Rat Liver D-β-Hydroxybutyrate Dehydrogenase. II. Lipid Requirement*

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ABSTRACT: The role of lipid in the activation of solubilized rat liver D-β-hydroxybutyrate dehydrogenase was investigated. The requirement for lipid was specific for phosphatidylcholine; cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, and sphingomyelin were all inactive and inhibited the activation by phosphatidylcholine. Several natural phosphatidylcholines were found to be effective activators of the enzyme. The removal of the plasmalogen from beef heart phosphatidylcholine reduced the effectiveness of the lipid as an activator. The addition of cholesterol to the lecithin micelles reduced the effectiveness of the lecithin as an activator but did not appear to interfere with the stoichiometry of binding between the micelles and the protein. The lipid was found to activate the enzyme more efficiently at lower pH. The results are interpreted in terms of lipid binding sites on the enzyme molecules that are specific with respect to both charge and hydrophobic composition of the lipid.

The previous paper (Gotterer, 1967) in this series discussed the isolation and general properties of rat liver mitochondrial βOHDH.[1] It was shown that the enzyme must be preincubated in the presence of pyridine nucleotide, lipid, and high concentrations of thiol in order to recover enzymatic activity from isolated enzyme preparations. The requirement for thiol was absolute over a wide pH range, but the requirement for added lipid and pyridine nucleotide became less as the pH was lowered. The specificity and other characteristics of the lipid requirement are described in this paper.

Experimental Procedures

Procedures for the isolation and assay of the enzyme preparation and for the addition of lipids to the preincubation medium have been described previously (Gotterer, 1967). Cholesterol was obtained from Pfanstiehl Laboratories, Inc., phosphatidylserine from Applied Science Laboratories, and egg and beef heart lecithin from Sylvana Chemical Co.

Lipids were extracted and washed by the method of Folch *et al.* (1957) and Ways and Hanahan (1964). Lipid classes were separated by column chromatography on silicic acid. Sphingomyelin was isolated from sheep red blood cells by the following procedure. The plasma from about 1 l. of citrated sheep blood was removed by centrifugation and the cells were washed with saline. The washed cells (360 ml) were blended with 4 and then 2 l. of acetone. The residue was recovered by filtration and extracted overnight with 1900 ml of ether and then reextracted with an additional 635 ml of ether. The combined ether extracts were taken to dryness *in vacuo* and the residue was extracted with 500 ml and then 200 ml of hexane–acetone (1:1, v/v). The residue was taken up in hot absolute ethanol and was predominantly sphingomyelin, with traces of phosphatidylethanolamine and lysophosphatidylcholine. The sphingomyelin was finally purified by chromatography on a silica gel G column, using chloroform–methanol–acetic acid–water (25:15:4:2, v/v) as eluting solvent.

Lysophosphatidylcholine, prepared from egg phosphatidylcholine by treatment with snake venom and purified by recrystallization, was kindly supplied by Dr. N. A. Gregson. Plasmalogen was removed from beef heart phosphatidylcholine by treatment at 37° for 16 hr with 90% acetic acid (v/v), neutralization with NaOH, and extraction of lipids by the method of Bligh and Dyer (1959). The phosphatidylcholine was repurified by column chromatography on silicic acid, eluting with chloroform–methanol (2:1, v/v). The purity of all lipids was verified by thin layer chromatography on silicic acid, using chloroform–methanol–water (95:35:4) and/or chloroform–methanol–acetic acid–water (25:15:4:2, v/v) as solvent systems.

Plasmalogen was determined by the method of Williams *et al.* (1962) except that ethyl acetate was used to extract iodine. Cholesterol was estimated by the Lieberman–Burchard reaction as described by Stadtman (1957). Total phosphorus was determined by the method of Gomori (1942) after prior digestion with H_2SO_4 and H_2O_2. Protein was determined by the method of Lowry *et al.* (1951).

