Studies on the Phospholipid Requirement of Glucose 6-Phosphatase*

(Received for publication, April 7, 1967)

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SUMMARY

The role of phospholipid in rat liver microsomal glucose 6-phosphatase has been investigated with the use of phospholipase A and phospholipase C to alter the microsomal phospholipids. The phospholipase C-treated preparation lost a maximum of 80 to 90% of the original activity, and 70% of the microsomal phospholipid was hydrolyzed. Addition of phospholipid completely restored the original activity. The soluble and insoluble products of the phospholipase C treatment had no effect on the inactivation of glucose 6-phosphatase or the subsequent reactivation by phospholipid. The relative effectiveness of various single and mixed phospholipids, with respect to reactivation of the lipid-deficient, phospholipase C-treated glucose 6-phosphatase, was compared. Lecithin, which represents 53% of the microsomal phospholipid, was essentially all hydrolyzed by phospholipase C, but it was ineffective in reactivation. Phosphatidyl ethanolamine, which represents 23% of the phospholipid, was 60% hydrolyzed by phospholipase C, and it was the most effective individual phospholipid in terms of maximal reactivation. Lysolecithin, a trace component in microsomes, was the most effective phospholipid in reactivation at low concentrations, but inhibited at higher levels.

In contrast to the phospholipase C-treated preparation, the phospholipase A-treated samples were quite unstable; i.e. the ability to reactivate glucose 6-phosphatase with phospholipid was rapidly lost. In the presence of bovine serum albumin, a fatty acid-binding agent, there was essentially no loss of activity during incubation with phospholipase A. Lysolecithin, another product of the phospholipase A action, was shown to satisfy the phospholipid requirement of the lipid-deficient phospholipase C-treated preparation; thus, the phospholipase A products have contradictory effects on glucose 6-phosphatase activity.

* This work was supported by United States Public Health Service Grant GM-06628.
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Hepatic glucose 6-phosphatase (phosphogluconate phosphohydrolase, EC 3.1.3.9) is an enzyme which is localized in the endoplasmic reticulum (1-3), a membrane system which is rich in phospholipid (4). Current efforts aimed at a further understanding of the membrane systems of living organisms include attempts to delineate the relative importance and specific contribution of the lipid and protein components, including their mutual interdependence. Glucose 6-phosphatase, by virtue of its enzymatic activity, furnishes an opportunity to investigate the relation of one protein to the other components of a particular membrane system.

Glucose 6-phosphatase catalyzes the reaction

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\text{Glucose 6-phosphate} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{P}_i
\]

and other hydrolytic and transfer reactions (5-11). In 1954, Beaufay and de Duve (12), prior to the general recognition and acceptance of lipid-requiring enzymes, reported that treatment of a microsomal fraction from rat liver, with either phospholipase C or organic solvents capable of extracting lipids resulted in a loss of glucose 6-phosphatase activity. Based on this information, they suggested that glucose 6-phosphatase is a lipoprotein and further proposed that the lipid stabilizes a particular conformation of the protein which is necessary for catalytic activity. Since then, studies with enzymes such as D-(-)-3-hydroxybutyrate dehydrogenase, an outer mitochondrial membrane enzyme (13), clearly indicated a functional role for lipid in the activity of a particular enzyme (14, 15). Rigorous criteria for establishing the essentiality of lipid in the mitochondrial electron transfer system have been proposed by Fleischer et al. (16). These criteria emphasize the necessity of correlating loss of enzymatic activity with alteration or removal of lipid and the restoration of activity on addition, i.e. rebinding, of lipid. This report deals with the modification of microsomal phospholipids with phospholipases A and C, and shows the essentiality of phospholipid for microsomal glucose 6-phosphatase activity. Preliminary reports on the phospholipase A treatment of glucose 6-phosphatase have appeared (17-21).

EXPERIMENTAL PROCEDURE

Materials

Glucose 6-phosphate was obtained as the disodium salt from Sigma. Lyophilized Crotalus adamanteus venom was obtained...
from Ross Allen's Reptile Institute. Lecithinase C (phospholipase C) prepared from *Clostridium welchii* was purchased from Sigma. The preparation is an ammonium sulfate fraction prepared from a culture filtrate as described by MacFarlane and Knight (22). Dithiothreitol was purchased from Calbiochem, and crystallized bovine serum albumin, lot No. 11, from Pentex. The fatty acid content was determined by titration, as described below, to be 1 mole per mole of bovine serum albumin. Asolectin, a mixture of soybean phosphatides, was purchased from Associated Concentrates. On thin layer chromatography (see "Methods"), it was seen that Asolectin contained phosphatidyl serine, phosphatidyl ethanolamine, and lecithin. There were also at least two unidentified neutral lipid spots. The following percentage composition of the phospholipid has been reported (23): 36.9% cephalin, 36.5% lecithin, 8.8% cardiolipin, and 17.8% others. Lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin, all bovine preparations, were obtained from Applied Science Laboratories. Egg lysolecithin was purchased from General Biochemicals and Applied Science Laboratories. Silicic acid (100 to 200 mesh) was obtained from Applied Science Laboratories. Egg lysolecithin was purchased from General Biochemicals and Applied Science Laboratories. The purified lipids were checked for identity and homogeneity by thin layer chromatography with samples of 60 to 100 µg. Silicic acid (100 to 200 mesh) was obtained from Clarkson Chemical Company; Silica Gel G and H were from E. Merck (Darmstadt, Germany).

