Lipid Dependence of Glucose-6-phosphate Phosphohydrolase: A Study with Purified Phospholipid Transfer Proteins and Phosphatidylinositol-Specific Phospholipase C†

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ABSTRACT: The nonspecific and phosphatidylcholine-specific transfer proteins from beef liver and the phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* were used to modify the phospholipid composition of microsomal membranes in order to study the dependence of glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) activity on membrane phospholipids. Incubation of microsomes with dipalmitoylphosphatidylcholine-containing unilamellar vesicles and either transfer protein produced a membrane in which this disaturated phospholipid contributed up to 43% of the total phosphatidylcholine. Incubation of microsomes and phosphatidylcholine unilamellar vesicles with nonspecific transfer protein caused a net increase in the membrane phosphatidylcholine content and a net decrease in the phosphatidylethanolamine and phosphatidylinositol levels, whereas in incubations with the phosphatidylcholine transfer protein, no change in phospholipid class composition occurred. Incubations of microsomes and phosphatidylcholine/phosphatidylethanolamine (3:1 mol/mol) unilamellar vesicles with the nonspecific transfer protein also caused an increased phosphatidylcholine content and a decreased phosphatidylinositol content but no change in the phosphatidylethanolamine level. Similar incubations in the presence of phosphatidylcholine-specific transfer protein had no effect on the phospholipid composition. Incubation of microsomes with 10 μg of phosphatidylinositol-specific phospholipase C caused hydrolysis of over 50% of the membrane phosphatidylinositol but no change in the other membrane phospholipids. These changes in membrane composition were correlated with changes in glucose-6-phosphate phosphohydrolase activity. It is concluded that removal of 55% of membrane phosphatidylinositol or 30% of the cholesterol contents has no effect on the enzyme activity. The fatty acid saturation of phosphatidylcholine has a small effect, inhibiting the enzyme activity (assayed at 30 °C) by 12% when 43% of the phosphatidylcholine was replaced by dipalmitoylphosphatidylcholine. A relationship was found between the level of phosphatidylethanolamine in the membrane and enzyme activity; very little inhibition occurred when no change in phosphatidylethanolamine level was produced whereas a 37% inhibition was produced by a 27% depletion of phosphatidylethanolamine.

Numerous membrane-bound enzymes have been found to have a functional dependence on the lipid bilayer (Sandermann, 1978). This was clearly demonstrated by Fleischer and co-workers (1962) who first showed a role of phospholipids in the mitochondrial electron transfer system by (a) removing phospholipid by acetone extraction, (b) correlating the removal of phospholipid with loss of activity, and (c) correlating restoration of enzyme activity with rebinding of phospholipid. Since then, a number of techniques have been used to study the lipid dependence of membrane function. Inhibition of enzyme activity has been correlated with hydrolysis of phospholipids by phospholipases (Martonosi et al., 1968) as well as with phospholipid removal by acetone extraction. The relationship between the physical state of the bilayer and enzyme activity has been inferred from breaks in Arrhenius plots (Londesborough, 1980). Indirect evidence for a role of fatty acid saturation on enzyme activity has been obtained with membranes prepared from animals whose diets were manipulated (Spector et al., 1980; Louis et al., 1976; Haeffner & Privett, 1975) and from changes in membrane properties caused by temperature acclimatization (Cossins, 1977; Kasai et al., 1976). More direct evidence has been obtained by reconstitution of solubilized enzymes in lipid vesicles (Gazzotti et al., 1975; O'Brien et al., 1977). Finally, in certain bacterial auxotrophs having defective fatty acid desaturase activity, the dependence of enzyme activity on phospholipid saturation has been clearly demonstrated (Hsu & Fox, 1970; Haslam & Fellows, 1975). Though extensive evidence for dependence of membrane enzyme activity on fatty acid saturation of phospholipid exists, there is relatively little evidence for the specific requirement for a particular phospholipid class.

Recently, modulation of the lipid composition with phospholipid transfer proteins has been explored as a means of manipulating the lipid environment of membrane enzymes (Dyatlovitskaya et al., 1977–1979). Protein-catalyzed transfer of phospholipid between lipid vesicles and biological membranes should allow modification of the lipid environment in situ under mild conditions and allow the study of the lipid dependence of membrane enzyme activity under conditions in which many of the drawbacks and limitations of previous methods are not encountered. In the present report, we describe the manipulation of the phospholipid composition of rat liver microsomal membranes with purified transfer protein and unilamellar lipid vesicles and with the phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*. The effect of the altered lipid composition on glucose-6-phosphate phosphohydrolase activity is examined and compared to reports concerning the lipid dependence of microsomal glucose-6-phosphate phosphohydrolase as measured by other techniques.

Materials and Methods

Lipids. ^{32}P-Labeled PC[1] was isolated from the liver of a

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rat injected intraperitoneally with 3 mCi of $^{32}P_i$ (New England Nuclear, Boston, MA) per 100 g of body wt. 16 h before sacrifice. It was extracted and purified as previously described (Bloj & Zilversmit, 1976). Nonradioactive PC was extracted from rat liver and purified on an alumina column (15 g of Woelm alumina, activity I, ICN Pharmaceuticals, Plainview, NY). Glycerol [9,10-$^3H_2$]trioleate (New England Nuclear) was purified by thin-layer chromatography on silica gel H with hexane/diethyl ether/acetic acid (60:40:1 v/v/v). The glycerol trioleate was eluted with chloroform and stored at −20 °C.

