Influence of Cephalin and Egg Lecithin on Pig Heart Malate Dehydrogenase Activity

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Abstract □ A requirement for phospholipids in the activity of a number of membrane-bound enzymes is recognized; *i.e.*, enzyme activity is reduced or lost entirely when the lipid is removed and is restored or increased when this specific lipid is added back to the system. In general, however, only the effects of relatively low lipid concentrations ($\sim 10^{-4}$ g/100 ml) have been reported. In this investigation, the influence of phospholipids (egg lecithin and bovine brain cephalin) at concentrations approaching normal human plasma levels was determined on the activity of malate dehydrogenase dispersed in water. The enzyme activity was measured by the rate of oxidation of nicotinamide adenine dinucleotide (reduced) in the presence of the substrate oxalacetic acid. On the addition of cephalin, malate dehydrogenase activity decreased with the increasing concentration of this phospholipid from 5×10^{-4} to 2×10^{-2} g/100 ml. Similarly, the addition of egg lecithin resulted in a decrease in enzyme activity. In no instance was any increase in activity observed. Moreover, at all concentrations of egg lecithin, the reduction in activity was less than that observed in the presence of equal concentrations of cephalin. Monolayer studies provided evidence that, whereas egg lecithin interacts to a significant degree with the substrate oxalacetic acid, cephalin shows relatively little interaction with that material. The difference observed in the inhibitory action of these two phospholipids may be related to the difference in their tendency to react with the substrate.

Keyphrases □ Malate dehydrogenase activity and stability—effect of phospholipids (cephalin and egg lecithin), substrate considerations □ Phospholipids (cephalin and egg lecithin)—effect on malate dehydrogenase activity and stability, substrate considerations □ Enzyme inhibition—effect of phospholipids (cephalin and egg lecithin) on malate dehydrogenase activity

The activity and stability of a number of membrane-bound enzymes have been shown to have a lipid requirement; *i.e.*, enzyme activity and stability are lost or reduced when the lipid is removed from the enzyme system. The mechanism of these effects is, in general, considered to involve either: *(a)* lipid interaction with and activation of the substrate; or *(b)* a direct lipid–enzyme hydrophobic group interaction, the result of which is a conformational change of the protein. This latter mechanism is believed to be operative with some dehydrogenases (1).

Usually, the lipid requirement exhibits some specificity. Sekuzu *et al.* (2) and Fleischer *et al.* (3) showed that phosphatidylcholine was the only lipid able to activate β-hydroxybutyrate dehydrogenase, whereas other lipids, including phosphatidylethanolamine, phosphatidylinositol, cardiolipin, sphingomyelin, and neutral lipids, were inactive. In the case of cytochrome c, it was reported that cardiolipin and phosphatidylethanolamine were the most effective activators in the mitochondrial electron-transfer system (4). Similarly, Callahan and Kosicki (5) observed an increase in activity and stability of a mitochondrial malate dehydrogenase in the presence of lipids extracted from the same tissue as the enzyme as well as an increase in enzyme activity in the presence of a series of saturated and unsaturated fatty acids. At higher concentrations of these fatty acids, however, enzyme inhibition was observed. It thus appears that both lipid specificity and lipid concentration are involved in the activity of lipid-requiring enzymes.

It was of interest, therefore, to study the activity and stability of malate dehydrogenase in the presence of phospholipids from sources different than that of the enzyme itself. Furthermore, plasma levels of phospholipids are considerably higher than those used in most previous studies on lipid-requiring enzymes. Therefore, studies were performed to determine the effect of lipids at concentrations approaching plasma levels on malate dehydrogenase activity and stability.

EXPERIMENTAL

Materials and Equipment—Malate dehydrogenase[1] (porcine heart), activity 260 units/mg, was supplied as a suspension in 70% saturated ammonium sulfate solution. The lipids used were egg lecithin[1], highly purified, and cephalin[2], bovine brain tissue. Oxalacetic acid (*cis*-enol) and nicotinamide adenine dinucleotide[1], reduced (NADH), 90%, were used to measure the activity of the enzyme. The *n*-hexane[3] was 99 mole % pure, and all other chemicals were reagent grade. The water used was first deionized by passing it through a mixed resin bed and then distilled from an all-glass distilling apparatus[4].