**Methods**

**Preparation of Microsomes** Albino Osborne-Mendel male rats weighing 150 to 200 g were fasted for 48 hours and killed, and the livers were removed immediately and immersed in ice-cold 0.25 m sucrose-0.01 m Tris (pH 8.0)-1 mM EDTA. The livers were weighed, cut into small pieces, and homogenized with a Teflon glass homogenizer in the sucrose-Tris-EDTA solution. The final dilution in the homogenate was 1 g, wet weight, of liver to 10 ml of sucrose-Tris-EDTA. Separation of subcellular fractions was done by a modification of the method of Schneider (24). The homogenate was centrifuged at 600 x g for 10 min, and the supernatant was collected and centrifuged at 15,700 x g for 10 min in a Servall centrifuge. This centrifugation was repeated on the resultant supernatant, or, more recently, following the 600 x g centrifugation, the resulting supernatant was centrifuged once at 15,700 x g for 15 min. The supernatant following the 15,700 x g centrifugation was carefully removed by suction, and a small residual amount was left in the tube. This supernatant was then centrifuged at 105,000 x g for 30 min for small quantities, or at 78,400 x g for 50 min for larger quantities in a Spinco preparative ultracentrifuge. The microsomal pellet was resuspended in sucrose-Tris-EDTA (with 2.5 to 5.0 ml per g of original tissue) and recentrifuged. The pellet was finally suspended in sufficient sucrose-Tris-EDTA to give a final protein concentration of 8 to 10 mg per ml. The sample was stored at 0° (in ice).

**Protein Determination**—Protein was determined by the method of Lowry et al. (25), as adapted for the Technicon AutoAnalyzer.1

**Inorganic Phosphate Determination**—Inorganic phosphate was determined by the method of Fiske and SubbaRow (26), as adapted for the Technicon AutoAnalyzer.1

**Glucose 6-Phosphatase Assay**—The glucose 6-phosphatase activity of the microsomes was measured by following the rate of orthophosphate appearance. Incubation mixtures with a final volume of 3 ml contained the following: 0.1 m sodium acetate-0.1 m sodium succinate buffer (pH 6.0), and 0.01 m glucose-6-P. The reaction was initiated by the addition of the desired aliquot of the microsomal fraction (usually in the range, 0.4 to 0.5 mg of protein). Incubation was at 38°. At 0, 5, and 10 min, 0.5-ml samples were withdrawn and added to 1.0 ml of 10% trichloroacetic acid. The samples were centrifuged, and the supernatant was analyzed for inorganic phosphate with the Technicon AutoAnalyzer as described above. One unit of enzyme activity corresponds to the release of 1 amole of inorganic phosphate in 10 min in the standard assay. Unless otherwise specified, the micromoles of inorganic phosphate released are the amount released in 10 min per ml of the incubation mixture or the percentage of the activity of the untreated microsomes in the normal assay. It should be emphasized that the activity of untreated microsomes equals 100%, and higher activity levels have been observed.

**Preparation of Phospholipase A (EC 3.1.1.4)**—Phospholipase A was purified from lyophilized *C. adamanteus* venom according to the procedure of Saito and Hanabusa (27). Chromatography on DEAE-cellulose was omitted.

**Preparation of Phospholipase C (EC 3.1.4.3)**—The commercial preparation (see "Materials") was dissolved in 0.01 m Tris buffer, pH 7.5 to 8.0, in a final concentration of 1 to 3 mg of protein per ml.

**Total Phosphorus Determination**—The samples were ashed according to the method of Ames and Dubin (28). The resulting inorganic phosphate was determined with the Technicon AutoAnalyzer, as described above, after the addition of 1 N HCl and heating for 15 min at 100°.

**Fatty Acid Extraction and Titration**—Fatty acids were extracted and titrated according to the procedure of Dole (29), as modified by Trout, Estes, and Friedberg (30), with the use of a Manostat microburette.

**Lipid Extraction**—Microsomal lipids were extracted by the method of Folch, Lees, and Sloane Stanley (31), or by the modification of Bligh and Dyer (32). In the latter case the microsomes were lyophilized prior to extraction. Microsomal phospholipid was separated from neutral lipid by elution on a silicic acid column (33). Silicic acid, 60 g, was washed with methanol and then chloroform and dried at room temperature. The silicic acid was suspended in chloroform, and a column, 25 x 28 cm, was poured. Approximately 250 to 300 mg of sample were applied to the column after it was washed with chloroform. The sample was first eluted with chloroform (5 volumes) to remove neutral lipid and then methanol (5 volumes) to remove phospholipid. The solvents were removed under reduced pressure and the samples were stored under nitrogen. Generally, the neutral lipid represented 15%, and the phospholipid 85%, by weight, of the total extracted microsomal lipid.

**Preparation of Diglyceride from Microsomal Phospholipid**—Microsomal phospholipid, 83 mg, prepared as described above, was incubated with 1 mg of phospholipase C in the presence of 0.0125 m CaCl₂ and 0.01 m Tris, pH 8.0, for 1 hour at 20°. The sample was mixed periodically. The lipids in the mixture were extracted as described above, and 72 mg were recovered. The diglycerides were separated from the phospholipid by means of the silicic acid column procedure described under "Lipid Extraction," and 35 mg of diglyceride were recovered. The identity of the diglyceride was checked by thin layer chromatography (see below).

1 William L. Byrne, unpublished procedure.
were added to the incubations at 20° in a final volume of 0.635 ml. For each experiment, were incubated in the presence of 2.5 μm of microsomes, in the amounts specified above. The eluting solvent used was chloroform-methanol-glacial acetic acid-water (25:15:10:20), by volume. The standard lanes were identified with ultraviolet light. The appropriate areas in the standard, and blank lanes were scraped, and the amount of lipid present was determined according to the method of Amenta (30). All samples, standards, and blanks were done in duplicate.