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[1] Abbreviations used: βOHDH, D-β-hydroxybutyrate dehydrogenase; NAD, nicotinamide–adenine dinucleotide.

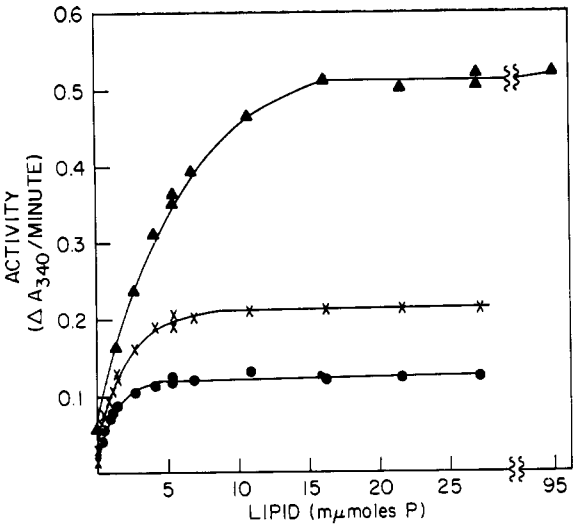

FIGURE 1: Activation as a function of lipid added to preincubation. Preincubation medium: Tris-Cl (pH 8.1) 60 mM; thioglycerol (pH 8.1), 200 mM; NAD, 4 mM; and mixed mitochondrial lipids as indicated; protein (●—●) 12.7 μg, (X—X) 25.4 μg, and (▲—▲) 50.8 μg in a total volume of 0.1 ml. Incubated at 29° for 40 min. Assayed in 2.0 ml under standard conditions.

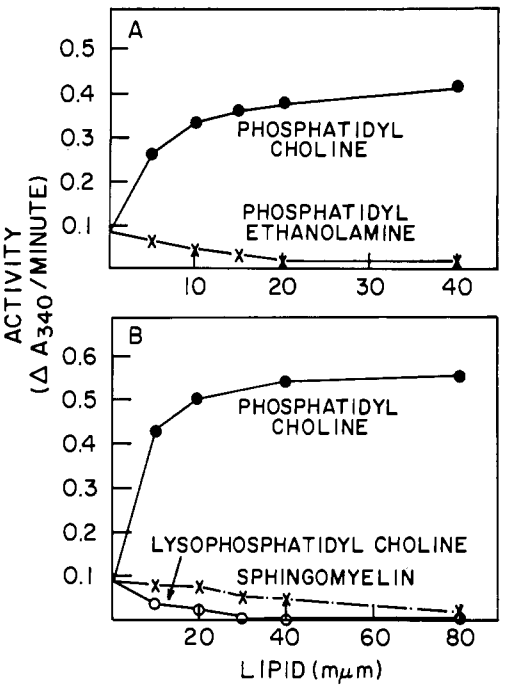

FIGURE 3: Inhibition by purified lipids. Preincubation medium: Tris-Cl, 55 mM; thioglycerol, 180 mM; NAD, 3.6 mM; lipid as indicated; and enzyme, 30 (expt A) and 50 μg (expt B) in a total volume of 0.11 ml. After preincubation at 29° for 30 min, activity was assayed in standard system.

Results

As seen in Figure 1, the addition of increasing amounts of lipid to the preincubation medium resulted in an increased recovery of enzymatic activity, until saturation was reached. The amount of lipid required to achieve maximum activation was related to the amount of protein present in the preincubation medium.