Spectrophotometric measurements were made using a spectrophotometer[5] equipped with a recorder[6]. Surface tension measurements were made by the Wilhelmy plate method (6) using a thin, roughened platinum plate suspended from a torsion balance[7]. Surface pressure, π, defined as surface tension of the subphase in the absence of the film minus the surface tension of the subphase in the presence of the film, was determined from these measurements. The film balance[8] consists of a 540-ml capacity Langmuir-type Teflon trough equipped with a Teflon movable barrier. The barrier could be disengaged from the lead screw for rapid sweeping of the surface. The entire film balance was enclosed in a Plexiglas cabinet to avoid possible contamination by dust and other airborne impurities.

All experiments were conducted at room temperature (25 ± 2°).

Preparation of Solutions—For the enzyme activity studies, stock solutions of oxalacetic acid were prepared each day by dissolving 20 mg of oxalacetic acid in 100 ml of a phosphate buffer (pH 7.4). The NADH, 12.4 mg, was dissolved in 25 ml of the phosphate buffer and used only for 1 hr, after which it was discarded. Both stock solutions were stored at 0° between experiments.

One gram of egg lecithin was dispersed in 10 ml of water, and the dispersion was stirred at high speed for 1 hr. Water was then added to make 100 ml, and the dispersion was stirred at high

[1] Schwarz/Mann, Orangeburg, N.Y.
[2] Natural Biochemical Laboratories.
[3] J.T. Baker Chemical Co., Phillipsburg, N.J.
[4] Model AG-1a, Corning Glass Works, Corning, N.Y.
[5] Perkin-Elmer 139, Perkin-Elmer Corp., Norwalk, Conn.
[6] Perkin-Elmer 159.
[7] Federal Pacific Electric Co., Newark, N.J.
[8] Frater Instrument Co., Corona, N.Y.

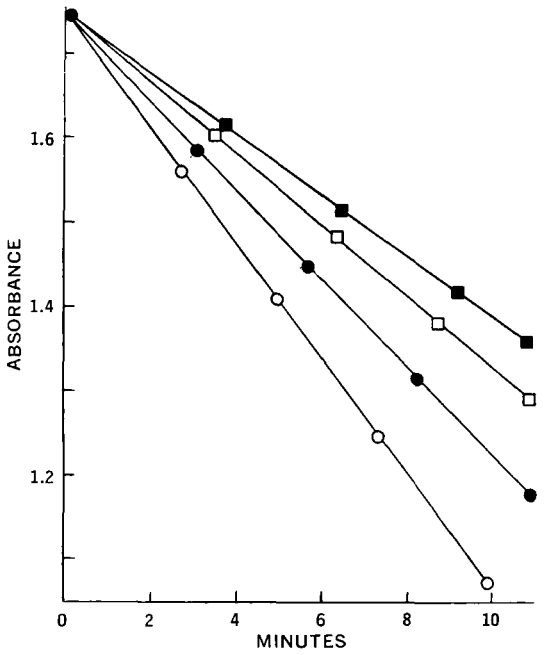

Figure 1—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase dispersed in water and incubated for various periods at 25 ± 2°. Key (incubation times prior to assay): ○, 0 min; ●, 15 min; □, 30 min; and ■, 60 min.*

speed for another hour. The resultant stock dispersion was prepared fresh each day. Dispersions of cephalin (1 g/100 ml) were prepared in the same way as the stock dispersions of egg lecithin. These were stored in the freezer and used for a maximum of 3 days.

In all instances where stock solutions or dispersions were stored as specified, no differences were observed between experiments using the stored and the freshly prepared materials.

One-hundredth milliliter of the enzyme was measured, using a 0.1-ml disposable pipet, and dispersed in 50 ml of water contained in a 50-ml volumetric flask. The flask was inverted twice to ensure complete mixing.