**RESULTS**

**Phospholipase A Treatment**—In order to study the phospholipid requirement of glucose 6-phosphatase activity, several methods of altering or removing phospholipids were examined. Phospholipase A alteration of phospholipid has been used in several cases to show a phospholipid requirement for enzymatic activity, e.g. mitochondrial Mg++-dependent ATPase (37), mitochondrial DPNH-cytochrome c reductase and DPNH-coenzyme Q reductase (38), and yeast succinate dehydrogenase (39). Fig. 1 shows that partially purified phospholipase A inactivated glucose 6-phosphatase, and that the inactivation was Ca++-dependent. Phospholipase A requires Ca++ for activity (27) and cleaves the α-fatty acid from phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine (40), which compose from 80 to 90% of the microsomal phospholipid (41, 42). It should be noted that the Ca++-activated protease normally present in *C. adamanteus* venom is largely removed during the purification of phospholipase A (27), and a comparison of crude and purified preparations showed that the inactivation of glucose 6-phosphatase was correlated with the phospholipase A activity (20). The loss in glucose 6-phosphatase activity was accompanied by an increase in titratable acidity (Fig. 2), presumably due to the release of fatty acid. In separate experiments the

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**Preparation of Phospholipid Micelles**—Phospholipids (approximately 4 mg per ml) were sonically disrupted at maximum setting on a Branson model LS 75 sonifier for 1 to 3 min in 0.02 m Tris (pH 8.0)-1 mM EDTA. Following sonication, the samples were centrifuged at 10,000 × g for 10 min in a Servall centrifuge, and the supernatant was stored at 4°. All samples were analysed for phospholipid-phosphorus by the total phosphorus method described above.

**Thin Layer Chromatography**—Thin layer chromatography for separation of phospholipids was carried out by the method of Skipski, Peterson, and Barclay (34), with one exception. 40 g of Silica Gel II were poured with 100 ml of 1 mM Na2CO3 and applied to plates, 20 × 20 cm, in the usual manner. The eluting solvent used was chloroform-methanol-glacial acetic acid-water (25:15:4:2, v/v). Neutral lipids were separated by thin layer chromatography according to the method of Brown and Johnston (35). Iodine vapor was used to detect lipid spots. When quantitative determinations were done, the method of Skipski et al. (34) was used, except that the phosphorus determinations were carried out as described under “Total Phosphorus Determination.”

**Phospholipase Treatment of Microsomes**—A similar incubation for alteration by either phospholipase A or phospholipase C was used. Microsomes and phospholipase, in the amounts specified for each experiment, were incubated in the presence of 2.5 m CaCl2 and 0.0125 Tris, pH 8.0, at 20°. The reaction with phospholipase was initiated by the addition of the appropriate amount of microsomes. The zero time sample, in those experiments in which it was determined, was removed immediately after mixing.

**Ether Extraction of Microsomes**—The method was a modification of that described by Beaufay and de Duve (12). Untreated and phospholipase C-treated microsomes (see above) were lyophilized. Following lyophilization, the samples were kept at −15° for the entire procedure. For approximately every 2 mg of microsomal protein, 10 ml of ether were added. The suspension was homogenized for 10 min and centrifuged for 15 min at 9000 × g in a Servall centrifuge refrigerated to −15°. The supernatant was poured off, and the pellet was resuspended in 10 ml of ether and again homogenized. The procedure was repeated four times. The time of homogenization was reduced to 5 min for the second, and 3 min for the third and fourth extractions. After the fourth extraction, the final traces of ether were removed from the pellets under reduced pressure, and the pellets were resuspended in 0.25 m sucrose-0.01 m Tris (pH 8.0)-1 mM EDTA. Recovery of protein and glucose 6-phosphatase activity was determined on the resuspended, control, and phospholipase C-treated pellets. The lipids from a sample of the resuspended ether-extracted microsomes, as well as from untreated and phospholipase C-treated microsomes which had not been extracted with ether, were extracted as described under “Lipid Extraction” above. The diglycerides from these samples were determined quantitatively and compared with the diglycerides found in the ether supernatants as described below.

**Diglyceride Determination**—The diglycerides from the ether-extracted microsomes and the ether supernatants (see above) were separated from other neutral lipids by thin layer chromatography on plates 0.25 mm thick spread with a slurry of 30 g of Silica Gel H and 70 ml of water. The developing solvent was ether-lignin-acetic acid (80:20:1), by volume. The standard lanes for identification were sprayed with 0.2% 2,7-dichlorofluorescein in methanol, and the location of the diglyceride spot was identified with ultraviolet light. The appropriate area in the standard, and blank lanes were scraped, and the amount of lipid present was determined according to the method of Amenta (30). All samples, standards, and blanks were done in duplicate.

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The valuable assistance of Dr. S. J. Friedberg, Mrs. Mary Ruth Greenfield, and Miss Helen Hilderman of the Co-operative Lipid Laboratory, Veterans' Administration Hospital, Durham, North Carolina, is gratefully acknowledged. The diglyceride data was analyzed by Dr. J. C. Friedberg from Dr. P. H. Matson, Proctor and Gamble, Cincinnati, Ohio.
fatty acids were extracted, determined quantitatively by titration, and characterized qualitatively by gas chromatography.

If phospholipid is indeed required for glucose 6-phosphatase activity, then activity loss due to alteration or loss of phospholipid should be regained on the addition of phospholipid, provided that irreversible denaturation has not occurred. Fig. 3 shows preliminary results which suggest such a requirement for glucose 6-phosphatase. Microsomes were exposed to phospholipase A for 10 min at 20°, and the reaction was terminated by the addition of EDTA. Aliquots of the treated mixture were then incubated with and without added phospholipid for 30 min, at which time glucose 6-phosphatase activity was assayed. The reaction with phospholipid has been shown subsequently to be essentially instantaneous, and 5-min incubations are currently used. Fig. 3 shows that as phospholipid was added in increasing amounts, there was a concomitant increase in glucose 6-phosphatase activity. At optimal phospholipid concentrations, up to 94% of the control activity was observed. Similar results were observed when Asolectin (see "Materials") was added under the same conditions. These results suggested a phospholipid requirement and indicated that there was a concentration dependence for phospholipid. However, the products of the phospholipase A action on microsomal lipid, i.e., fatty acids and lysophosphatides, were still present. In other lipid-requiring enzyme systems, these products have varying effects. Fatty

![Fig. 2. Relation between phospholipid hydrolysis by phospholipase A and glucose 6-phosphatase activity](image)

acids have been reported to inhibit (a) DPNH-cytochrome c reductase activity (38), and (b) the reactivation by phospholipid of Mg++-activated ATPase and Ca++ transport of muscle microsomes (43), while fatty acids have been reported to activate the Mg++-dependent ATPase from lipid-depleted mitochondria (37). Lysolecithin has been reported to reconstitute a preparation of cytochrome-oxidase (44), the above mentioned Mg++-activated ATPase, and phospholipid have been reported to activate the Mg++-dependent ATPase from lipid-depleted mitochondria (37). The preliminary incubation with phospholipid was carried out at 20° for 30 min, at which time glucose 6-phosphatase activity was assayed as described in "Methods," except that the reaction was initiated by adding a mixture of substrate, succinate-acetate buffer, and EDTA at 38°. The final concentration of EDTA in the assay was 1 mM. Total microsomal lipid was prepared as described under "Methods" by the procedure of Bligh and Dyer (32).

