Effect of Phospholipids on Induced Enzyme Release from Mitochondria*

Sergio Estrada-O.,† A. Carabez T., and A. Cabeza G.

ABSTRACT: When incubated in an appropriate medium, mitochondria release glutamic aspartic transaminase (GOT) and glutamic dehydrogenase activities to the supernatant solution in a temperature-dependent fashion. This phenomenon is inhibited by chromatographically pure phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and sphingomyelin. A relationship seems to exist between the blocking activity of these molecules and the unsaturation of their constituent fatty acids, since saturated phospholipids do not prevent release of enzymes. Some free unsaturated fatty acids (*e.g.*, oleic acid) induce the release of GOT activity from mitochondria, whereas some saturated fatty acids moderately protect or are ineffective in this process. Phospholipids are ineffective in preventing the oleic acid induced release of GOT. If lysolecithin or lecithinase D is added to the incubation medium, the release of enzyme activity from the mitochondria is enhanced. The lysolecithin-induced release of glutamic aspartic transaminase is prevented by preincubation of mitochondria with phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphorylcholine, or a synthetic lecithin analog (2,3-distearoyloxypropyl)dimethyl-β-hydroxyethylammonium acetate. No preventive effect is found if these compounds are added simultaneously with or after the addition of lysolecithin. The effect of lecithinase D is blocked if the above lecithin analog is preincubated with the enzyme.

Preincubation of mitochondria with bovine serum albumin prevented the GOT releasing activity of lysolecithin, lecithinase D, and oleic acid. The significance of these findings in relation to the possible role of phospholipids and some of their derivatives in the regulation of lecithinase and lysolecithin activity in mitochondria is discussed.

As more knowledge has accumulated on the structure and function of the mitochondrial membrane, it has become clear that phospholipids are significantly involved at different levels of its molecular organization. It has been pointed out (Fleischer *et al.*, 1962) that phospholipids play an important role in mitochondrial electron transport. In mitochondrial β-hydroxybutyric dehydrogenase (Jurtshuk *et al.*, 1961), cytochrome oxidase, succinate cytochrome *c* reductase, and succinate coenzyme Q reductase activities (Brierley *et al.*, 1962), phospholipids also seem to be essential components. The isolation of a phosphorylated derivative of DPNH[1] (Griffiths and Chaplain, 1963) which requires a low dielectric constant for its stability suggests that phospholipids probably facilitate the formation of high energy compounds.

Wojtczak *et al.* (1963) have presented evidence that certain phospholipids are involved in the ATP-induced contractile mechanism of mitochondria. A similar requirement for phospholipid seems to exist for the expression of the contractile ability of actin- and myosinlike proteins of mitochondria (Vignais *et al.*, 1963). Further demonstration of phospholipid involvement in the water-extrusion mechanism of mitochondria has been pointed out by Rendi (1964). Also, it has been suggested that phospholipids may participate in the accumulation of divalent metal ions such as Mn^{2+} and Ca^{2+} (Chappell *et al.*, 1963; Slater and Cleland, 1963) in the mitochondrial membrane.

More specifically related to the results to be presented in this work is the finding of Rossi *et al.* (1964) that the decreased oxidative phosphorylation efficiency occurring during aging of mitochondria is consistently accompanied by a breakdown of mitochondrial phospholipids. It has also been demonstrated that it is possible to prevent both the decay of oxidative phosphorylation and the decrease of mitochondrial phospholipids by the addition of phosphorylcholine, phosphorylethanolamine, and phosphorylserine to the incubation medium. It was suggested that this protective effect was mediated through inhibition of mitochondrial lecithinases by these compounds. The recent demonstration of the presence of lecithinase A activity in mitochondria (Rossi *et al.*, 1965; Scherphoff and Van Deenen, 1965) together with the reported competitive inhibitory activity of these compounds

* From the Departamento de Bioquimica, Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Ciudad Universitaria, Mexico 20, D. F. *Received November 8, 1965.*

† Present address: The Institute for Enzyme Research, University of Wisconsin, Madison, Wis. Postdoctoral fellow supported by a research fellowship (FOS-TW-965-01) of the National Institutes of Health.

[1] Abbreviations used in this work: GOT, glutamic aspartic transaminase; GD, glutamic dehydrogenase; BSA, bovine serum albumin; ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide.

on a lecithinase A preparation obtained from cobra venom (Rossi *et al.*, 1964) supports this idea.

Although the above data emphasize the importance of phospholipase activities on the regulation of mitochondrial metabolism, little is known about the possible regulation of the detergent effects of free lysolecithin. It is the purpose of this work to show: (a) that lysolecithin's detergent activity in mitochondria may be regulated by the presence of phospholipid molecules and some phospholipid derivatives independently of the inhibition of lecithinase activities; (b) that the temperature-dependent release of mitochondrial GOT or GD could be mediated in part through the activation of mitochondrial lecithinases and the subsequent liberation of lysolecithin molecules; and (c) that the association of phospholipids, phospholipid derivatives, and some protein molecules could constitute part of a system which controls the activity of both lysolecithin and lecithinases in mitochondria. In order to demonstrate our proposals, the authors studied the effects of phospholipids, phospholipid derivatives, and some proteins on the spontaneously induced as well as on the lysolecithin and lecithinase D induced release of GOT from mitochondria. This induced release of enzyme was used as a measure of the detergent activity of these compounds on mitochondrial membranes. In some experiments, the release of GD was also measured.

