Separation of Phospholipids from Glucose-6-phosphatase by Gel Chromatography. Specificity of Phospholipid Reactivation†

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ABSTRACT: A particulate glucose-6-phosphatase preparation, stable as a lyophilized powder and purified about 40-fold from a rat liver homogenate, was freed of 90% or more of phospholipid by gel chromatography on a Sepharose 4B column in the presence of deoxycholate. The activity of the eluted enzyme could be fully restored by the addition of phospholipid in the presence of a low concentration of detergent. Phosphatidylcholine (monounsaturated) was the most effective phospholipid, giving full activation when about one-half of the original phospholipid content of the particles had been added back. Dipalmitoylphosphatidylcholine was inactive under all conditions tested, while dioleylphosphatidylcholine reactivated fully without detergent when first sonicated. These observations are discussed in terms of the micellar structure of the activating phospholipid. Bile salts and nonionic detergents were found to inhibit the purified enzyme. Some preliminary results on the kinetics of this inhibition are reported.

Glucose-6-phosphatase (EC 3.1.3.9), a microsomal enzyme found in liver, kidney, and small intestine, has been shown by previous workers to require phospholipid for activity (Beaufay and de Duve, 1954; Duttera *et al.*, 1968; Zakim, 1970; Poulter *et al.*, 1971). In these studies, microsomal particles were treated with phospholipases in order to show dependence of enzyme activity on exogenous phospholipids.

In the studies reported here on a partially purified glucose-6-phosphatase from rat liver, we have found that gel chromatography at 4° in the presence of deoxycholate removes 90% or more of the phospholipids quickly and conveniently. The specific activity of these lipid-depleted particles was 10–40% that of the original sample. Although we have never achieved more than 80% recovery of total activity when phospholipids were added back, the specific activity of our most active fractions was generally equal to or greater than that of the starting material.

This method has the advantage of not requiring Ca^{2+}, as do phospholipases C and D, which irreversibly inactivates up to 40% of the enzyme in our preparations, and of avoiding the release of products by phospholipases A_1 and A_2 which can interfere with reactivation and the interpretation of results (Cater and Hallinan, 1971).

Materials and Methods

Glucose-6-phosphate was purchased from Boehringer or Sigma as the sodium salt and was further purified to remove inorganic phosphate by the following procedure. To 50 ml of water was added 5.5 g of $MgCl_2 \cdot 6H_2O$, 10 g of NH_4Cl, and 10 ml of 15 M ammonia with enough additional water to bring the volume to 100 ml; 1 ml of this magnesia mixture corresponds to 270 μmol of inorganic phosphate. Glucose 6-phosphate was dissolved in water to a concentration of around 0.3 M, and the inorganic phosphate was measured by the method of Ames (1966). To this solution was added a tenfold excess of magnesia mixture over the determined amount of inorganic phosphate, and the ammonia concentration was adjusted to 1.5 M. After standing overnight at 4°, the precipitated $MgNH_4PO_4$ was removed by centrifugation and filtration and the solution was brought to pH 7.7 with HCl. The glucose 6-phosphate was precipitated with an excess of $BaCl_2$ and two volumes of ethanol. The precipitate was washed twice with methanol–H_2O (2:1), dried, weighed, dissolved in water, and treated with an equimolar amount of Na_2SO_4 so that the final volume gave a glucose 6-phosphate concentration of 0.2 M. After removal of $BaSO_4$ by centrifugation, the supernatant was adjusted to pH 6.8 with HCl and stored at −20° in small aliquots.

Sodium barbital was obtained from Mallinckrodt, sodium deoxycholate from Schwarz-Mann, sodium glyco- and sodium taurodeoxycholate from Calbiochem and Na_2EDTA from Fisher. Egg phosphatidylcholine, phosphatidylethanolamine, lysolecithin, phosphatidylserine, and sphingomyelin were obtained from General Biochemicals. Asolectin, a purified mixture of phospholipids, was from Associated Concentrates. Synthetic L-α-dioleylphosphatidylcholine was purchased from the Hormel Institute and synthetic L-α-dipalmitoylphosphatidylcholine, from Schwarz-Mann. All the phospholipids were found to be essentially pure by thin-layer chromatography in two solvent systems. They were stored as aqueous suspensions at −20°.

Sepharose 4B and Sephadex G-200 were purchased from Pharmacia. Amberlite XAD-4 was kindly donated by Rohm and Haas. Triton X-100 was purchased from Sigma.

A Beckman L2-50 preparative ultracentrifuge was used to prepare microsomes and "M" fractions. All centrifugations were at 3°.

Sonication was performed with a Bronwill Biosonik III using the small probe tip.