![Fig. 3. Apparent lipid requirement of phospholipase A-treated glucose 6-phosphatase. Microsomes, 4.4 mg of protein, were incubated with 0.4 mg of phospholipase A for 10 min at 20° in a mixture containing 2.5 mM CaCl₂ and 0.0125 M Tris, pH 8.0, in a final volume of 0.9 ml. At 10 min, 0.01 M EDTA, final concentration, was added to inhibit the phospholipase activity. Aliquots corresponding to 0.44 mg of microsomal protein were transferred to incubation mixtures containing 0.0125 M Tris, pH 8.0, and the indicated concentrations of phospholipid. The preliminary incubation with phospholipid was carried out at 20° for 30 min, at which time glucose 6-phosphatase activity was assayed as described in "Methods," except that the reaction was initiated by adding a mixture of substrate, succinate-acetate buffer, and EDTA at 38°. The final concentration of EDTA in the assay was 1 mM. Total microsomal lipid was prepared as described under "Methods" by the procedure of Bligh and Dyer (32).](image)
somes were incubated at 20° before addition to the incubation phospholipids were determined quantitatively as described in "Methods." The microsomes, 13 mg of protein, were incubated with or without 1.7 mg of phospholipase C in a final volume of 4.0 ml, as described under "Methods." The microsomes were incubated at 20° before addition to the incubation mixture at 20°. At fixed time intervals, an aliquot corresponding to 0.84 mg of microsomal protein was removed and added to 10% trichloracetic acid for total acid-soluble phosphorus determination; at the same time, an aliquot corresponding to 0.42 mg of microsomal protein was added to 0.01 M EDTA, final concentration, cooled to 0°, and held at 0° until assayed for glucose 6-phosphatase activity. The assay was carried out, as described under "Methods," within 5 min after the addition of EDTA. The samples for total acid-soluble phosphorus determination were decanted and recentrifuged. The supernatant was again carefully decanted and recentrifuged. The supernatant was again carefully decanted, and duplicate 0.1-ml aliquots were removed for total phosphorus determination as described under "Methods." The results are summarized in Table I.

Table I

| Phospholipid Composition of Control and Phospholipase C-Treated Microsomes |
|--------------------------|--------------------------|--------------------------|
| Phospholipid             | Control Microsomes (µmole) | Phospholipase C-Treated Microsomes (µmole) |
| Lysosomeerylthanolamine   | 0.017                    | 0                        |
| Sphingomyelin            | 0.017                    | 0.004                    |
| Lysolecithin             | 0.0030                   | 0.008                    |
| Lecithin                 | 0.034                    | 0.032                    |
| Phosphatidylserine       | 0.086                    | 0.083                    |
| Phosphatidyl ethanolamine| 0.015                    | 0.005                    |
| Unknown                  | 0.034                    | 0.032                    |

* Phosphatidyl serine and phosphatidylinositol have similar Rf values (34) and, although the spot was ninhydrin-positive (determined in a separate experiment), phosphatidylinositol could be present.

* The unknown or undetermined fraction corresponds to a combination of the phosphorus found in the solvent front fraction and at the origin.

Fig. 4 shows that loss of glucose 6-phosphatase activity in the presence of phospholipase C was accompanied by an increase in acid-soluble phosphorus. The reaction was essentially complete in 15 min, and the average increase in acid-soluble phosphorus was 0.40 µmole per mg of microsomal protein. The increase in acid-soluble phosphorus is presumably due to the release of soluble phosphorylcholine and phosphorylethanolamine. To determine whether the increase in acid-soluble phosphorus corresponded to the loss of phospholipid, the following experiment was carried out. The lipids from control microsomes and phospholipase C-treated microsomes were extracted by the method of Folich et al. (31), and the phospholipids were determined quantitatively following thin layer chromatography as described under "Methods." The results are summarized in Table I.

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A generous gift from Dr. Sidney Fleischer.
The sum of the individual phospholipids was 0.62 µmole of phospholipid-phosphorus per mg of microsomal protein for the untreated microsomes in both experiments. For the phospholipase C-treated preparations, 0.17 µmole and 0.22 µmole of phosphorus per mg of microsomal protein were recovered in Experiments 1 and 2, respectively. The average loss of phospholipid was 0.42 µmole of phospholipid-phosphorus per mg of protein (70% of the phospholipid), and this value agrees well with the observed increase in acid-soluble phosphorus, 0.40 µmole per mg of protein (Fig. 4). Essentially all of the sphingomyelin and lecithin and approximately 60% of the phosphatidyl ethanolamine of the microsomes were hydrolyzed under the conditions used.

As stated previously, if the correlation of phospholipid modification with loss of activity represents a phospholipid requirement, it should be possible to reactivate the phospholipase C-treated preparation with phospholipid. Preliminary incubation of phospholipase C-treated microsomes with Asolectin resulted in apparent glucose 6-phosphatase reactivation (Fig. 5). Experiments designed to distinguish reactivation from stabilization are described below. The concentration dependences of a 10- and 50-min preliminary incubation of microsomes with phospholipase C are compared in Fig. 5. The maximum activity was the same, but a higher concentration of added phospholipid was required for the sample first incubated for 50 min with phospholipase C. Maximum phospholipid alteration was probably not achieved in the 10-min preliminary incubation (cf. Fig. 4). The phospholipase C products were still present and their effects, if any, are described below.