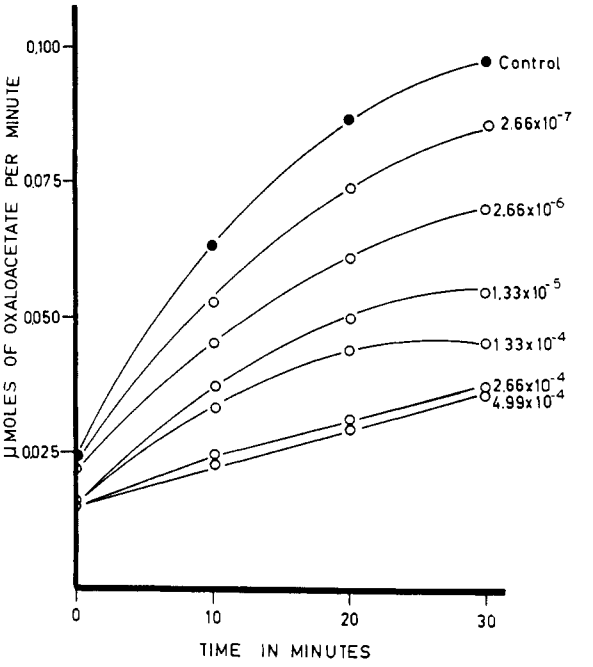

FIGURE 1: Effect of chromatographically pure phosphatidylethanolamine on the temperature-dependent release of GOT from liver mitochondria. Mitochondria equivalent to 6 mg of liver protein were incubated in 7.5 ml of 0.02 M Tris–HCl–0.0125 M KCl buffer, pH 7.4. Zero time was taken at an incubation temperature of 4°; next aliquots were taken each 15 min at an incubation temperature of 28° and, after centrifuged at 18,000 g for 5 min, the supernatants were assayed for GOT activity. Mean values from three individual experiments are presented.

Experimental Procedure

Rat liver mitochondria were prepared as described by Schneider and Hogeboom (1950) and suspended in 0.25 M sucrose to give 1 ml of suspension/g of original liver. Aliquots of the mitochondrial suspension containing 6 mg of mitochondrial protein were incubated in 7.5 ml of the indicated assay medium with or without the different compounds described in the tables and figures. Before transfering the incubation vessels from an ice bath (4°) to a water bath (30°), a zero-time aliquot was taken. Beginning after 3 min of temperature equilibration at 30°, 1.5-ml aliquots were removed for analysis every 15–20 min. The samples were centrifuged in the cold at 18,000*g* for 5 min and the supernatant solutions were assayed for GOT or GD activity. Swelling of mitochondrial particles was recorded by measuring changes in optical density at 520 mμ in a M4-Q II Zeiss spectrophotometer.

Analytical Methods. GOT activity in 0.5–1.0 ml of mitochondrial supernatant solution was assayed according to the method of Kun *et al.* (1960), as modified by Estrada and Cordoba (1963). One unit equals 0.0011 μmole of oxaloacetate formed/min. The GD activity of 0.5 ml of mitochondrial supernatant solution was measured spectrophotometrically as described by Pena *et al.* (1963). Mitochondrial protein was determined by the method of Lowry (1951). Crystalline bovine serum albumin was used as a standard for this method.

Phospholipids and Related Molecules. Chromatographically homogeneous phospholipids from three different sources were used throughout this work. (a) Phospholipids were obtained chromatographically pure (verified by thin layer chromatography) from General Biochemical Laboratory (GBI Laboratory, Chagrin Falls, Ohio). (b) Commercial preparations were repurified by silicic acid column chromatography as described below. (c) Phospholipids were separated and purified from rat liver mitochondria. Regardless of the source, each phospholipid had essentially the same effect in our experiments.

Phosphatidylethanolamine, 1-α-cephalin of animal origin, phosphatidylinositol, egg lecithin, phosphatidylserine (*l* form), chromatographically pure synthetic DL-α-cephalin, saturated synthetic lecithin (β_1,γ-dipalmitoyl-DL-α-lecithin), cardiolipin, phosphorylcholine (Ca^{2+} salt), phosphorylethanolamine, and lecithinase D were obtained from Nutritional Biochemicals; chromatographically pure 1-α-lecithin; 1-α-cephalin, saturated synthetic lecithin (dipalmitoylglycerophosphorylcholine), and lysolecithin were purchased from GBI Laboratories. The structural lecithin analog (2,3-distearoyloxypropyl)dimethyl-β-hydroxyethylammonium acetate was a generous gift from Dr. A. F. Rosenthal.