Various concentrations of egg lecithin and cephalin were prepared by measuring the required amounts of the stock solution into a 50-ml volumetric flask and adding sufficient distilled water. These dispersions were stirred for 30 min. Enzyme (0.01 ml) was then added to the suspension, and the resulting dispersion was mixed for 1 min by inverting the flask twice.

Spreading solutions for the monomolecular film studies were prepared as follows: 38.8 mg of egg lecithin or 62.5 mg of cephalin was dissolved in a 1:99 (v/v) ethanol–hexane solvent mixture to make 100 ml of the respective spreading solution.

Procedure—The absorbance of various concentrations of NADH in water (from 6.25 to 25 mg/100 ml) was determined at 340 nm and a Beer's law plot was prepared.

The enzyme activity was measured by adding 0.3 ml of the stock solution of the malate dehydrogenase to 2 ml of the NADH solution and 2 ml of the oxalacetic acid solution contained in a test tube. The solution was immediately transferred to a 1-cm quartz cell, and its absorbance was recorded for 9–11 min. The slope of the absorbance–time curve, which represents the rate of enzyme-induced oxidation of NADH to NAD, was used as a measure of malate dehydrogenase activity.

In determining the effect of incubation time on enzyme stability, the enzyme dispersion, with or without lipid added, was stored for various periods at 25 ± 2° prior to measuring enzyme activity.

In the monolayer experiments, the trough was filled with a 0.1 *M* phosphate buffer (pH 7.4) alone or with varying concentrations of oxalacetic acid. The surface was then swept several times with the barrier to remove surface-active contaminants; suction was used to adjust the level of the subphase and to remove any remaining traces of dust or other contaminants. The platinum plate was then lowered into position and allowed to remain beneath the surface while the films were applied from their appropriate spreading solvents with the aid of a micrometer syringe[9]. This syringe can deliver accurately volumes as small as 0.001 ml. Generally, 0.07 ml of the spreading solution was applied onto the subphase by allowing small drops to fall from the syringe held a few millimeters away from the subphase surface. Fifteen minutes was allowed for complete evaporation of the spreading solvent from the surface before manual compression of the film was initiated.

RESULTS AND DISCUSSION

The effect of incubating the malate dehydrogenase enzyme for various time periods in water before the reaction was initiated is shown in Fig. 1. These plots represent incubation periods of 0, 15, 30, and 60 min at room temperature (25 ± 2°). As shown by the change in the slope of the absorbance *versus* time curve from 0.064 at 0 min to 0.034 at 60 min, about one-half of the activity of the enzyme was lost after 1 hr of incubation. This demonstrates that the enzyme is quite unstable when dispersed and incubated in water.

The enzyme was then dispersed in water in the presence of varying concentrations of cephalin, and its activity was determined at 0- and 60-min incubation times (Fig. 2). At concentrations of cephalin as low as 5×10^{-4} g/100 ml at zero incubation time, the activity of the enzyme decreased markedly. Increasing concentrations of cephalin under this condition resulted in further decreases in the activity of the malate dehydrogenase, reaching almost zero activity at a concentration of 2×10^{-2} g/100 ml of cephalin. Incubation of the enzyme for 60 min with all concentrations of cephalin (from 5×10^{-4} to 2×10^{-2} g/100 ml) resulted in total inactivation of the enzyme. By comparison, significant enzyme activity remained on incubation in water alone for the same period (Fig. 2).

The enzyme also showed decreasing activity at zero incubation time with increasing concentrations of egg lecithin (Fig. 3). However, the effect was not as marked as it was with cephalin. Even at high concentrations of egg lecithin (4×10^{-2} g/100 ml), the enzyme still retained about two-thirds of the activity observed in water alone. Furthermore, this reduction in enzyme activity was not observed until the concentration of egg lecithin reached 2.5 ×