**Fig. 5.** Apparent phospholipid requirement of phospholipase C-treated glucose 6-phosphatase. Two separate incubations of microsomes, 3.42 mg of protein, with 0.39 mg of phospholipase C, in a final volume of 0.9 ml, were carried out as described under “Methods” for 10 and 50 min, respectively. At these times 0.01 M EDTA, final concentration, was added to the mixtures, and the mixtures were chilled. Aliquots corresponding to 0.046 mg of microsomal protein were withdrawn and assayed for phospholipid-phosphorus as described under “Methods.” The sample in EDTA alone was assayed immediately for glucose 6-phosphatase activity. The assay was carried out as described under “Methods.”

Reactivation versus Stabilization—The apparent concentration-dependent requirement of glucose 6-phosphatase for phospholipid is suggested by the results in Figs. 3 and 5, but the experiments do not distinguish between reactivation and stabilization. In order to show reactivation, the lipid deficient form must be sufficiently stable so that the inactivation and reactivation can be followed as separate phenomena. Fig. 6 shows the loss of activity associated with phospholipase C treatment, and complete reactivation for all samples on the addition of Asolectin. In the time beyond 5 min, where maximal inactivation had occurred, it is evident that the added phospholipid was capable of reactivating glucose 6-phosphatase. Fig. 7, on the other hand, shows that the loss of activity with phospholipase A treatment was relatively slow under the conditions used, and the degree of reactivation by phospholipid decreased with time. Apparently, phospholipase A action led to an unstable form of the enzyme which irreversibly lost activity. Again, it should be noted that the products of the phospholipase action were still present.

**Fig. 6.** Reactivation of glucose 6-phosphatase activity by phospholipid at various times during phospholipase C treatment. Microsomes, 0.8 mg of protein, were incubated with 1.0 mg of phospholipase C in a final volume of 5.0 ml, as described under “Methods.” At the times indicated, aliquots corresponding to 0.44 mg of microsomal protein were withdrawn and added to 0.01 M EDTA, final concentration, or to 0.01 M EDTA, final concentration, plus Asolectin (40 µg of phospholipid-phosphorus). The latter was incubated for an additional 5 min at 20° before the glucose 6-phosphatase assay was initiated. The sample in EDTA alone was assayed immediately for glucose 6-phosphatase activity. The assay was carried out as described under “Methods.”

Effect of Products of Phospholipase Action—In the experiments described above, no attempts were made to remove the products of the phospholipase action on microsomal lipids. Previous experiments in our laboratory (20) suggested that the released
fatty acids and the lysophosphatides from phospholipase A action inhibited glucose 6-phosphatase or the phospholipid reactivation, or both. Salomon, James, and Daginawala (50) have reported that fatty acids and lysolecithin inhibited intestinal glucose 6-phosphatase. Therefore, in an attempt to evaluate potential interference by phospholipase A products, bovine serum albumin was included in an incubation with phospholipase A (Fig. 8). Fatty acid-poor bovine serum albumin can be used effectively as a binding agent for fatty acids (51). The serum albumin prevented the normal loss of glucose 6-phosphatase activity seen in the presence of phospholipase A. The serum albumin alone had little, if any, effect on the control glucose 6-phosphatase activity. The fatty acid release was the same in both the presence and absence of bovine serum albumin, which proves that phospholipase A was not inhibited in the presence of serum albumin. These results suggest either that alteration of the phospholipid by phospholipase A did not cause loss of glucose 6-phosphatase activity (negating a phospholipid requirement) or that perhaps, in the absence of fatty acid inhibition, the other products of the phospholipase A reaction, i.e. lysophosphatides, could reactivate or stabilize the phospholipase A-treated preparation.

An experiment to determine whether the lysophosphatides, e.g. lysolecithin, could, indeed, reactivate a lipid-deficient glucose 6-phosphatase preparation is illustrated in Fig. 9. Microsomes were first incubated with phospholipase C. After the reaction was terminated with EDTA, aliquots were removed and incubated in the presence of graded amounts of lysolecithin and finally assayed for glucose 6-phosphatase activity. As illustrated in Fig. 9, glucose 6-phosphatase activity was regained, and 0.31 to 12 µg of phospholipid-phosphorus were required to give approximately 100% of the original untreated activity. These results suggest that the absence of any significant loss of glucose 6-phosphatase activity, when microsomes were incubated with phospholipase A in the presence of bovine serum albumin, was probably due to the formation of lysophosphatides. The amount of total lysophosphatides released by treatment of a comparable sample of microsomes with phospholipase A, 9 to 12 µg of phospholipid-phosphorus, may be calculated from fatty acid release data, and this quantity of lysophosphatides is apparently sufficient to achieve maximum reactivation of the lipid-deficient glucose 6-phosphatase.

As pointed out above, the routine procedure for examining the phospholipid requirement after phospholipase C treatment did not include removal of phospholipase C products. Therefore, it was important to evaluate the possible effect of these products

Fig. 7. Reactivation of glucose 6-phosphatase activity by phospholipid at various times during phospholipase A treatment. This experiment was carried out with the procedure described in Fig. 6, except that 12.4 mg of microsomal protein were incubated with 1.7 mg of phospholipase A, in a final volume of 4.8 ml. Aliquots corresponding to 0.41 mg of microsomal protein were removed at the indicated times and added to 0.01 M EDTA, final concentration, or 0.01 M EDTA, final concentration, plus Asolectin (35 µg of phospholipid-phosphorus). The latter was incubated an additional 5 min at 30° before being assayed for glucose 6-phosphatase activity.