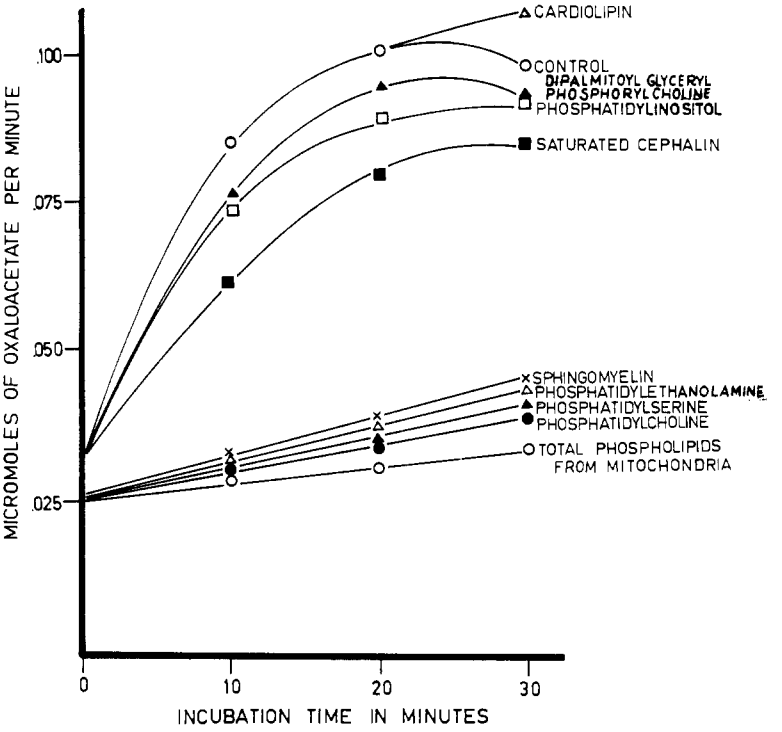

FIGURE 2: Effect of different phospholipid molecules on the temperature-dependent release of GOT from mitochondria. Experimental conditions were essentially the same as described in Figure 1. Final molarity of 2.6×10^{-4} M was assayed for each individual phospholipid molecule. Values from five different experiments are presented.

Separation of Individual Phospholipids. A freshly prepared mitochondrial pellet was extracted three times with 20, 20, and 10 volumes of chloroform–methanol mixture (2:1, v/v) per g of liver tissue (Folch *et al.*, 1958). The extraction was carried out in an atmosphere of purified nitrogen. The extracted lipids were rapidly concentrated in a flash evaporator, homogenized in chloroform, and then applied to a column (1.3 × 48 cm) of silicic acid. The latter had been activated by heating overnight at 110° and packed by suspending it in chloroform. The phospholipids were eluted as described by Fleischer *et al.* (1962); 1500 ml of chloroform was used to separate neutral lipids, 1000 ml of chloroform–methanol (4:1) to separate phosphatidylethanolamine (sometimes with some traces of cardiolipin), 1000 ml of chloroform–methanol (3:2) to separate phosphatidylinositol, and finally, 1000 ml of chloroform–methanol (1:2) to elute phosphatidylcholine. The individual phospholipids were then identified by thin layer chromatography.

Identification of Phospholipids by Thin Layer Chromatography. An aliquot of each methanol–chloroform eluate (10–50 μl) was applied to a thin layer of silica gel G (Merck) previously activated for 1 hr at 120°. For the chromatographic run the solvent chloroform–methanol–water (70:30:5, v/v) was used. The spots of separated phospholipids, detected by exposure to iodine vapor, were identified by comparison of their R_F values with those of authentic phospholipids.

Assay for Total Fatty Acids. In some experiments the total amount of free fatty acids was measured in an aliquot of chloroform–methanol eluate by the KOH microtitration procedure of Dole (1956) as modified by Gordon and Sunderman (1960). The few preparations found to be contaminated with fatty acids were not used in the experiments to be described.

Repurification of Phospholipids. Commercial preparations of phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were repurified and freed of a number of contaminants by dissolving known amounts of each phospholipid in chloroform, applying these samples individually to silicic acid columns, and eluting with large volumes (1500 ml) of the previously described methanol–chloroform solvent pairs. The homogeneity of these preparations was established by thin layer chromatography. In some instances the fraction containing phosphatidylethanolamine was sometimes contaminated with cardiolipin and other unidentified phospholipids. By rechromatographing the fraction and by increasing the ratio of chloroform to methanol these contaminants were removed.

Preparation of Phospholipid Micelles. Phospholipid micelles in aqueous solution were prepared according to Lester and Smith (1961) in 0.25 M sucrose–0.01 M Tris–HCl, pH 7.4, or in 0.125 M KCl–0.01 M Tris–HCl, pH 7.4. Mixtures of phospholipid were homogenized in the cold with a Teflon pestle and the resulting suspensions were treated by sonic oscillation in a MSE

TABLE I: Effect of Phospholipid Constituents on Mitochondrial GOT and GD Spontaneous Release.[a]

Assay Compound	Act. of GOT[b] Released from Mitochondria to the Supernatant	Act. of GD[c] Released from Mitochondria to the Supernatant
Control without addition	0.051 ± 0.005	0.72 ± 0.05
Phosphatidylcholine	0.016 ± 0.002	0.230 ± 0.01
Phosphatidylethanolamine	0.012 ± 0.001	0.210 ± 0.01
Phosphorylcholine	0.031 ± 0.004	0.46 ● 0.02
Phosphorylethanolamine	0.034 ± 0.003	0.480 ± 0.01
Choline	0.050 ± 0.006	0.70 ± 0.04
Ethanolamine	0.053 ± 0.007	0.76 ± 0.04
Glycerol	0.033 ± 0.004	0.52 ± 0.06
Palmitic acid	0.042 ± 0.002	0.61 ± 0.05
Stearic acid	0.048 ± 0.005	—
Oleic acid	0.140 ± 0.017	—
Oleic acid + BSA	0.025 ± 0.004	—
Linoleic acid	0.138 ± 0.013	—
Linoleic acid + phosphatidylcholine	0.140 ± 0.016	—
Total mitochondrial phospholipids	0.014 ± 0.001	0.20 ± 0.01