Glucose-6-phosphatase was assayed at 30 or 4° in a 0.5-ml reaction mixture containing 0.02 or 0.04 M glucose 6-phosphate

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TABLE I: Fractionation of Rat Liver Homogenate.[a]

Fraction	Vol (ml)	Total Protein (mg)	Total Act. (μmol/min)	Sp. Act. (μmol/mg per min)	Purifcn	% Act. Recovd
Homogenate	160	3875	134	0.035	1	100
11,000*g*, S	140	1800	78	0.043	1.2	58
(11,000*g*, P)	160	2100	51	0.024	0.7	38
105,000*g*, P[b]	100	647	77	0.119	3.4	58
Same + 0.24% DOC	110	647	176[c]	0.272	7.8	131
(105,000*g*, S)	98	322	37	0.115	3.3	28
(105,000*g*, P)	20	134	30	0.224	6.4	22
105,000*g*, M	7	141	90	0.638	18.2	67
580 mg of lyophilized M						
(pH 10 extract)	116	93	22	0.236	6.7	16
(pH 7.5 extract)	145	7	0			
Lyophilized residue, M_1	12	40	60	1.50	42.9	45

[a] The fractions which are discarded are shown in parentheses. S = supernatant fluid, P = precipitate, M = fluffy layer, and M_1 = first purified fraction. [b] Supernatant not analyzed. [c] In presence of 0.024% DOC.

(pH 6.8) as substrate and buffer. The reaction was started with enzyme and was terminated by the addition of 0.5 ml of a 4% perchloric acid solution which was 10% in trichloroacetic acid. Added phospholipids were completely precipitated after chilling for 15 min followed by centrifugation. Supernatant (0.3 ml) was assayed for inorganic phosphate by the method of Ames (1966) when the incubation had been at 4°, and by the Fiske–Subbarow method (1925) following an incubation at 30°. The enzymatic rate at 30° is about six times greater than at 4°.

Total phosphate was determined by the Ames method after ashing in the presence of ethanolic $Mg(NO_3)_2$ and hydrolysis in a steam bath for 15 min with 0.5 N HCl.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Deoxycholate concentrations were determined by the method of Mosbach *et al.* (1954).

Sodium dodecyl sulfate disc electrophoresis was performed by the method of Shapiro *et al.* (1967) except that the samples were dialyzed against distilled water before sodium dodecyl sulfate treatment and were not dialyzed again before electrophoresis.

Results

Preparation of "M_1" Fraction. Livers were removed from two or three male rats (200–250 g each) which had been fasted overnight. After weighing, they were minced in a beaker over ice, and washed three times with cold 0.25 M sucrose and suspended in a 1:10 ratio (w/v) in the sucrose solution. This suspension was dispersed in a Potter-Elvehjem glass homogenizer, while on ice, with a motor-driven Teflon pestle. The homogenate was centrifuged at 11,000*g* for 10 min, and the supernatant was recentrifuged at 105,000*g* for 85 min. The microsomal pellets were resuspended in 100 ml of 0.25 M sucrose with brief homogenization and cooled on ice. Ten milliliters of a 2.6% solution of sodium deoxycholate was added and the contents were mixed by several inversions. This partially clarified suspension was divided into four tubes and recentrifuged at 105,000*g* for 130 min. Three fractions were obtained which have been described by Ernster *et al.* (1962). The clear supernatant (S fraction) was removed with a Pasteur pipet taking care not to disturb the fluffy layer (M fraction), which was then removed the same way. The glassy pellet (P fraction) was resuspended by homogenization in a small volume of 0.25 M sucrose for assay. The fractions were kept on ice during these procedures.

The M fraction was freeze-dried and stored at −20°. It was quite stable for several months.

To make the M_1 fractions which were used in the subsequent experiments, 160 mg of powdered M was suspended with a glass rod in 32 ml of 0.25 M sucrose containing 0.01 M Tris-HCl (pH 10.0). This suspension was divided equally into eight heavy-walled glass tubes and centrifuged for 1 hr at 4° in a Sorvall SS-1 rotor at top speed (approximately 31,000*g*). The supernatant material was discarded and each precipitate was resuspended in 5 ml of 0.02 M Tris-HCl (pH 7.5) and recentrifuged for 1 hr. After decanting, the precipitate was freeze-dried and stored at −20°. Freeze-drying did not cause loss of enzyme activity. These M_1 fractions were prepared as needed, since 20–30% of their activity was lost in 1 month during storage at −20°. Each M_1 tube contained approximately 1 mg of protein and 1.5 μmol of phospholipid. The phospholipid content was determined by ashing and phosphate analysis as described under Methods. This seemed permissible since the RNA–DNA content as determined by ultraviolet (uv) analysis of clear alkaline solutions of these preparations corresponded to less than 0.1 μmol of P/mg of protein. The purification procedure is summarized in Table I.