Fig. 8. Effect of bovine serum albumin on phospholipase A treatment of microsomes. Microsomes, 8.1 mg of protein, were incubated with or without 0.83 mg of phospholipase A, as described under "Methods." Serum albumin, 90 mg, was included in the incubations where indicated. The final volume of all incubations was 6.0 ml. At the indicated times, aliquots corresponding to 0.50 mg of microsomal protein were withdrawn and added to 0.01 M EDTA, final concentration, chilled, and assayed for glucose 6-phosphatase activity as described under "Methods." BSA, bovine serum albumin.
and, when the resuspended pellet was incubated with phospholipase C-treated, centrifuged and uncentrifuged microsomes. 911 of the potential activity was recovered in the pellet fraction, and this value was compared with untreated microsomes which had been centrifuged in the same manner. A difference (control minus phospholipase C-treated) of 0.55 μmole of phosphorus per mg of microsomal protein was obtained. The results indicated that there was also some solubilization of microsomal protein on treatment with phospholipase C, but this did not appear to include glucose 6-phosphatase. Preliminary experiments on the selective extraction of diglyceride were carried out with other extraction of lyophilized preparations (see "Methods"), since this allows retention of glucose 6-phosphatase activity (12). A quantitative determination of the diglyceride regions from thin layer plates indicated that ether extraction did not remove a significant fraction, 40%, of the diglyceride, and this by comparing the properties of these preparations in the presence and absence of products and by adding a several-fold excess of one of the products, a diglyceride fraction prepared from microsomal phospholipid (see "Methods"). Centrifugation of phospholipase C-treated microsomes at 93,000 × g for 35 min indicated that the glucose 6-phosphatase was isolated in the pellet fraction, and this made it possible to separate those products which were soluble. However, the results of Finean and Martonosi (32) with phospholipase C-treated microsomes from skeletal muscle indicated that the diglyceride product was released as uniformly dense droplets and that the droplets remained "entrapped in the vesicular material." Evidence that the released diglyceride remained in the sedimented liver microsomes following centrifugation was obtained when the lipids were extracted from the pellet and supernatant. The extracted lipids were separated by thin layer chromatography by the method of Brown and Johnston (35) and were visualized by iodine staining (see "Methods"). As the time of incubation with phospholipase C increased from 0 to 20 min, the diglyceride region, primarily in the sample extracted from the pellet fraction, increased in intensity.

Table II shows the results of phospholipid reactivation of the phospholipase C-treated, centrifuged and uncentrifuged microsomes. All of the potential activity was recovered in the pellet and, when the resuspended pellet was incubated with phospholipid, complete activity was restored. The uncentrifuged sample showed the same results, i.e. complete restoration of activity on addition of phospholipid, an indication that the presence of the soluble products did not affect the activity or reactivation of glucose 6-phosphatase. Release of soluble phosphorus to the supernatant was confirmed in a separate but similar experiment. Phospholipase C-treated microsomes were centrifuged as described in Table II, the pellet was resuspended, the phosphorus content was determined, and this value was compared with untreated microsomes which had been centrifuged in the same manner.

Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>(100)</td>
<td>%</td>
</tr>
<tr>
<td>Untreated plus Asolectin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Phospholipase C-treated</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Phospholipase C-treated plus Asolectin</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>Phospholipase C-treated pellet (following centrifugation at 93,000 × g for 35 min)</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>Phospholipase C-treated pellet plus Asolectin</td>
<td>103</td>
<td>154</td>
</tr>
</tbody>
</table>
Microsomal protein, 16.2 mg, was incubated with 2.4 mg of phospholipase C for 20 min at 20°, as described in "Methods," in a final volume of 4.9 ml. After 20 min, 0.01 M EDTA, final concentration, was added and the mixture was chilled. Equivalent amounts of untreated and treated microsomes were lyophylized and extracted with ether as described under "Ether Extraction." The extracted microsomes were resuspended in 1.0 ml of 0.25 M sucrose-0.01 M Tris (pH 8.0)-1.0 mM EDTA. One-tenth milliliter was used for the glucose 6-phosphatase assay, and the specific activities recovered are reported in the table. One-tenth milliliter of each sample was incubated for 5 min at 20° with 80 pg of phospholipid-phosphorus (Asolectin), where designated, prior to assay for glucose 6-phosphatase activity. The diglyceride in the fractions, expressed as micrograms per mg of microsomal protein, was: untreated, -150; phospholipase C-treated, -500; phospholipase C-treated after four ether extractions, -200; untreated after four ether extractions, -90.

### Table III

**Glucose 6-phosphatase activity following ether extraction of lyophilized microsomes**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity (μg/mg)</th>
<th>% untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Treated for 20 min with phospholipase C</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Phospholipase C-treated microsomes after four ether extractions</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Extracted phospholipase C-treated microsomes + Asolectin</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Control microsomes after four ether extractions</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Extracted control microsomes + Asolectin</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

did not appear to influence the activity or the phospholipid requirement (Table III).

Furthemore, it was shown (Table IV) that the addition of a 5-fold excess of diglyceride had no effect on the glucose 6-phosphatase activity of control or of microsomes treated for 5 and 20 min with phospholipase C. Also, when 1- or 2-fold excess diglyceride was included in the incubation with Asolectin, following phospholipase C treatment, there was no significant difference in activity. At a 5-fold excess some decrease was noted in the phospholipid reactivation (cf. Table IV). At the maximum level of phospholipid used, the presence of 5-fold excess products resulted in 88% of the reactivation observed with no excess products added; however, this activity with phospholipid in the presence of 5-fold products represented 98% of the original untreated activity. Consequently, it would appear that removal of diglyceride from phospholipase C-treated enzyme had no observable effect on activity. It can be added to preparations which were untreated, phospholipase C-treated, or phospholipase C plus phospholipid-treated in as much as 5-fold excess with no major changes in activity.