[a] Mitochondria obtained from 16 mg of wet liver tissue were incubated for 30 min at 28° in 7.5 ml of 0.125 M KCl–0.02 M Tris–HCl, pH 7.4, with the above-mentioned compounds and centrifuged in the cold at 18,000*g* for 5 min after the end of this incubation time. Each individual supernatant (1 ml) was assayed for GOT activity. [b] GOT activity values are expressed in micromoles of oxaloacetate produced per minute by 1.0 ml of mitochondrial supernatant. [c] GD activity was assayed in a reaction mixture with: 0.166 M sodium phosphate, pH 7.4; 0.0166 M α-ketoglutarate–Tris, pH 7.4; 0.0166 M NH_4Cl; 10^{-4} DPNH; and 1.0 ml of mitochondrial supernatants. Values represent changes in OD at 340 mμ by 1.0 ml of supernatants. Compounds were assayed at the same final concentration of 2×10^{-4} M, except with fatty acids tested at 2×10^{-5} M, and phosphorylcholine and phosphorylethanolamine, assayed at 2.3 μmoles/ml. Standard deviation values from six individual experiments are presented.

ultrasonic power unit Model 60 W at 20 kcycles for 10 min. After this step, phospholipids were freely soluble in the aqueous solution.

Results

In this work, mitochondrial phospholipids purified as described by Fleischer *et al.* (1962), as well as commercial preparations of phospholipids repurified by silicic acid column chromatography, showed reproducible and similar effects on the spontaneous and induced release of mitochondrial enzymes. All phospholipids and related molecules tested had no effect on the enzymatic activity of either glutamic dehydrogenase or glutamic–oxalacetic transaminase solubilized from rat liver mitochondria.

As previously mentioned, the incubation of mitochondria in KCl–Tris medium, pH 7.4, at 30° produces a definite increase in the enzymatic activity of GOT and GD in the mitochondrial supernatant solution. The authors interpret the progressive increase in the enzymatic activity of the supernatant solutions as an actual release of enzyme molecules whose only possible source is the mitochondria.

Eight phospholipids were tested for their effects on the spontaneous, temperature-dependent liberation of GOT and GD from mitochondria. Five were found to inhibit these phenomena and three were inactive. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and spingomyelin produced maximal inhibition at 2.6×10^{-4} M, and all were found to be active even at a concentration of 2.6×10^{-7} M. Figure 1, which depicts the data obtained with phosphatidylethanolamine, is illustrative of the temporal response obtained with these four phospholipids. Phosphatidylinositol, saturated lecithin (dipalmitoylglycerophosphorylcholine), and saturated cephalin at concentrations of 2.6×10^{-4} M caused only very slight inhibition of the temperature-dependent release of GOT; cardiolipin was inactive (Figure 2).

It was considered of interest to determine which part of the phospholipid molecule was responsible for its inhibitory effect on the temperature-dependent enzyme release. Accordingly, the following compounds were assayed at various concentrations (see Table I): phosphorylcholine, phosphorylethanolamine, choline, ethanolamine (as chloride salts), glycerol, phosphate, and free fatty acids. Of these compounds, glycerol, phosphorylcholine, and phosphorylethanolamine inhibited spontaneous enzyme release from mitochondria by about 40%; phosphatidylcholine and phosphatidylethanolamine inhibited by about 85%. Choline and

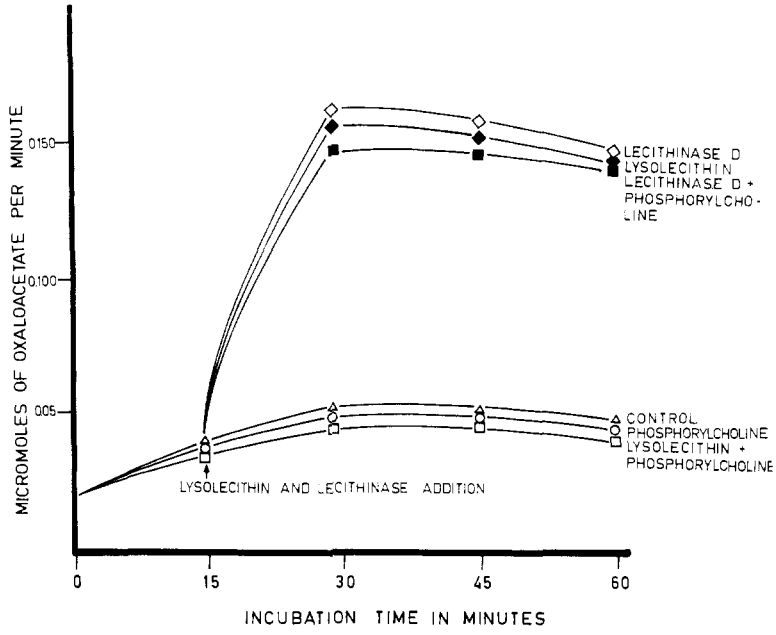

FIGURE 3: Effect of phosphorylcholine on mitochondrial GOT release induced by lecithinase D and lysolecithin. Mitochondria were preincubated 10 min at 25° with 2.3 μmoles of phosphorylcholine prior to the addition of 30 μg/ml of lysolecithin and 60 μg/ml of lecithinase D to the incubation media. Experimental conditions were as those described in Figure 1, except that lysolecithin and lecithinase D were added 15 min after the mitochondria were started to incubate. Representative pattern of six different individual experiments is presented.

ethanolamine were inactive in preventing the release of GOT or GD. The effect of phosphate could not easily be studied because this anion by itself modified GOT activity (Morino and Wada, 1963). Oleic and linoleic acids were found to be extremely active in inducing the liberation of GOT activity to the mitochondrial supernatant solutions. On the other hand, palmitic acid protected slightly against enzyme release while stearic acid had no effect (Table I).