Effect of Detergents on Glucose-6-phosphatase Activity. It has long been known that addition of detergents to liver homogenates or crude microsomal fractions increases enzyme activity (Beaufay and de Duve, 1954). An example is shown in Table I where the presence of 0.024% deoxycholate in the reaction mixture more than doubles the enzymatic rate of the resuspended 105,000*g* precipitate. In contrast, the purified M_1 fraction was never stimulated—only inhibited—by deoxycholate, a number of other bile acids, and the nonionic detergent, Triton X-100. Figure 1a shows the decrease in enzymatic activity with increasing concentrations of glycodeoxycholate during incubation with glucose 6-phosphate at 4 and 30°. In these experiments the reaction rate was shown to remain linear with time indicating that the inhibition at a given concentration of bile salt was not increasing during the course

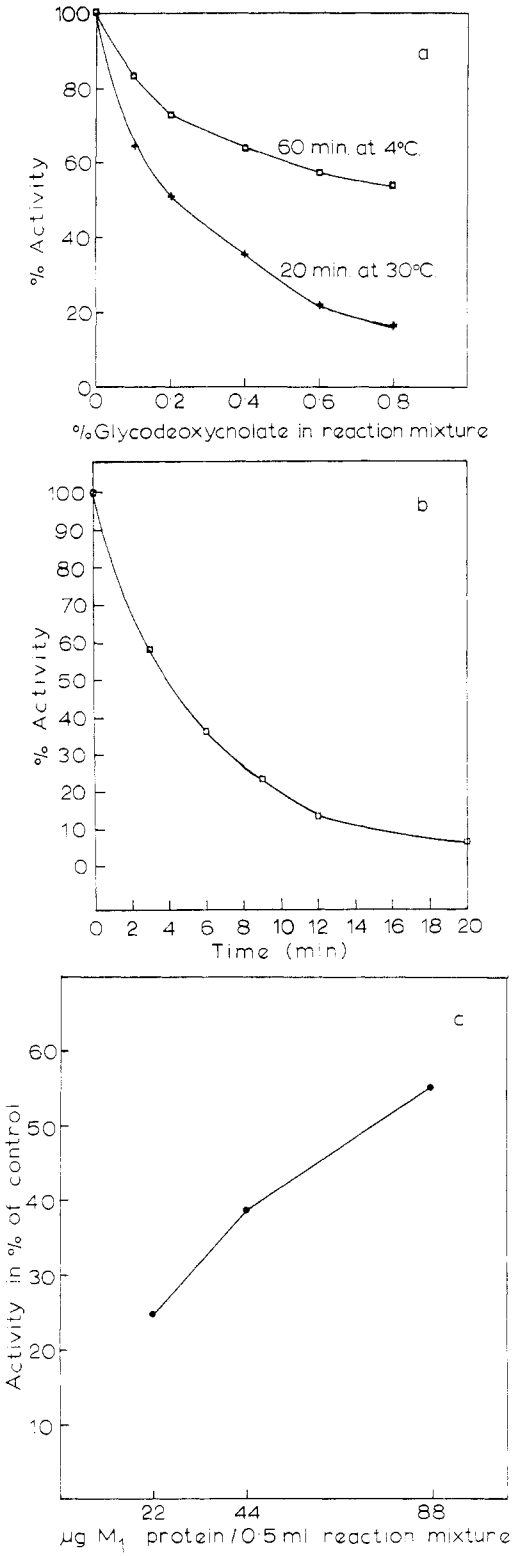

FIGURE 1: The inhibition of glucose-6-phosphatase by glycodeoxycholate. (a) One M_1 tube containing 0.9 mg of freeze-dried protein was sonicated in 1 ml of 0.02 M citrate–1 mM EDTA (pH 6.8). The reaction mixture contained 0.04 M glucose 6-phosphate (pH 6.8) and varying amounts of glycodeoxycholate. The reaction was started by adding 0.1 ml of M_1 (final volume 0.5 ml). Incubation was for 20 min at 30° or for 60 min at 4°. (b) 0.045 mg of M_1 protein was preincubated at 30° for the time indicated in 0.4 ml containing 0.01 M citrate (pH 6.8) and 0.05% glycodeoxycholate. The enzyme reaction was then started by addition of 0.1 ml of 0.2 M glucose 6-phosphate (pH 6.8) and the incubation continued for 20 min at 30°. (c) Varying amounts of M_1 protein were added to reaction mixture containing 0.04 M glucose 6-phosphate (pH 6.8) and 0.2% glycodeoxycholate (final volume 0.5 ml). Incubation was for 20 min at 30°. Controls without glycodeoxycholate containing the same amount of protein were incubated simultaneously. Activity is expressed as % of the control value.

of the reaction. At 30° 50% inhibition was obtained at 0.2% glycodeoxycholate, but at 4° even 0.8% produced less than 50% inhibition. If substrate is not present, the inhibition increases progressively with time (Figure 1b). It should be noted that in this experiment the concentration of bile salts (0.05%) was too low to cause a separation of protein and phospholipid during column chromatography on Sepharose 4B (see below). The degree of inhibition by detergents is also dependent on the concentration of M_1 particles in the reaction mixture (Figure 1c). These factors play a role in the recovery of enzyme activity in the experiments to be described in the next section. A more detailed analysis of the effect of detergents on purified microsomal particles will be presented in another paper.