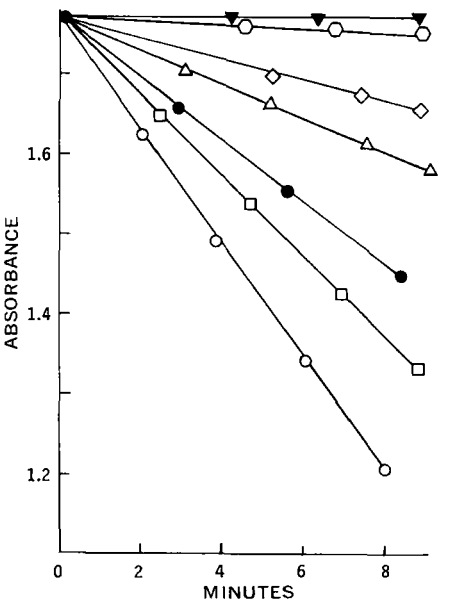

Figure 2—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase and cephalin dispersed in water and incubated for 0 (open symbols) and 60 (closed symbols) min. Key (amount of cephalin/100 ml of dispersion): ○ and ●, 0; □, 5×10^{-4} g; △, 1×10^{-3} g; ◊, 2×10^{-3} g; ⬡, 2×10^{-2} g; and ▼, all concentrations after 60 min incubation.*

[9] Agla.

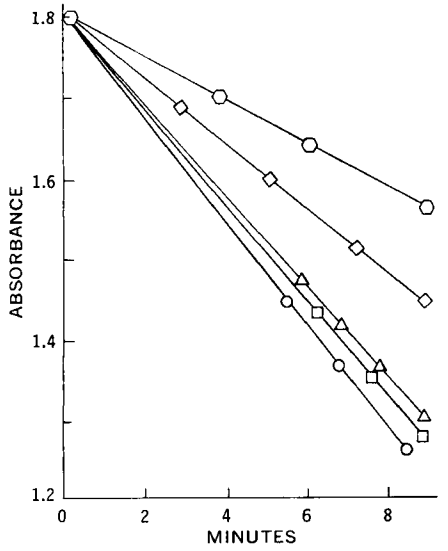

Figure 3—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase and egg lecithin dispersed in water and incubated for 0 min. Key (amount of egg lecithin/100 ml of dispersion): ○, 0; □, 2.5 × 10^{-3} g; △, 5 × 10^{-3} g; ◊, 2 × 10^{-2} g; and ⬡, 4 × 10^{-2} g.*

10^{-3} g/100 ml as compared to 5 × 10^{-4} g/100 ml for cephalin. However, as was the case with cephalin, all enzyme activity was lost after incubation for 60 min in the presence of all concentrations of egg lecithin studied.

The difference in the concentration of these two lipids needed to decrease the enzyme activity can be seen in Fig. 4, which shows plots of lipid concentration *versus* percent of NADH oxidized per minute. These differences, as well as the difference in the shape of these two curves, suggest that the mechanism of this effect may not be the same.

Surface studies were used to determine whether these differences were due, in part, to a difference in the interaction between the lipid and the substrate. This interaction was determined by spreading the lipids as monolayers over a subphase containing oxalacetic acid and measuring changes in the surface pressure-

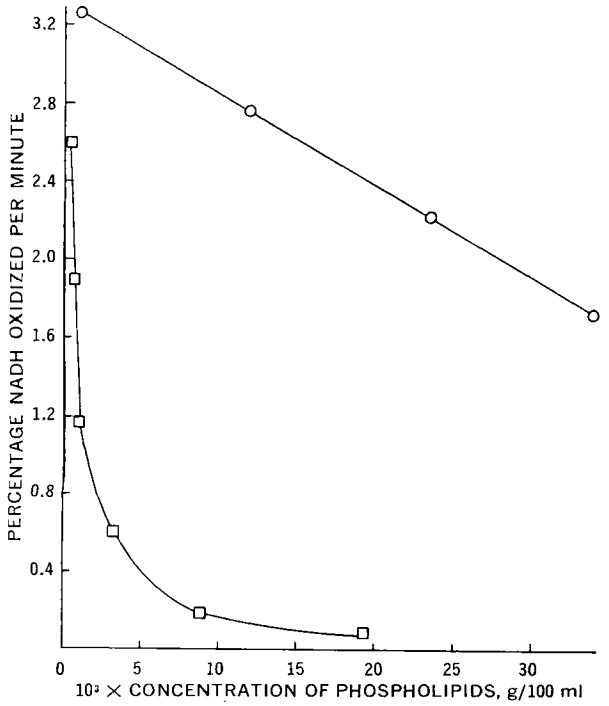

Figure 4—*Percentage of NADH oxidized per minute as a function of the concentration of phospholipid (g/100 ml). Key: □, cephalin; and ○, egg lecithin.*