As the results obtained with phospholipids could not be quantitatively reproduced with the separate components of these molecules, it may be suggested that the protective effect of phospholipids is not localized in one part of the molecule. As will be seen below, both the constituent fatty acids and the phosphorylated esters seem to be important for the inhibitory activity of these molecules. After these results were obtained, an attempt was made to study the possible effect of phospholipids and structurally related molecules on the mitochondrial release of GOT induced by lysolecithin and lecithinase D.

Although both lysolecithin and lecithinase D enhance the release of GOT from mitochondria (Figures 3 and 4), their mechanism of action appears to be different as indicated by the effect of phospholipids on the enhanced release of enzyme activity. None of the phospholipids tested was able to block the lecithinase D induced release of mitochondrial GOT even at concentrations of 10^{-3} M (Table II). However, the synthetic lecithin analog (2,3-distearoyloxypropyl)-dimethyl-β-hydroxyethylammonium acetate, as expected from the observations of Rosenthal and Geyer (1962) prevented the stimulation of release of GOT by lecithinase D (Table II). For the synthetic lipid to block the action of lecithinase D, the enzyme and the lecithin analog must be mixed prior to incubation with mitochondria; if the synthetic lecithin analog is preincubated with mitochondria and then lecithinase D is added, no protective effect is observed under any conditions tested.

In contrast to the absence of an effect of phospholipids on the lecithinase D induced enzyme release, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at 10^{-5} M inhibited the liberation of enzyme induced by lysolecithin (Table II). Phosphorylcholine and the lecithin analog also prevented lysolecithin from inducing the release of GOT. Phospholipids inhibited the lysolecithin-induced release of GOT only at low concentrations of lysolecithin (5–25 μg/ml), while the synthetic lecithin as well as phosphorylcholine showed a protective effect even at high concentrations of lysolecithin (30–50 μg/ml), as seen in Figures 3 and 4. In all cases it was necessary to incubate the phospholipids, phosphorylcholine, or the lecithin analog with the mitochondria prior to the addition of lysolecithin to demonstrate inhibition of the action of lysolecithin. If these compounds were preincubated with lysolecithin and this mixture was added to the mitochondria, no detectable inhibitory effect was found. Other studies demonstrated that the phospholipids that were inactive in inhibiting the temperature-dependent release of GOT were also ineffective in

TABLE II: Effect of Phospholipids, Structurally Related Compounds, and Protein Molecules on Lysolecithin and Lecithinase D–GOT-Induced Release from Mitochondria.[a]

	Lysolecithin	Lecithinase D
No addition	0.140 ± 0.016	0.162 ± 0.017
Phosphatidylcholine	0.042 ± 0.007	0.160 ± 0.019
Phosphatidylserine	0.063 ± 0.006	0.160 ± 0.019
Phosphatidylethanolamine	0.048 ± 0.008	0.165 ± 0.016
Phosphorylcholine	0.040 ± 0.005	0.060 ± 0.015
Lecithin analog	0.025 ± 0.002	0.052 ± 0.007
Phosphatidylinositol	0.146 ± 0.011	0.164 ± 0.019
Cardiolipin	0.146 ± 0.018	0.162 ± 0.012
EDTA	0.144 ± 0.022	0.036 ± 0.002
Bovine serum albumin	0.025 ± 0.003	0.014 ± 0.003
α_2-Globulin	0.022 ± 0.002	—
β_3-Globulin	0.017 ± 0.002	—
γ_2-Globulin	0.138 ± 0.016	—

[a] All the assayed compounds were preincubated in a small volume with mitochondria, 10 min at 25°, prior to their addition to the incubation media, ice bath 4°, in which lysolecithin and lecithinase D were added. In the case of lecithinase D values the compounds assayed were preincubated with the enzyme prior to its addition to mitochondria; only BSA was preincubated with mitochondria, before the addition of lecithinase. The samples were then transferred to a water bath 28°. The experimental conditions were essentially the same as those described in Table I. Phospholipid molecules were assayed at 2×10^{-4} M; phosphorylcholine at 2.3 μmoles/ml; lecithin analog at 0.018 μmole/ml; EDTA, 6×10^{-3} M; protein molecules at a final concentration of 2 mg/ml; lysolecithin 25 μg/ml; and lecithinase D at 60 μg/ml. Values above represent GOT activity expressed in micromoles of oxalacetate formed per minute by 1.0 ml of mitochondrial supernatants. Standard deviation values from five individual experiments are presented.

blocking the lysolecithin-induced leakage of enzyme (Table II).

The effect of phospholipids and the synthetic lipid on lecithinase D induced mitochondrial swelling was also studied. In apparent agreement with the data obtained on the lecithinase D induced release of enzyme from mitochondria, phospholipids were found to be inactive in protecting against lecithinase D induced swelling.

The lysolecithin-induced mitochondrial swelling was also not affected by the presence of phospholipids; however, the lecithin analog showed a protective effect when preincubated with mitochondria prior to the addition of lysolecithin. Although phosphorylcholine is a potent inhibitor of the lysolecithin-induced release of enzymes if preincubated with mitochondria, it does not appreciably protect against lysolecithin-induced swelling. These results signify that mitochondrial swelling and enzyme release are independent phenomena.