Separation of Phospholipids by Gel Filtration. The freeze-dried material of one M_1 tube (*ca.* 1 mg of protein) was suspended in 0.2 or 0.3 ml of 0.03 M Veronal–HCl (pH 7.5) containing 0.1–1.0% deoxycholate and a few crystals of sucrose to increase the density. After three 5-sec bursts of sonication (the tube was returned to the ice bucket between bursts), 0.15 or 0.20 ml of the sample was layered over the top of the gel column under the buffer layer. (It was determined that more sonication did not change the elution pattern of the column fractions.) The early void volume fractions were pooled, after which 1-ml fractions were collected. For the activity assay, 0.05 or 0.1 ml of the fractions was added to the reaction mixture which usually contained added phospholipid for reactivation. The column runs and enzymatic reactions were carried out at 4°.

In some experiments, the enzyme, which appeared in the void volume, was passed through a small (0.9 × 5.0 cm) column of polystyrene beads (XAD-4) to remove deoxycholate (Shechter and Bloch, 1971). The residual deoxycholate was 0.05–0.07% by analysis.

The results of three column experiments with Sepharose 4B, equilibrated with 0.03 M Veronal–HCl (pH 7.5), 1 mM EDTA, and different amounts of deoxycholate, are shown in Figure 2a–c. Under these conditions, low concentrations of deoxycholate did not separate phospholipids or substantial amounts of protein from the bulk of the activity, which was found in the void volume (Figure 2a). (Sepharose 4B excludes proteins with molecular weights over 20 million.) When the buffer contained 0.2% deoxycholate (not shown), the large particles were partially disrupted, with most of the phospholipid and about half of the protein retarded, due to entering the gel beads. With 0.4% (Figure 2b), almost two-thirds of the protein and more than 90% of the phospholipid was retarded. When these two higher concentrations of deoxycholate were used, phospholipid had to be added to the reaction mixture to recover maximum activity. With 1% sodium deoxycholate most of the protein and activity was eluted after the void volume, with peaks in tubes 10–11. However, the recovery of activity was only 21%. Another effective way to remove phospholipid is to add KCl to a low concentration of detergent. The best separation was achieved with 0.1% deoxycholate and 0.3 M KCl (higher concentrations of either component caused the deoxycholate to gel). In this case (Figure 2c), the void volume particles were so large that they partially settled out on standing. The greater size can also be inferred from the total exclusion of the active particles from the gel, *i.e.*, there is no protein in the fractions immediately following the void volume. When this fraction was centrifuged before the addition of phospholipid, and the precipitate resuspended, the recovery of activity was not satisfactory. In contrast, when M_1 was chromatographed with 0.4% deoxycholate, without

TABLE II: Reactivation of M_2 with Exogenous Phospholipids.[a]

Additions	Final Concn (mg/ml)	OD_{820}
None		0.026
Asolectin	0.4	0.115
	0.8	0.115
	1.2	0.117
Phosphatidylcholine (egg)	0.9	0.122
Phosphatidylethanolamine	0.8	0.101
Phosphatidylserine	0.8	0.102
Sphingomyelin	0.8	0.038
Lysolecithin	0.8	0.011

[a] The M_2 used was from the void volume fraction prepared as described in the legend for Figure 2c. The reaction mixture contained 0.02% sodium deoxycholate and 0.06 M KCl (transferred with 0.1 ml of M_2) and 0.02 M glucose 6-phosphate (pH 6.8) in a volume of 0.5 ml. Incubation was for 30 min at 4°.

KCl, centrifugation of the clear void volume fraction after removal of detergent gave a pellet which could be fully reactivated (see below).

In these experiments there was an increase in specific activity in the void volume fraction, after the addition of phospholipid, over the starting material. When 0.08% deoxycholate was used, the total recovery of activity was greater than with higher concentrations of detergent, resulting in a comparable purification although less protein was removed. The failure to recover all of the protein could be due to denaturation and entrapment of material in the column.