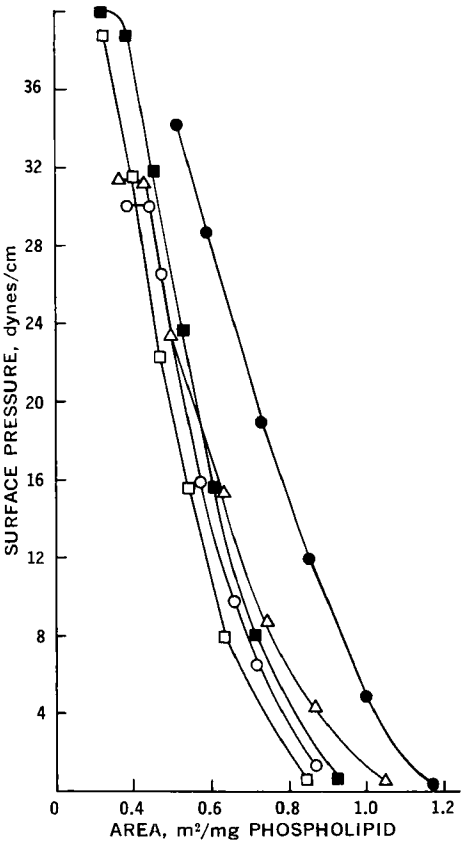

Figure 5—*Surface pressure-surface area curves of the phospholipids spread on a phosphate buffer subphase containing oxalacetic acid. Key (cephalin spread on following concentrations of oxalacetic acid): □, 0, 0.2, and 0.4%; and ■, 2.0%. Key: (egg lecithin spread on following concentrations of oxalacetic acid): ○, 0 and 0.2%; △, 0.4%; and ●, 2.0%.*

surface area (π-A) curves. Figure 5 shows the π-A curves of cephalin spread on a buffer solution and in the presence of various concentrations of oxalacetic acid. Only in the presence of very high concentrations of oxalacetic acid (10 times the amount used in the enzyme activity studies) was there a shift in the π-A curves to larger areas. Even at high concentration, the shift was small and the collapse pressure remained the same, indicating only a weak interaction between oxalacetic acid and cephalin.

In contrast, a comparison of the π-A curves of egg lecithin spread on buffer solution alone and in the presence of various concentrations of oxalacetic acid shows a significant shift in the π-A curves to larger areas (Fig. 5). This effect was observed even at low concentrations of oxalacetic acid (identical to that used in the enzyme activity studies). An increase in collapse pressure of the film also was observed at all concentrations of oxalacetic acid in the subphase. These data suggest that a strong interaction between egg lecithin and oxalacetic acid develops at the concentration levels used in the enzyme activity studies. It appears, however, that there is no significant interaction between oxalacetic acid and cephalin at similar concentrations.

Siegel and Englard (7) suggested that malate dehydrogenase activity is dependent on the oxalacetic acid concentrations and that there is an optimum concentration of oxalacetic acid at which the malate dehydrogenase activity is at a maximum. Any deviation from this optimum concentration decreases the enzyme activity. It appears then that the interaction between oxalacetic acid and egg lecithin may be the basis for the observed decrease in the reaction rates of malate dehydrogenase, while in the case of cephalin the decreased reaction rate may be due to the interaction between the enzyme and the phospholipid.

The activity of the enzyme dropped almost to zero after 30 min of incubation at room temperature (25 ± 2°) at all concentrations of cephalin and egg lecithin. This finding indicates that egg lecithin and cephalin not only do not stabilize or potentiate the activity of malate dehydrogenase but actually reduce its activity.