In contrast to the apparent specificity of the compounds discussed above, it is shown in Table II that preincubation of mitochondria with bovine serum albumin, β_3-globulin, or α_2-globulin gives complete protection against the GOT leakage induced by oleic acid, lysolecithin and lecithinase D.

Discussion

It is of interest that phospholipid molecules and their derivatives affect almost identically the spontaneous or induced release of both GD and GOT (see Tables I and II). These results, together with previous observations that other compounds (uncouplers of oxidative phosphorylation, ATP, EDTA) that modify the release of GOT also modify in an identical manner the release of GD (Estrada, 1964), indicate that GD and GOT are located in the same compartment of the mitochondrion.

Previous work has shown that the release of GOT and GD under certain conditions may be energy dependent (Estrada, 1964; Estrada and Montal, unpublished data). However, the results presented in this paper indicate that the release of these enzymes may also occur passively as a result of structural changes in the mitochondrial membrane caused by phospholipases or lysophosphatidyl compounds.

It has been suggested that lecithinase activity in mitochondria may be inhibited by phosphorylcholine (Siliprandi, 1963), phosphorylethanolamine, and phosphorylserine (Rossi *et al.*, 1964). The data presented above demonstrated that as in the case of lecithinases, lysolecithin activity in mitochondria may be controlled by compounds that naturally exist as constituents of the mitochondrial membrane.

The temperature-dependent release of GOT from mitochondria was inhibited by phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine, which contain some unsaturated fatty acids in the molecule, while dipalmitoylglyceryl

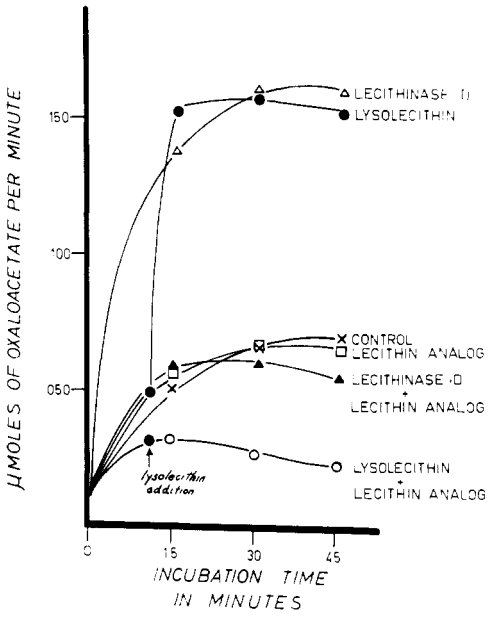

FIGURE 4: Effect of 0.16 μmole/ml of the synthetic lecithin analog (2,3-distearoyloxypropyl)dimethyl-β-hydroxyethylammonium acetate on the mitochondrial release of GOT induced by lecithinase D and lysolecithin. Experimental conditions were the same as those described in Figure 3. The mean values from four different individual experiments are presented.

phosphorylcholine and a saturated cephalin which contain only saturated fatty acids were inactive in inhibiting the leakage of the mitochondrial enzyme.

Apparently the polarity and the free electrical charges of a phospholipid do not contribute to the ability of the molecule to inhibit changes in the mitochondrial membrane leading to the release of GOT and GD. The polarity and electrical charges of phosphatidylcholine, an active compound, are identical with those of dipalmitoylglycerylphosphorylcholine, an inactive lipid. Polarity and charges are also similar for phosphatidylethanolamine, phosphatidylserine, and chemically saturated cephalin, but only the latter is inactive. Thus the ability of phospholipids to inhibit the aforementioned phenomenon would seem to be due to the presence of unsaturated fatty acids in the molecule. A requirement for unsaturation of phospholipids for the phospholipid-dependent activation of β-hydroxybutyric dehydrogenase purified from mitochondria has also been observed by Jurtshuk *et al.* (1961). The enzyme is activated by phosphatidylcholine but not by a saturated lecithin (synthetic L-α-dimyristoyl derivative).

The effect of free fatty acids *per se* is shown in Table I. Unsaturated fatty acids cause release of GOT activity, whereas saturated fatty acids are inactive in this respect. One might suggest a relationship of these observations to those of other authors who have studied the effects of free fatty acids on mitochondrial metabolism. Pressman and Lardy (1956) noted an activation of the latent ATPase activity of fresh mitochondria under the influence of *cis* unsaturated fatty acids. Zborowski and Wojtczak (1963) described an unsaturated fatty acid induced swelling of mitochondria. It has also been reported that some uncouplers of oxidative phosphorylation produce a release of GD and GOT activities from mitochondria (Estrada, 1964) and that unsaturated fatty acids are uncouplers of oxidative phosphorylation (Pressman and Lardy, 1955; Hulsmann *et al.*, 1960). However, the release of GOT induced by fatty acids as described in this paper occurs immediately upon the addition of these molecules and is obtained at a temperature of 0–4°. Thus it is most probable that this particular type of GOT release is due to the detergent action of the fatty acids and not to metabolic alteration of mitochondrial function. In this respect it is interesting to point out that phospholipids showed a negligible protective effect on fatty acid induced GOT release (Table I).

The instantaneous release of GOT from mitochondria after addition of lysolecithin (Figures 3 and 4) is probably due to the latter's known lytic effect on most types of cell membranes. The stimulation of the release of enzyme by low concentrations of lysolecithin can be completely inhibited by phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphorylcholine, and the synthetic lecithin analog; at higher concentrations of this lytic molecule only phosphorylcholine and the synthetic lecithin analog could prevent the induced enzyme release. For inhibition to occur, the active lipid preparations must be incubated with the mitochondria prior to addition of lysolecithin. On the other hand, mitochondrial swelling, as induced by this compound, is not affected by the five above-mentioned lipid molecules. This differentiation between swelling and enzyme release with respect to the action of phospholipids suggests that these phenomena are independent.