In some experiments Sephadex G-200 was used, which is faster than Sepharose 4B. Since the enzyme loses activity on standing in the presence of detergent, speed is essential in recovering maximum activity. Although this gel excludes most of the protein, the phospholipid is sufficiently retarded to allow a clean separation from the enzyme. When the void volume fraction was freeze-dried after passing through a XAD-4 column to remove detergent, it could be reactivated with the appropriate reactivation mixture (see below).

Reactivation of M_2 fractions. In a preliminary experiment, the void volume fraction from a Sepharose 4B column ("M,"), prepared as in Figure 2c, was reactivated with different phospholipids including Asolectin, a mixture in which phosphatidylcholine and phosphatidylethanolamine predominate (Kagawa and Racker, 1966) (Table II). The reaction mixture contained 0.02% deoxycholate and 0.06 M KCl. Under these conditions, egg phosphatidylcholine was the single most effective phospholipid of those tried for reactivation of glucose-6-phosphatase and was used in subsequent experiments.

In order to study the effect of deoxycholate on reactivation, an M_2 fraction was passed through a XAD-4 column to remove the detergent. Without added deoxycholate there was essentially no increase in activity, even when 0.9 mg/ml of phosphatidylcholine was added (Table III). Sonication of the phosphatidylcholine suspension for three minutes prior to its addition to enzyme resulted in about 30% reactivation. The other results shown in this table will be discussed later.

To determine more precisely the deoxycholate dependence of reactivation, aliquots of a XAD-4-treated M_2 were added to the reactivation mixtures containing fixed levels of phosphatidylcholine, but varying amounts of detergent (Figure 3). When 0.1 mg/ml of phosphatidylcholine was included in the reaction mixture, maximum activity was obtained with 0.02–0.05% deoxycholate. At higher detergent concentrations, the