**Relative Effectiveness of Various Phospholipids in Reactivating Glucose 6-Phosphatase**—Because of the marked stability of the phospholipase C-treated glucose 6-phosphatase activity and the negligible effect of the presence of the products of the phospholipase action, the phospholipase C-treated fraction has been used for a study of the relative effectiveness of a series of phospholipids in reactivating glucose 6-phosphatase. Reactivation, as opposed to stabilization, has been verified in all cases described below. It should be noted here that one of the criteria described as necessary for the demonstration of a phospholipid requirement (16) is to show that binding of phospholipid takes place concomitantly with the reactivation observed on the addition of phospholipid to the phospholipid-deficient enzyme. In the case of a purified enzyme, this type of binding-reactivation criteria would be meaningful but, in a case such as the glucose 6-phosphatase activity of microsomal fractions or comparable studies with unfraccionated membrane-derived samples, the percentage of the total protein present as a single protein, e.g. glucose 6-phosphatase, is unknown. Consequently, binding of lipid per se cannot be interpreted as binding of lipid in direct association with the lipid-deficient glucose 6-phosphatase moiety.

Preliminary studies of phospholipid binding have been carried out with phospholipase C-treated microsomes. The amount of phospholipid bound is a function of the amount of lipid added (Fig. 10), and the activity increased up to 100% of the original activity.

### Table IV

**Effect of excess diglycerides on glucose 6-phosphatase activity**

<table>
<thead>
<tr>
<th>Treatment of microsomes</th>
<th>Excess diglyceride</th>
<th>% original activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td>Phospholipase C-treated</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Phospholipase C-treated + Asolectin (60 μg of phospholipid-phosphorus)</td>
<td>112</td>
<td>117</td>
</tr>
</tbody>
</table>

* Microsomal protein was incubated with phospholipase C for 5 min.

** Microsomal protein was incubated with phospholipase C for 20 min.
untreated activity when 0.90 μmole or 28 μg of phosphorus were bound per mg of microsomal protein. The amount bound is determined by subtracting the micrograms of phosphorus originally present from the micrograms of phosphorus recovered after incubation with phospholipid. Fig. 10 is based on the addition of lysolecithin, and a qualitatively similar relationship of binding and reactivation was observed when Asolectin was used. However, as the amount of lysolecithin is increased beyond the amount needed for maximum activity, the amount of phospholipid bound continues to increase. The binding of lipid, as judged by the activity of glucose 6-phosphatase, appeared to have saturation type kinetics, but the directly determined binding of lysolecithin and Asolectin continued to increase at the highest increment of phospholipid tested. This would indicate that the contribution of lipid binding to lipid-deficient glucose 6-phosphatase is quantitatively a minor component of the total binding. In addition, the limited value of a binding-reactivation criterion for a microsomal enzyme is also indicated by preliminary binding studies in which control microsomes were compared with phospholipase C-treated microsomes. Untreated microsomes were found to bind Asolectin in amounts comparable to the phospholipase C-treated microsomes. In these studies, it was also shown that the Asolectin centrifuged alone, under the same conditions, did not sediment. The Asolectin had been centrifuged, prior to these binding studies, at conditions used to sediment microsomes. It should also be pointed out that lysolecithin, at all concentrations tested, was completely bound to phospholipase C-treated microsomes. Thus, binding studies have not been carried out routinely. The relative effectiveness of various phospholipids in reactivating glucose 6-phosphatase activity could be a reflection of binding specificities of the phospholipid for the glucose 6-phosphatase moiety; however, binding studies with the microsomal preparation may not reflect these differences.

Fig. 11 shows reactivation of phospholipase C-treated glucose 6-phosphatase by total microsomal lipid and Asolectin. Microsomal lipid was less effective than Asolectin in terms of the...
amount required and the maximal activity. Also, a comparison of the results illustrated in Fig. 11 with those in Fig. 7 made it clear that a 6-fold increase in phospholipid-phosphorus was required for maximal reactivation with microsomal lipid as compared with lysolecithin. The reactivation of glucose 6-phosphatase by a variety of purified phospholipids is illustrated in Fig. 12. The lipids used are those which are components of the microsomal fraction, except for Asolectin which was included for comparison. For these experiments the purified lipids were commercial preparations from bovine brain. Lysolecithin was the most effective in terms of phospholipid required, and phosphatidyl ethanolamine was most effective in terms of maximal reactivation. A surprising result of this study was the ineffective reactivation attained with lecithin, which makes up approximately 45 to 60% of the microsomal phospholipid (References 41, 42 and 53; see Table I). Reactivation with sphingomyelin was also low.

The results in Fig. 13 include a comparison of reactivation by individual phospholipids and mixtures of lecithin and lysolecithin. The mixtures were 3.3:1 and 1.7:1 (mole:mole) for lysolecithin and lecithin, respectively, which had been sonically treated together. As the relative proportion of lecithin in the mixture was increased, a higher concentration of total phospholipid was required to obtain the same level of activity as that attained with lysolecithin alone. In fact, below the 80% level of reactivation, the restored activity can be accounted for as being due simply to the concentration of lysolecithin present in the mixture. Above the 80% level, however, the activity attained was greater than that expected from lysolecithin alone. The additional activity is less than that predicted by summing the individual effects, but it does indicate that lecithin has at least a partial effect in the presence of lysolecithin.

**DISCUSSION**

The first suggestion that a lipoprotein complex might be required for glucose 6-phosphatase activity was made by Beaufay and de Duve (12) in 1954. Their report was prior to the general acceptance of a functional role of lipid in enzyme activity, and the evidence was restricted to a correlation of lipid modification and activity loss. The present report has attempted to show the phospholipid requirement of glucose 6-phosphatase. The criteria set for establishing the essentiality of phospholipid for glucose 6-phosphatase were loss of activity on alteration or removal of phospholipid and the subsequent restoration of activity on the addition of phospholipid. Fleischer et al. (16), who first established the criteria for demonstration of a phospholipid requirement for enzymatic activity, also suggested a third criterion: the demonstration of phospholipid rebinding to the lipid-deficient preparation with concomitant reactivation of activity. However, a reactivation-binding criterion for a heterogeneous preparation such as microsomes is of limited value.
Several methods for removing the phospholipid from microsomes were used in this study. Removal of phospholipid by acetone-water treatment yielded suggestive evidence for a phospholipid requirement for glucose 6-phosphatase. However, the incomplete inactivation and only partial recovery observed with added phospholipid were not amenable to further experiments in terms of a detailed study of the phospholipid requirement.