Phosphatidylinositol and cardiolipin did not inhibit the lysolecithin-induced liberation of GOT from mitochondria. This points to the possible specificity of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, the synthetic lecithin analog, and phosphorylcholine in blocking the lysolecithin-induced GOT release. The common structural feature of most of these inhibitory molecules is the highly polar radical formed by a nitrogenous base and phosphate. One is tempted to suggest that this part of the molecule, which is similar to the radical present on the α_3 carbon of lysolecithin, can occupy the site in the mitochondrial membrane which lysolecithin requires to produce its effect. The fact that preincubation of the inhibitory phospholipid molecules with mitochondria is required to block release of GOT induced by lysolecithin would support this idea. However, as emphasized above, unsaturation of the phospholipid molecule is also important in conferring upon the molecule the ability to protect against leakage of enzymes. The chromatographic criteria of purity followed in this work establish only the gross homogeneity of each family of phospholipids. Consequently, further speculation on the

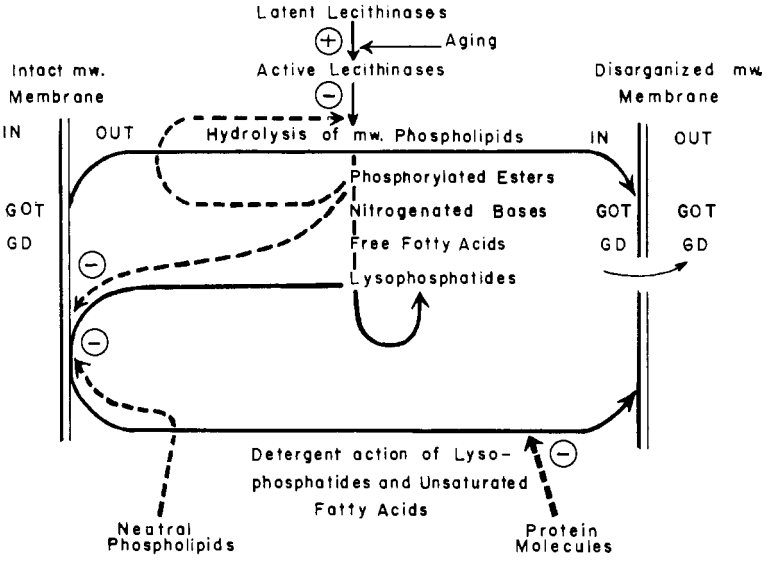

FIGURE 5: A scheme attempting to correlate the results of the present investigation in terms of their possible significance for the maintenance of the structural and functional integrity of the mitochondrial membranes *in vivo*.

structural basis of the specificity of the phospholipids in maintaining the integrity of the mitochondrial membrane will require detailed information on the structure of phospholipids, active or inactive, in this process.

It also should be emphasized that the release of GOT induced by lecithinase D was not blocked by phospholipids under any experimental conditions. However, the synthetic lecithin analog, when preincubated with the enzyme, inhibited the lecithinase D induced release of GOT and, as well, mitochondrial swelling. This analog is known to competitively inhibit the enzyme *in vitro* (Rosenthal and Geyer, 1962).

As noted in Tables I and II, some proteins (*e.g.*, BSA), if preincubated with the mitochondria, inhibit strikingly the release of GOT induced by lysolecithin or lecithinase D or oleic acid. The protective effect of BSA on the lysolecithin, or oleic acid induced release of enzyme is presumably the result of the formation of a nonspecific complex between the protein and the detergent molecule. This assumption is supported by the fact that oleic acid and lysolecithin were inactive if they were incubated with BSA before addition to the mitochondria. Similarly, the inhibitory effect of BSA on the lecithinase D induced release of enzyme can reasonably be explained by the complexing action of BSA on the products of lecithinase activity.

Our data lead us to suggest a mechanism for the spontaneous, temperature-dependent release of GOT. Associated with the aging process of mitochondria is the activation of phospholipases which hydrolyze membrane phospholipids to produce unsaturated free fatty acids and lysophosphatides. The detergent activity of the latter compounds results in structural alterations in the mitochondrial membrane which permit the leakage of certain enzymes. In support of this hypothesis is the good agreement between the time courses of the release of GOT (Estrada, 1964) and of the hydrolysis of phospholipids when mitochondria are incubated at 30° (Rossi *et al.*, 1964). Additional evidence for the hypothesis is that phosphorylcholine and the lecithin analog, both of which inhibit the temperature-dependent release of GOT, also inhibit competitively phospholipase A (Siliprandi, 1963; Rossi *et al.*, 1965) and phospholipase D (Rosenthal and Geyer, 1962), respectively. The presence of phospholipase A activity in mitochondria has been demonstrated by Rossi *et al.* (1965) and by Scherphoff and Van Deenen (1965).