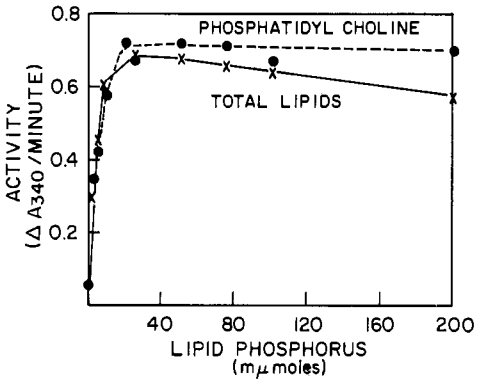

FIGURE 2: Activation by purified phosphatidylcholine and by mixture of lipids. Preincubation medium: Tris-Cl (pH 8.1) 60 mM; NAD, 4 mM; thioglycerol (pH 8.1), 200 mM; lipid as indicated; and enzyme, 30 μg in a total volume of 0.1 ml. Preincubated at 29° 30 min. Assayed in total volume of 2.0 ml under standard conditions.

In the experiment illustrated in Figure 1, about 0.32 μmole of lipid phosphorus/mg of protein was required to achieve full saturation. The addition of lipid caused approximately an eightfold increase in activity over that obtained by preincubation in the absence of added lipid. This particular enzyme preparation contained about 0.043 μmole of endogenous lipid phosphorus/mg of protein. The results indicate, therefore, that the endogenous lipid is present in sufficient amounts to account for the enzymatic activity of the preparation obtained in the absence of added lipid. Both the amount of lipid required to saturate the enzyme and the increase in activity obtained by saturating amounts of lipid were found to vary from one preparation to the next, but remained constant for any one enzyme preparation.

At pH 8.1, where maximum activity is recovered, maximum activation was obtained with a ratio of lipid to protein of 0.08–0.33 μmole of lipid phosphorus/mg of protein. The usual value was around 0.2 μmole. The amount of endogenous activity varied between 1 and 25% of that obtained after activation in the presence of saturating amounts of lipid. However, values between 5 and 10% were obtained with more than 80% of the preparations. There appeared to be no correlation between these variables (amount of lipid required and the increased activation observed) and the specific activity of the preparation.