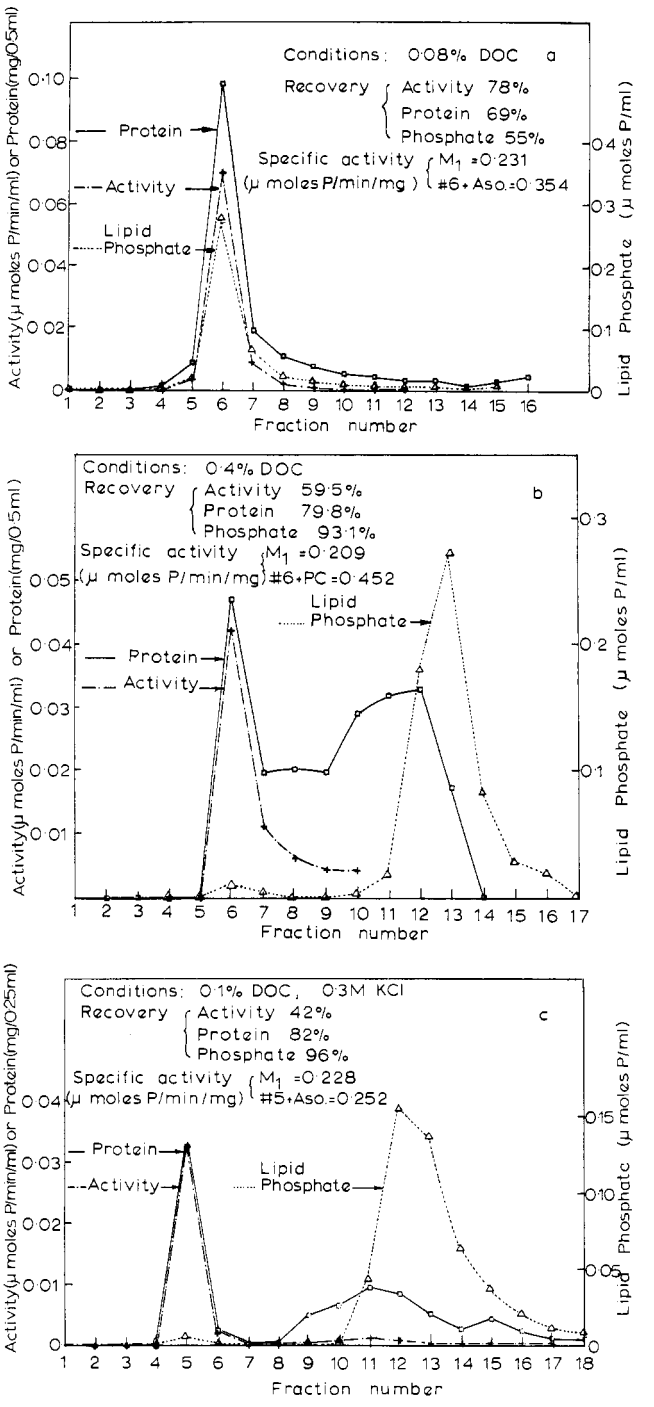

FIGURE 2: Effect of detergent concentration on the removal of phospholipid from M_1 particles. A Sepharose 4B column (0.9 × 25 cm) equilibrated with 0.033 M Veronal–HCl (pH 7.5) with additions as noted on each figure was used in these experiments. Fractions of 1 ml were collected and were used for determination of protein, ashed phosphate, and enzyme activity. Specific activities are shown for fraction 6 in parts a and b and 5 in part c containing material eluted in the void volume. The reaction with 0.02 M glucose 6-phosphate was run at 4° for 30 min. Each assay mixture was brought to a concentration of 0.2 mg/ml with phospholipid as noted. The activity is given as μmol of P/min per ml. Aso, Asolectin; PC, phosphatidylcholine.

TABLE III: Summary of Miscellaneous Reactivation Experiments.[a]

Additions	Phospho-lipid (mg/ml)	Deter-gent (%)	Temp (°C)	Reactivn (%)
Egg PC	0.02–0.9		4	1
	0.2		30	0–4
Sonicated	0.2		30	32
Dipalmitoyl-PC + DOC	0.2	0.05	4	0
	0.2	0.05	30	8
Egg PC + Triton X-100	0.1	0.05	4	100
Dioleyl-PC + DOC	0.2	0.08	4	90
Dioleyl-PC	0.5		4	3
	0.5		30	0
Sonicated	0.5		4	29
Sonicated	0.5		30	77–140
M_1 phospholipids + DOC	0.1	0.1	4	59
	0.1	0.05	30	111

[a] 0.1 ml of XAD-4-treated M_2 was added to a reaction mixture containing 0.02 M glucose 6-phosphate (pH 6.8) and the additions shown in the table (final volume, 0.5 ml). The reactivation with optimal amounts of egg PC (phosphatidylcholine)–DOC (deoxycholate) (see Figure 4) was taken as 100%.

enzyme was strongly inhibited. With 0.4 mg/ml of phosphatidylcholine, maximum reactivation was observed between 0.05 and 0.1% deoxycholate.

On the basis of the above results, 0.05% deoxycholate was chosen as the fixed concentration with which to test the optimal level of phosphatidylcholine for reactivation (Figure 4). In this case, maximum activity was obtained with 0.1 mg/ml of phosphatidylcholine. Larger amounts, up to a tenfold excess, did not appreciably change the activity.

From these data, an optimal ratio of one volume of 0.5% deoxycholate to two volumes of 1.0 mg/ml phosphatidylcholine (0.17% deoxycholate and 0.67 mg/ml of phosphatidylcholine) was selected for the standard reactivating mixture, and it was determined that 0.02–0.2 ml of this solution in a 0.5-ml reaction mixture gave maximum reactivation (Figure 5).

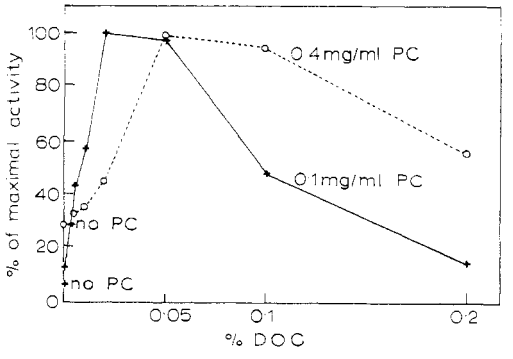

FIGURE 3: Effect of deoxycholate concentration on the reactivation of lipid-depleted M_2 particles. Each curve represents the results when a constant amount of egg phosphatidylcholine was included in the reaction mixture (final volume, 0.5 ml). Inorganic phosphate was determined after incubation with 0.1 ml of M_2 in the presence of 0.02 M glucose 6-phosphate (pH 6.8) for 30 min at 4°.

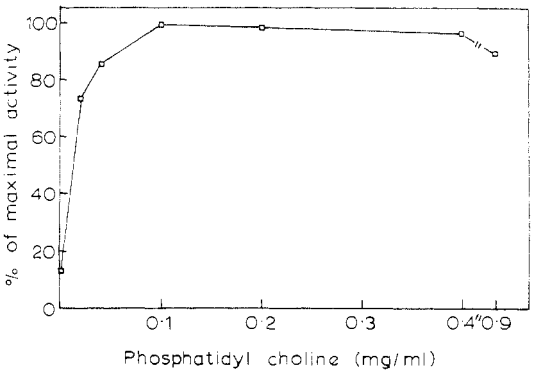

FIGURE 4: Effect of egg phosphatidylcholine concentration on the reactivation of M_2 in the presence of 0.05% deoxycholate. The other conditions were the same as in Figure 3.

