fig. 2). Total mitochondrial lipids which are predominantly phospholipids, or mitochondrial phospholipids devoid of neutral lipids (Fleischer et al., 1961b), are most efficient in activating the enzyme. Of the purified phospholipids tested, lecithin alone is effective. Lipid-like materials such as Triton X-100, Tween-80 and fatty acids are not effective when added alone and are inhibitory when added together with mitochondrial phospholipid.

About 50% of the enzyme is released by the action of phospholipase A under optimal conditions (Table I). However, even the enzyme which is not released shows a complete dependence upon added phospholipid for activity, and indeed shows the same specificity for lecithin.

TABLE I. Distribution of β-Hydroxybutyric Apodehydrogenase after Treatment of Beef Heart Mitochondria with Phospholipase A

	Recovery (%)	Specific activity**
Original mixture*	100	0.11
Residue*	49	0.049
Supernatant*	51	0.22
50% saturated ammonium sulfate precipitate	48	1.18

** Assayed in the presence of mitochondrial phospholipid. The rate is expressed as μmoles DPN red/min/mg protein at 37° C.

* The experimental procedures are described in the text. The original mixture was sedimented at 30,000 rpm for 15 minutes (Spinco No. 40 rotor) to yield a supernatant and a residue.

The released enzyme can be partially purified from the supernatant by precipitation with 50% saturated ammonium sulfate. The enzyme in this purified form, as well as in the original supernatant, loses most

of its activity when stored in the frozen state for several days. It is considerably stabilized by reprecipitation with 50% saturated ammonium sulfate in the presence of a 1% crystalline bovine plasma albumin solution (Fleischer et al., 1964). When reprecipitated twice in this manner, the preparation is devoid of cytochromes, exhibits no malic or isocitric dehydrogenase activity, and no DPNH or succinate-cytochrome c reductase activity. The α-ketoglutaric and pyruvic dehydrogenase activities, however, are enriched in our purified β-hydroxybutyric dehydrogenase preparation.

The more highly purified enzyme of Sekuzu et al., (1963) exhibits a complete dependence on added sulfhydryl containing reagents. In contrast, our preparation, which has been stabilized by reprecipitation, does not show such a requirement. Twenty to thirty percent enhancement of activity is observed when the assay mixture contains 50 mM cysteine.

The specific activity of the partially purified preparation described in the present paper is equivalent to that of fraction ID previously reported (Sekuzu et al., 1963) and is achieved in a much more rapid and simple manner. The specific requirement for lecithin previously reported for this enzyme is now confirmed by an independent isolation procedure which does not involve the use of bile acids. The release of β-hydroxybutyric dehydrogenase during breakdown of the phospholipid component of the mitochondrial membranes provides strong evidence for binding of enzyme to phospholipid in the membranes as well as for the involvement of lipid in the function of the enzyme in situ.

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