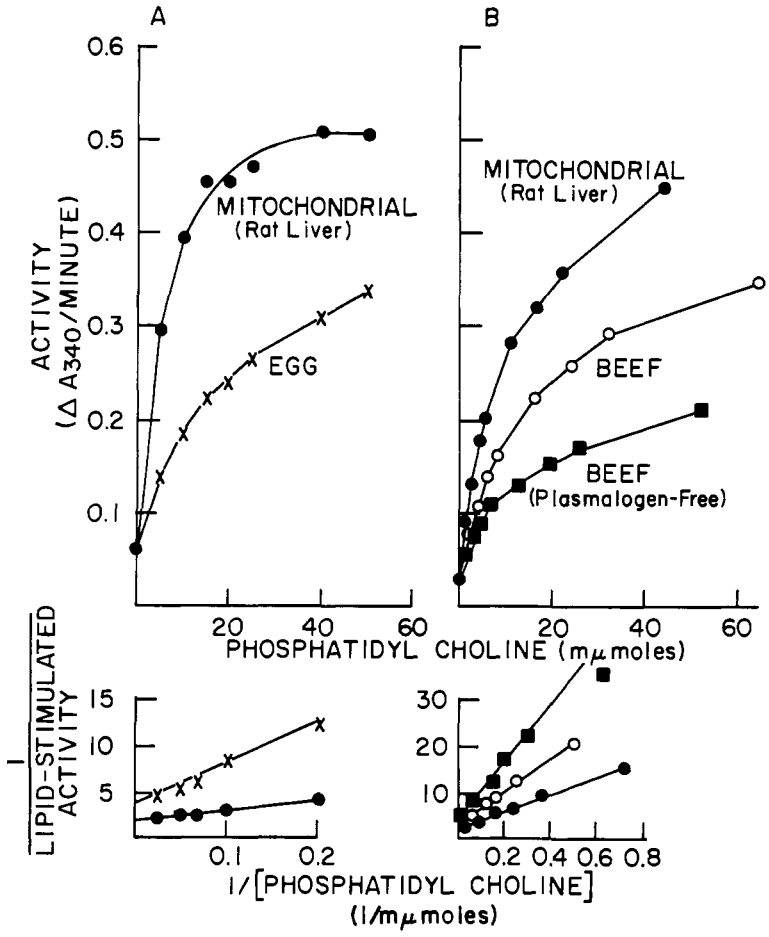

FIGURE 4: Comparison of phosphatidylcholines. Preincubation medium: Tris-Cl (pH 8.1), 60 mM; NAD, 4 mM; thioglycerol (pH 8.1), 200 mM; lipid as indicated; enzyme (A) 30 and (B) 75 μg, in a total volume of 0.1 ml. Preincubated 29° for 30 min. Assayed in 2.0 ml under standard conditions.

The lipid requirement was found to be highly specific for phosphatidylcholine or lipid preparations containing phosphatidylcholine, as had been found for the detergent-solubilized enzyme from beef heart (Sekuzu *et al.*, 1961). Activation by a crude mixture of mitochondrial lipids and by a preparation of phosphatidylcholine isolated therefrom is shown in Figure 2. It is seen that both the extent of activation and the absolute requirement, on a molar lipid phosphorus basis, are the same for both the mixture, which contained about 30% phosphatidylcholine, and the purified phosphatidylcholine. Such results have been obtained consistently and agree with the observations of Sekuzu *et al.* (1961) with the beef heart enzyme. Attempts to find an additional activating factor in the lipid extract have been unsuccessful. It would appear, then, that the phosphatidylcholine present within a mixed micelle containing a complete complement of mitochondrial lipids is more active on a molar basis than the phosphatidylcholine present in a pure phosphatidylcholine micelle.

No other class of phospholipids isolated either from mitochondria or from other sources could replace phosphatidylcholine in activating the enzyme. The following purified lipids were tested and found to be inactive: cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, and sphingomyelin. As seen in Figure 3, the addition of purified lipids other than phosphatidylcholine to the preincubation medium in fact resulted in the inhibition of the low-level endogenous activity of the enzyme preparation.

On the other hand, phosphatidylcholines from several natural sources were all effective in activating the enzyme, as seen in Figure 4. Phosphatidylcholine isolated from the same source as the enzyme (rat liver mitochondria) was more effective than the other phosphatidylcholines, in that a lower amount of lipid was required to activate the enzyme. When the data for low levels of lipid are replotted according to the method of Lineweaver and Burk (1934), extrapolation to infinite lipid concentration would suggest that the difference in the effectiveness of the phosphatidylcholine

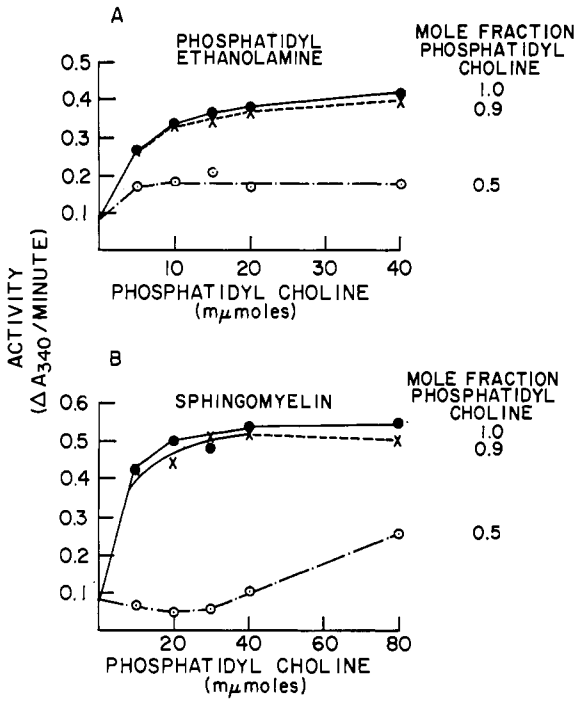

FIGURE 5: Effect of addition of other phospholipids to phosphatidylcholine micelles. Preincubation medium: Tris-Cl (pH 8.1), 60 mM; NAD, 4 mM; thioglycerol (pH 8.1), 200 mM; lipid as indicated; and enzyme, 30 μg, in a total volume of 0.11 ml. Preincubated at 29° for 30 min. Assayed under standard conditions in total volume of 2.0 ml.