Callahan and Kosicki (5) observed that the use of lipids extracted from the same source as that of the malate dehydrogenase increased the activity and stability of this enzyme. They concluded that this action was due to hydrophobic interactions between the lipid and the enzyme and that any agent capable of participating in this type of interaction should serve to stabilize malate dehydrogenase.

This effect was not observed with the phospholipids (egg lecithin and cephalin) used in this study. Instead, a decrease in the activity and stability of the enzyme was observed; at concentrations approaching normal serum levels of phospholipid, total inhibition of activity resulted. This suggests that nonspecific hydrophobic interactions may not be the principal factors involved in the stability and activity of this enzyme. The enzyme probably has very specific requirements that must be met for participation in such an interaction. These requirements would be expected to be met by lipids from the same organ as the enzyme. Furthermore, high concentrations of lipid appear to be inhibitory (5).

The effect of self-association of the phospholipids, which might occur at the higher concentrations used in this study, must also be considered. By reducing the availability of interacting sites, self-association could reduce the possibility of a hydrophobic interaction between the lipid and the enzyme. This could eliminate the stabilizing effect observed by Callahan and Kosicki (5). However, it would not be expected to decrease enzyme activity below that observed in the absence of the lipids, particularly at the lower lipid concentrations where self-association would be minimal. Therefore, while this effect could be responsible for some experimental observations, self-association of the lipids could not in itself explain the reduced enzyme activity observed in this study.

In addition, it was observed that the enzyme did not lose any activity during the reaction, even after 15 min. This can be seen from the linearity of the absorbance *versus* time plots over the full course of the reaction (Figs. 1–3). But when the enzyme was incubated in water alone for 15 min prior to initiation of the reaction, the activity of the enzyme was decreased by about 20%. Furthermore, when the enzyme was incubated for 15 min in the presence of oxalacetic acid prior to the initiation of the reaction, the activity decreased to the same extent as was observed when the enzyme was incubated in water alone. When the experiment was repeated using NADH in place of oxalacetic acid, the same results were obtained. Thus, neither oxalacetic acid nor NADH alone stabilizes the enzyme in water, and the presence of both the coenzyme and the substrate is necessary for the stabilization of malate dehydrogenase. Since these two components are always present in the body along with the enzyme and the reaction is a continuous process, this interaction may be an important factor in maintaining stability of malate dehydrogenase *in vivo*. Whether high concentrations of lipid act to inhibit enzyme activity *in vivo* cannot be ascertained from this study. However, the possibility of such an effect should receive further consideration.

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Improved Methods for Quantitative Determination of Methadone

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Abstract □ A spectrophotometric method is described that permits the rapid analysis of methadone in urine or tissues at concentrations corresponding to therapeutic, maintenance, or toxic doses of that drug. As little as 5 μg may be detected in a biological specimen. The procedure involves an alkaline extraction into *n*-hexane and subsequent back-extraction into a ceric sulfate–sulfuric acid solution. The acid extract is refluxed with *n*-heptane for 30 min, oxidizing methadone to benzophenone which, in contrast to the unchanged drug, has a high molar absorptivity in the UV region. A GLC method is also described. Both procedures require fewer manipulations and less analysis time than previously reported methods for determining methadone.

Keyphrases □ Methadone in urine and tissues—UV and GLC analyses □ UV spectrophotometry—analysis, methadone in urine and tissues □ GLC—analysis, methadone in urine and tissues

The low molar aborptivity of methadone renders spectrophotometric methods that determine unchanged methadone (1) unsatisfactory for analyzing the drug in biological specimens. Wallace *et al.* (2) recently developed a spectrophotometric method sufficiently sensitive to analyze therapeutic doses of methadone in urine and tissue specimens. The method is based upon the oxidation of methadone to benzophenone, a compound possessing a molar absorptivity approximately 34 times that of methadone.

This report describes an adaptation of the previously reported spectrophotometric method (2). The