To determine whether microsomal phospholipids would be more effective in reactivation than the other preparations, a chloroform–methanol (2:1) extract of several M_1's was prepared, evaporated under N_2, and suspended in water at a concentration of 1 mg/ml. This extract at 4° was not as effective as egg phosphatidylcholine in reactivating the enzyme (Table III), although analyses by thin-layer chromatography in this laboratory of chloroform–methanol extracts of M_1 demonstrate the presence of typical microsomal phospholipids (Dallner *et al.*, 1970), with phosphatidylcholine predominating. However, at 30° the same material gave full reactivation.

The effect of double bonds in the fatty acid side chains of phosphatidylcholine was examined by substituting the saturated, dipalmitoyl, and the doubly unsaturated, dioleyl, derivatives in the standard reactivating mixture (Table III). While the results show that the fully saturated compound does not reactivate, the dioleyl derivative is no more effective than egg phosphatidylcholine, which is predominately the monooleyl compound (Johnson and Stocks, 1971). However, when dioleylphosphatidylcholine is sonicated for 3 min, it can fully reactivate at 30° and partially reactivate at 4° without added detergent.

We were also interested in the specificity of the detergent requirement for reactivation with egg phosphatidylcholine. The results with Triton X-100 show that this nonionic deter-

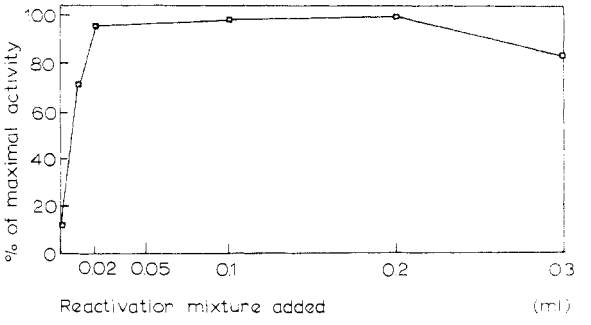

FIGURE 5: Reactivation of glucose-6-phosphatase activity in M_2 particles by the addition of increasing amounts of a deoxycholate (0.17%)–egg phosphatidylcholine (0.67 mg/ml) mixture to the reaction mixture (final volume, 0.5 ml). The other conditions are described in the legend for Figure 3.

gent is just as effective as the bile acid when added in optimal amounts (Table III).

Since we routinely found a small amount of phospholipid in the void volume fraction, we were interested to see if this could be removed by additional chromatography. When an M_2 preparation was rechromatographed on a freshly prepared Sephadex G-200 column, the ashed phosphate was reduced from 0.16 to 0.098 μmol per mg of protein. If this is residual phospholipid, it sticks very tightly to the protein. When the enzyme activity of these and similar preparations was plotted against the total phosphate present (the sum of that originally present plus phospholipid phosphate added), the curve in Figure 6 was obtained. It would appear that the activity without added phospholipid (marked with arrows) fits the phospholipid reactivation curve. Full activity of the M_2 fraction, equal to that of the M_1 fraction, is obtained at about 0.6 μmol of lipid phosphate/mg of protein. Since M_1 fractions contain from 1.2 to 1.9 μmol of phosphate per mg of protein, it would appear that one-half or more of the phospholipid in M_1 is not directly involved in enzyme activity. In fact, in one column fractionation with rather incomplete removal of phospholipid, the M_2 fraction with 0.44 μmol of phosphate/mg of protein (arrow) was fully active without addition of phospholipid.

Disc Electrophoresis. M_1 and M_2 particles are too large to enter polyacrylamide gels unless disrupted by strong detergents. After sodium dodecyl sulfate gel electrophoresis, these preparations exhibit several bands very close together which migrate just behind bovine serum albumin, and one band which migrates more rapidly than cytochrome *c*. In addition, a diffuse band, faster than albumin, is found with M_1 and, to a lesser extent, with M_2. It is the only band found after electrophoresis of the retarded fraction obtained from deoxycholate chromatography of M_1 on Sepharose 4B.

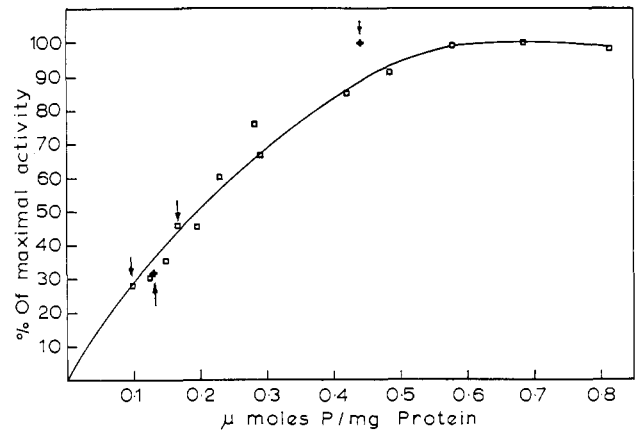

FIGURE 6: Activity of lyophilized M_2 fractions tested without additions (arrows) or after addition of varying amounts of egg phosphatidylcholine.