preparations was in part due to the differences in their binding constants. Figure 4B also demonstrates that beef heart phosphatidylcholine freed of its plasmalogen component was still an effective activator of the enzyme, but not quite as effective as the untreated, plasmalogen-containing preparation.

Activation of the enzyme by mixed micelles containing phosphatidylcholine and another purified lipid was not as effective as activation by pure phosphatidylcholine micelles, as seen in Figure 5. The degree of inhibition was inversely related to the phosphatidylcholine content of the lipid preparation. Of special significance is the effect of cholesterol on the activation by phosphatidylcholine. Cholesterol has no effect on the charge characteristic of the micelle and presumably interacts with the hydrophobic portion of the phosphatidylcholine molecule in the interior of the micelle structure (Price, 1933) (van Deenen *et al.*, 1962). In Figure 6 it is shown that cholesterol reduced the effectiveness of the phosphatidylcholine activation. The data are plotted as a function of the phosphatidylcholine added to the preincubation medium along the abscissa. As the mole fraction of phosphatidylcholine was decreased the maximum activity recoverable also decreased. It is to be noted that the enzyme was saturated with phosphatidylcholine at the same equivalent point for each phosphatidylcholine–cholesterol micelle preparation, regardless of the relative content of the two components. It would appear that the cholesterol interferes not so much with the stoichiometric aspects of the binding between phosphatidylcholine and enzyme, but with the effectiveness with which the bound phosphatidylcholine is able to activate the enzyme.

Activation as a function of the amount of lipid added was studied at several pH values. Figure 7 demonstrates that maximum activation occurred at pH 8. At higher pH values the lipid was less effective in activating the enzyme, but approached that at the optimum pH. At lower pH values (*i.e.*, 6.25) maximum activation achieved was far less than at the optimum pH and was not increased by using higher levels of lipid. In this experiment, with a mixed lipid preparation, activation at pH 6.25 is seen to have fallen off after the maximum was reached. In comparable experiments with pure lecithin micelles the lower maximum occurred at pH 6.25, but the activity remained at that maximum level without being inhibited by higher concentrations of lipid.

The effect of lipid on the kinetic properties of the enzyme was examined. The enzyme was activated to different levels of activity by varying the ratio of lipid to protein present during the preincubation and assaying the activated complex with varying concentrations of substrate. Figure 8 indicates that as the enzyme became increasingly saturated with lipid there occurred not only an increase in the V_{max}, but also an increase in the K_m for both β-hydroxybutyrate and NAD. Such findings would indicate that a profound alteration in the catalytic site of the enzyme occurred as the enzyme became activated by lipid.

Discussion

The lipid requirement is clearly highly specific for phosphatidylcholine. All other lipid classes were inactive when tried alone or inhibited when tried in combination with phosphatidylcholine. The charge and composition of the hydrophilic part of the lipid are, therefore, important in determining specificity.

It has been shown that phosphatidylcholine micelles are isoelectric over a wide pH range, from 1 to 13 (Bangham and Dawson, 1958). In the light of studies such as those illustrated in Figure 7, which demonstrate the altering effectiveness of phosphatidylcholine as an activator as a function of the pH of the preincubation medium, it can be concluded that the charge variations on the protein component of the lipoprotein complex plays the dominant role in controlling the extent of activation of the complex.