Protection against the spontaneous, temperature-dependent release of GOT can occur not only by inhibition of mitochondrial phospholipase activity but also through interference with the detergent activity of the unsaturated fatty acids and lysophosphatides produced by the action of phospholipases. The inhibition of the release of GOT by proteins such as BSA is due presumably to the formation of complexes between protein molecules and the unsaturated fatty acids and lysophosphatides. Several unsaturated phospholipids and also the lecithin analog inhibited not only the spontaneous release of enzymes caused by detergent molecules of endogenous origin, but also inhibit leakage of enzymes induced by lysolecithin added to the system. The authors suggest that this protective effect may be mediated at the site(s) in the mitochondrial membrane where the detergent molecules have their effect.

The scheme depicted in Figure 5 is an attempt to correlate and interpret the results of the present investigation in terms of their possible significance for the maintenance of the structural and functional integrity

of the mitochondrial membranes *in vivo*. The scheme shows that the activation of latent mitochondrial phospholipases (lecithinases), which occurs *in vitro* when mitochondria are aged, but which may occur *in vivo* under the influence of unknown stimuli, results in the hydrolysis of membrane phospholipids. The damage done to the mitochondrial membrane by the hydrolysis of its constituent phospholipids is increased by the detergent action of two of the hydrolytic products, fatty acids and lysophosphatides. However, as shown in the scheme, several mechanisms may operate to control or prevent destruction of the membrane by phospholipases and detergent compounds. One of these mechanisms is the product inhibition of phospholipases exhibited by phosphorylated esters. In addition, the phosphorylated esters appear to interact with the membrane and in some way prevent the detergent action of lysophosphatides. Neutral phospholipids in the membrane may also participate in the inhibition of the detergency of lysophosphatides. The ability of certain proteins in the mitochondria or in the soluble fraction of the cell to complex lysophosphatides and fatty acids may constitute a third mechanism for inhibiting the detergent activity of these compounds.

Acknowledgments

The authors are indebted to Dr. F. Rosenthal for supplying us with the lecithin analog used in this work. The authors are also grateful to Dr. A. Gomez Puyou, Miss Shelagh Ferguson, Dr. David O. Foster, and Dr. Henry A. Lardy for their help in preparing the manuscript. The stimulating encouragement of Dr. José Laguna G. is also sincerely appreciated.

References

Brierley, G., Merola, A. J., and Fleischer, S. (1962), *Biochim. Biophys. Acta 64*, 218.

Chappell, J. B., Cohn, M., and Greville, G. D. (1963), *in* Energy Linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 219.

Dole, V. P. (1956), *J. Clin. Invest. 35*, 150.

Estrada-O., S., (1964), *Arch. Biochem. Biophys. 106*, 498.

Estrada-O., S., and Cordoba, F. A. (1963), *Biochem. Biophys. Res. Commun. 12*, 487.

Fleischer, S., Brierley, G., Klouwen, H., and Slautterback, B. (1962), *J. Biol. Chem. 237*, 3264.

Folch, J., Lees, M., and Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Gordon, R. S., and Sunderman, W. (1960), *in* Lipids and the Steroid Hormones in Clinical Medicine, Philadelphia, Pa., J. B. Lippincott, p 45.

Griffiths, D. E., and Chaplain, R. A. (1963), *Biochem. Biophys. Res. Commun. 8*, 945.

Hulsmann, W. C., Elliot, W. B., and Slater, E. C. (1960), *Biochim. Biophys. Acta 39*, 267.

Jurtshuk, P., Jr., Sekuzu, I., and Green, D. E. (1961), *Biochem. Biophys. Res. Commun. 76*, 191.

Kun, E., Fanishier, D. W., and Graselli, D. R. (1960), *J. Biol. Chem. 235*, 416.

Lester, R. L., and Smith, A. (1961), *Biochim. Biophys. Acta 47*, 475.

Lowry, O. H. (1951), *J. Biol. Chem. 193*, 265.

Morino, Y., and Wada, N. (1963), *in* The Chemical and Biological Aspects of Pyridoxal Catalysis, London, Pergamon, p 181.

Peña, A. D., Gomez, A. P., and Guzman, J. G. (1963), *Arch. Biochem. Biophys. 100*, 426.

Pressman, B. C., and Lardy, H. A. (1955), *Biochim. Biophys. Acta 18*, 482.

Pressman, B. C., and Lardy, H. A. (1956), *Biochim. Biophys. Acta 21*, 458.

Rendi, R. (1964), *Biochim. Biophys. Acta 84*, 694.

Rosenthal, A. F., and Geyer, R. P. (1962), *Arch. Biochem. 96*, 240.

Rossi, C. R., Sartorelli, L., Tato, L., Baretta, L., and Siliprandi, N. (1965), *Biochim. Biophys. Acta 98*, 207.

Rossi, C. R., Sartorelli, L., Tato, L., and Siliprandi, N. (1964), *Arch. Biochem. Biophys. 107*, 170.

Scherphoff, G. L., and Van Deenen, L. L. M. (1965), *Biochim. Biophys. Acta 98*, 204.

Schneider, W. C., and Hogeboom, G. H. (1950), *J. Biol. Chem. 183*, 123.

Siliprandi, N. (1963), *in* Biochemical Problems of Lipids, Frazer, A. C., Ed., Amsterdam, Elsevier, p 346.

Slater, E. C., and Cleland, K. W. (1963), *Biochem. J. 55*, 556.

Vignais, P. V., Vignais, P. M., and Lehninger, A. L. (1963), *Biochem. Biophys. Res. Commun. 11*, 313.

Wojtczak, L., Wlodawer, P., and Zborowski, J. (1963), *Biochim. Biophys. Acta 70*, 290.

Zborowski, J., and Wojtczak, L. (1963), *Biochim. Biophys. Acta 70*, 596.