Discussion

The use of detergents to disrupt membranes and to solubilize their components has become a widely used technique (Tzagoloff and Penefsky, 1971; MacLennan, 1970; Razin, 1972). The purification of many loosely bound enzymes, and even some insoluble proteins which appear to be part of the membrane, have depended on this kind of treatment. In this respect, the interaction of detergents with the phospholipid bilayer may be no more important than that with the protein itself (Tzagoloff and MacLennan, 1965). In general, the more lipophilic the protein, the more detergent is bound to it during column chromatography in the presence of deoxycholate (Helenius and Simons, 1972). In our studies, the concentration of protein in the eluted fractions was too low, compared to the background level of deoxycholate, to establish binding ratios of detergent to protein or phospholipid. However, our impression is that it binds to some extent to both components. Further work is planned on this problem.

The dependence of glucose-6-phosphatase activity on bound phospholipid is confirmed by this work.[1] The suggestion that phospholipid is required mainly for thermal stability (Zakim, 1970) is not supported by our results, since full activity could be restored by replacement of lipid at 4° to particles which had lost activity after column fractionation at the same temperature.

The phospholipid requirements of glucose-6-phosphatase reported here are different from those found by other workers with crude microsome preparations (Duttera *et al.*, 1968; Zakim, 1970; Poulter *et al.*, 1971). We attribute this to the use of more purified starting material and to the preparation of delipidated protein by gel chromatography rather than with phospholipases. Another factor may be the use of detergents, which was found necessary for reactivation with some phospholipids. In any case, the specificity of the phospholipid requirement for reactivation does not necessarily reflect the organization of the membrane itself. *In vivo*, microsomes are associated with lipid-synthesizing enzymes (Wilgram and Kennedy, 1963; Bremer *et al.*, 1960; Gibson *et al.*, 1961) which can perhaps "insert" their products into the membrane. *In vitro*, however, the physical state of the added phospholipid becomes an important factor in its ability to associate with delipidated protein. Fully saturated dipalmitoylphosphatidylcholine, which does not activate the enzyme, exists in solution as large aggregates, even after extensive detergent treatment (Fleischer and Klouwen, 1961). Sonication for 5 min with or without deoxycholate does not clarify cloudy suspensions of this material. Van Deenen has noted the very low permeability of multilamellar "lipsomes" of this phospholipid (de Gier *et al.*, 1968) at temperatures below 41°, the melting point of its palmitate side chains (Chapman *et al.*, 1967). Others have also noted the failure of saturated phosphatidylcholine to activate phospholipid-dependent apoenzymes (Sebuzu *et al.*, 1963; Rothfield and Pearlaan, 1966; Pitlick and Nemerson, 1970). Small has demonstrated that bile acids disperse multilamellar aggregates of egg phosphatidylcholine into micellar solution (Small *et al.*, 1966). Since micelle formation of this phospholipid by sonication alone (Finer *et al.*, 1972)[2] results in partial activation of glucose-6-phosphatase, part of the bile salt requirement can be attributed to this effect.

It can also be inferred that, once formed, phospholipid micelles must be sufficiently labile to permit interaction with apoprotein. Detergent may be necessary to destabilize egg phosphatidylcholine micelles so that they can more easily

[1] Nakai *et al.* (1969) chromatographed the "M" fraction as described by Ernster *et al.* (1962) from rat liver in the presence of 0.5% deoxycholate at 4° on Sepharose 4B, and reported a severalfold purification of glucose-6-phosphatase. These authors did not present a phospholipid analysis of the eluted fractions, nor did they add phospholipid to reactivate the enzyme. This is in contrast to our finding with the M_1 fraction that phospholipid is required to show significant activity in the eluate.

[2] Finer *et al.* (1972) has described these sonicated micelles as spherical vesicles, 230 Å in diameter, consisting of a single bilayer of phospholipid.

bind to M_2 protein, thereby reversing the mechanism by which phospholipid was removed.

$$\text{protein–PL} + \text{DOC} \rightleftharpoons \text{protein} + \text{PL–DOC}$$

(DOC, deoxycholate; PL, phospholipid, endogenous or exogenous)

Similarly, it may be that dioleylphosphatidylcholine micelles, formed by sonication, are sufficiently labile to interact with M_2 protein at 30° and to some extent at 4° without added detergent.

In conclusion, we believe that gel chromatography in the presence of deoxycholate lends itself particularly well to the study of the phospholipid requirements of glucose-6-phosphatase and perhaps other enzymes.

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