The interaction of phosphatidylcholine with its binding sites on the protein must be quite extensive, however, and must involve more than a superficial interaction of charged groups. The enzyme is able to discriminate between phosphatidylcholine and sphingomyelin, a lipid which has the same charged characteris-

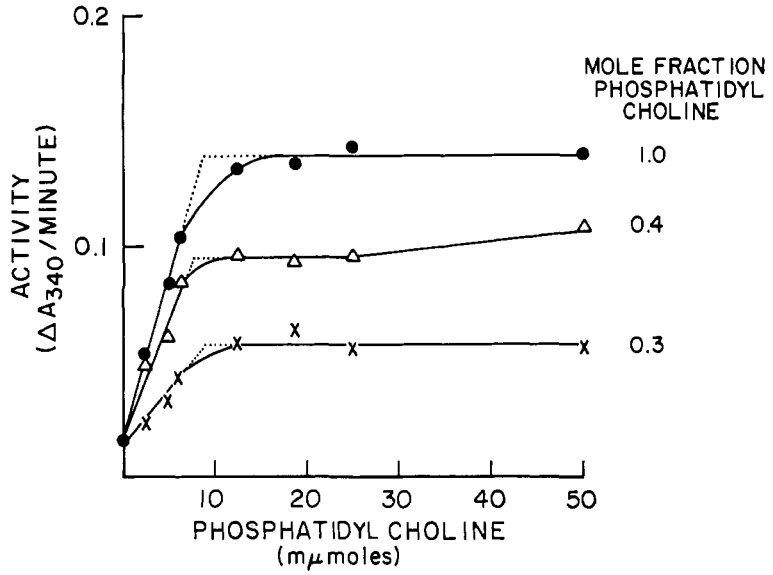

FIGURE 6: Effect of cholesterol on phosphatidylcholine activation. Preincubation medium: Tris-Cl (pH 8.1), 60 mM; NAD, 4 mM; thioglycerol, 200 mM; lipid as indicated; and enzyme, 30 μg, in a total volume of 0.11 ml. Preincubated at 29° for 30 min. Assayed in total volume of 2.0 under standard conditions.

tics as phosphatidylcholine, and as pointed out by Vandenheuvel (1963), has a three-dimensional conformation similar to phosphatidylcholine. Also, the enzyme was more effectively activated by purified beef heart phosphatidylcholine containing plasmalogen than by a preparation from which the plasmalogen had been removed. Here again, there is discrimination beyond the charged lipid–protein interface, in the region of the more hydrophobic portion of the lipid molecule. The results with mixed phosphatidylcholine–cholesterol micelles supports the conclusion of a deep interpenetration of phosphatidylcholine and enzyme. In such mixed micelles the cholesterol is thought to line up not at the charged surface of the micelle, but among the hydrophobic tails of the phospholipid (Price, 1933; van Deenen *et al.*, 1962; Vandenheuvel, 1963). In the experiments presented in this paper it was found that the cholesterol did not alter the ratio of phosphatidylcholine to protein at which maximum activation of βOHDH was achieved. The cholesterol did, however, decrease the level of activation obtained. It would appear that interaction between phosphatidylcholine and the enzyme by ionic binding alone will not result in activation of the enzyme. Interaction of the more deeply located hydrophobic portions of the micelle and protein must also take place.

The time dependence of the activation process, demonstrated in the previous paper (Gotterer, 1967), may in part be explained by the need for such interpenetration. Studies of the interaction of proteins and lipids in surface films (Matalon and Schulman, 1949) have demonstrated a biphasic character to the interaction. This has been interpreted as a rapid phase of ionic binding and a slower phase of interpenetration of hydrophobic groups. Analogous events would appear to be occurring during the activation of βOHDH