The apparent inactivation and reactivation of glucose 6-phosphatase by phospholipase A and subsequent addition of phospholipid were consistent with a phospholipid requirement for glucose 6-phosphatase. However, the fatty acids, a product of the phospholipase A action, were removed by including bovine serum albumin in the phospholipase incubation, there was no significant loss of activity. This was explained by experiments in which lysolecithin, at levels comparable to those formed during phospholipase A treatment, reactivated phospholipase C-treated preparations. It appears that, in the absence of released fatty acids, the lysolecithin released by phospholipase A is capable of satisfying the phospholipid requirement.

In contrast to treatment by acetone-water and phospholipase A, phospholipase C treatment appeared to be a satisfactory method for studying the phospholipid requirement of glucose 6-phosphatase. In this case, it was shown that the soluble products of the phospholipase C action had no effect on the inactivation of glucose 6-phosphatase or the subsequent reactivation with phospholipid. The insoluble diglycerides were more difficult to deal with, and the possibility existed that the apparent phospholipid requirement could be simple reversal of inhibition resulting from the presence of the released diglyceride; however, diglyceride prepared from microsomal phospholipid did not appear to be an inhibitor. The addition of excess diglyceride had little or no effect on untreated microsomes, phospholipase C-treated preparations at different stages of inactivation, or preparations which had been inactivated with phospholipase C and reactivated with phospholipid. The possible role of diglyceride as an inhibitor was also evaluated by ether extraction as a means of decreasing the diglyceride content of the phospholipase C-treated preparations. Approximately 40% of the diglyceride was removed, and there was no increase in activity. The unchanged activity was not due to a balanced removal of an inhibitor and inactivation by ether extraction since the ether-extracted preparation was still reactivated by phospholipid. Since loss of activity is correlated with product release (cf. Fig. 4), removal of 40% of the diglyceride product should give a readily detectable increase in activity if the products were inhibiting. Thus, loss of phospholipid, and not diglyceride or other product inhibition, is responsible for the loss of glucose 6-phosphatase activity on treatment with phospholipase C. For these reasons, the removal of the released products is not essential to the demonstration of a phospholipid requirement when the effectiveness of various phospholipids is surveyed.

Study of the relative effectiveness of various phospholipids revealed that, when maximal inactivation was achieved with phospholipase C, subsequent reactivation was observed on the addition of total microsomal lipid or a mixture of soybean phospholipids (Asolectin). However, this reactivation by two different mixtures of lipids should not be interpreted as evidence for a nonspecific reactivation phenomenon. For instance, lecithin and sphingomyelin were the least effective in reactivating the treated sample, and phosphatidyl ethanolamine was the most effective in regaining maximum activity.

Experiments were carried out which showed that Asolectin or lysolecithin was bound to phospholipase C-treated preparations under the conditions used for reactivation. However, control and phospholipase C-treated preparations bound approximately the same amount of Asolectin per mg of protein. Lipid rebinding and reactivation, as an additional criterion for a phospholipid requirement in a heterogeneous preparation such as microsomes, is of little value, and the binding of equal amounts of Asolectin by untreated or treated preparations makes it even less important.

The complete recovery of glucose 6-phosphatase activity on the addition of certain phospholipids to the phospholipase C-treated microsomes was possible because of the stability of the phospholipase C-treated enzyme. It is interesting to note that none of the glucose 6-phosphatase activity is solubilized following phospholipase C treatment; this could account for the marked stability of the phospholipase C-treated enzyme as compared to the phospholipase A-treated enzyme, which is partially solubilized under comparable conditions (20). It should be noted that Finess and Martonosi (52) found, on examining the microsomal pellet by electron microscopy following phospholipase C treatment of muscle microsomes, that the vesicular structure was maintained although there was evidence of shrinkage. Loss of ATPase activity, as well as loss of Ca++ transport, was associated with the phospholipase C treatment in their preparation. It is quite possible that maintenance of glucose 6-phosphatase in a particulate structure, perhaps with retention of specific protein-protein interactions, prevented irreversible denaturation when the microsomes were treated with phospholipase C and made it possible to recover the original activity when phospholipids were added.

A recent report in abstract form (64), in apparent contradiction to the above results, has indicated that, "Phospholipases A, C or D acting on liver microsomes in vitro reduced glucose 6-phosphatase and ... added phospholipid from liver microsomes ... or brain or egg phospholipids did not return enzyme activity to the original level before phospholipase action." This report may be partially explained by the results presented here for the phospholipase A-treated samples. The rapid irreversible loss of glucose 6-phosphatase activity in the presence of phospholipase A, unless fatty acids are trapped, could prevent reactivation to the original activity in the addition of phospholipids. The lack of reactivation of phospholipase C-treated preparations is more difficult to understand, but a more detailed comparison must wait for the availability of information on the experimental procedure used by these authors.

The phospholipid requirement of glucose 6-phosphatase has afforded an opportunity to study in part the interrelation of two important components of membrane systems, protein and lipid. A role for lipid has been implicated for other enzymes of the endoplasmic reticulum: aromatic hydroxylase (55), phosphatidic acid phosphatase (56), DPNH-cytochrome c reductase (53, 57, 58), and fatty acid desaturase (59). The exact physiological role of phospholipid for these enzymes, as well as for glucose 6-phosphatase, is not known. The possibility of specificity in the particular phospholipid required has been suggested in the present paper, and it is conceivable that changes in the specific phospholipid environment could result in changes in activity or specificity of the enzyme, or both. It is also possible that the affinity of the enzyme for phospholipid may be important in localizing
the enzyme in or on the endoplasmic reticulum where it could function to extrude glucose to the exterior of the cell (60).

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Studies on the Phospholipid Requirement of Glucose 6-Phosphatase
Sue M. Dutlera, William L. Byrne and M. Clelia Ganoza


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