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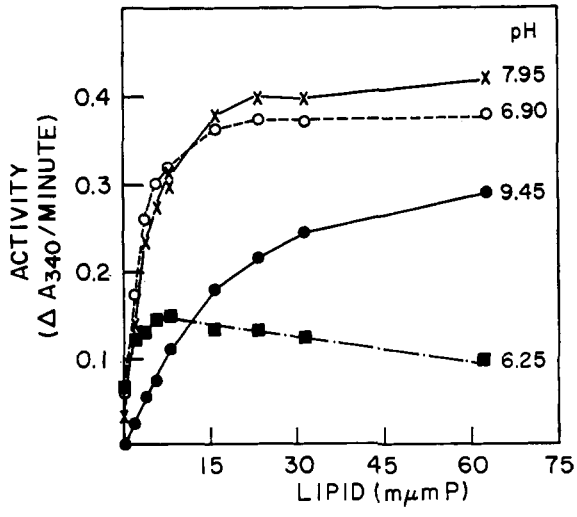

FIGURE 7: Activation as a function of added lipid at different pH values. Preincubation medium: Tris-phosphate–carbonate, 20 mM, adjusted to appropriate pH by addition of minimum amount of NaOH or HCl; NAD, 4 mM; thioglycerol, 200 mM, adjusted to appropriate pH with NaOH; lipid (mixed mitochondrial) as indicated; and enzyme, 50 μg, in a total volume 0.11 ml. Preincubated at 29° for 30 min. Assayed in total volume of 2.0 ml under standard conditions.

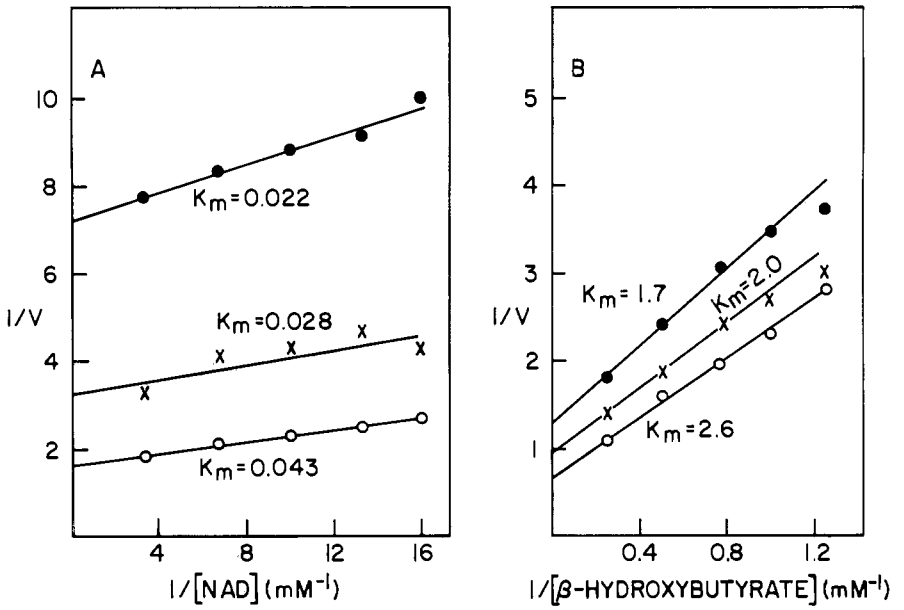

FIGURE 8: Effect of lipid activation on kinetic properties of enzyme. Preincubation medium: (A) Tris-Cl (pH 8.1), 60 mM; thioglycerol (pH 8.1), 200 mM; mixed mitochondrial lipids, (●) 45, (X) 90, and (○) 180 mμmoles; and enzyme, 1.37 mg, in a total volume 0.6 ml. (B) Tris-Cl (pH 8.1), 60 mM; NAD, 4 mM; thioglycerol (pH 8.1), 200 mM; mixed mitochondrial lipid (●) 90, (X) 120, and (○) 180 mμmoles; and enzyme, 840 μg, in a total volume 0.6 ml. Preincubated at 29° for 40 min. Medium kept at 0° while 0.1-ml aliquots were assayed in 2.0 ml of assay medium, containing 0.075 M Tris-Cl (pH 8.1). (A) BOH (10 mM) and NAD (as indicated); (B) NAD (1 mM) and BOH (as indicated).

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