^{14}C-Labeled DPPC (New England Nuclear), found to be greater than 95% pure by thin-layer chromatography, was diluted in chloroform and stored at −20 °C. Unlabeled egg PC, egg PE, ox heart diPG (Lipid Products, South Nutfield, England), and DPPC (Sigma Chemical Co., St. Louis, MO) were diluted in chloroform and stored at −20 °C. Unlabeled cholesterol was purified as the dibromide derivative, crystallized from methanol, and stored at −20 °C in chloroform. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, OH) was dissolved in chloroform without further purification.

Small sonicated unilamellar vesicles were prepared as described by Johnson & Zilversmit (1975) by sonication above the phase transition temperature of the lipid. Butylated hydroxytoluene (0.1% by weight) was included as an antioxidant. A trace of glycerol [^3H]trioleate was included as a nonexchangeable marker.

Microsomes. Microsomes were prepared from the livers of male rats (250–400 g) fasted 16 h. After two rats were decapitated and exsanguinated, the livers were excised and placed in 75 mL of ice-cold buffer containing 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0. The minced livers were washed with cold buffer, suspended in 2 volumes of buffer, and homogenized by four passes at 500 rpm in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted with buffer to give a 10% homogenate. Mitochondria, nuclei, and intact cells were sedimented by centrifugation 10 min at 8000*g*. Any unsedimented mitochondria and lysosomes were removed by centrifugation of the supernatant at 15000*g* for 10 min. Microsomes were sedimented by centrifugation for 60 min at 113000*g*. The pellets were suspended in 0.25 volume of 150 mM Tris-HCl, pH 8.0, and microsomes were resedimented by centrifugation for 30 min at 113000*g*. The pellet was suspended in 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0, at a final protein concentration of 30–40 mg/mL.

The homogeneity and membrane integrity of our microsomal preparations were examined by a modified procedure of Leskes et al. (1971). Microsomal membranes (7.4 mg of protein) were incubated at 21 °C in 20 mL of 175 mM sucrose, 100 mM sodium acetate, 2 mM $Pb(NO_3)_2$, and 1 mM Glc-6-P, pH 6.5. Controls were incubated with $Pb(NO_3)_2$ but without Glc-6-P. After 60 min, the reaction was stopped by transferring the sample to an ice bath. Pb^{2+} and $Pb_3(PO_4)_2$ bound to the outside surface of membranes were removed by incubating at 4 °C with 4 mM EDTA. After 90 min, 10 mL of the above was layered on 10 mL of 2 M sucrose, and the microsomes were sedimented by centrifugation at 64000*g* for 60 min. No pellet formed when control microsomes were centrifuged, but samples containing Glc-6-P and $Pb(NO_3)_2$ sedimented with less than 20% of the phospholipid remaining at the interface. The sedimented microsomal membranes were homogenized and extracted. Their phospholipid compositions were the same as those of microsomes before sedimentation. Membrane integrity was also tested by te latency of Man-6-P phosphohydrolase (Arion et al., 1972). Latency in our preparations was greater than 95%.

Analytical Procedures. Microsomal protein was measured by the biuret method with bovine serum albumin as standard (Gornall et al., 1949). Microsomal lipids were extracted by the method of Bligh & Dyer (1959). Free cholesterol was measured by an enzymatic method (Gallo et al., 1978) as modified by Pattnaik & Zilversmit (1980). Phospholipids were separated by thin-layer chromatography on silica gel 60 plates (E. Merck, Darmstadt, Germany) with chloroform–methanol/acetic acid/water (25:15:4:2 v/v). They were eluted twice by heating silica gel scrapings for 60 min at 65 °C with 4 mL of methanol/concentrated HCl (98:2 v/v) (Crain et al., 1978). Lipid phosphorus was measured by the method of Bartlett (1959). Inorganic phosphorus produced by phosphohydrolase activity on Glc-6-P was measured by a modification of the method of Lowry & Lopez (1946).

Protein Purifications. Partially purified PC-specific exchange protein was isolated from beef liver as described by Kamp et al. (1973). The protein was stored in 50% glycerol at −20 °C after chromatography on carboxymethylcellulose, achieving a 500-fold purification compared to the pH 5.1 supernatant. The nonspecific transfer proteins from beef liver were purified as previously described by Crain & Zilversmit (1980a). In the present study, we used the protein fraction after chromatography on an octylagarose column with a purification factor greater than 1200 compared to the postmicrosomal supernatant. One unit of transfer activity is defined as the transfer of 1 nmol of PC per min at 37 °C (Zilversmit & Hughes, 1976) between PC unilamellar vesicles and heat-treated mitochondria (Crain & Zilversmit, 1980a). PI-specific phospholipase C from *Staphylococcus aureus* was purified as described by Low & Finean (1977) as modified by Low & Zilversmit (1980).

Protein-Catalyzed Transfer of Phospholipid between Unilamellar Vesicles and Microsomes. Microsomes (2–6 mg of protein, 1000–3000 nmol of phospholipid) were incubated for 60 min at 37 °C in a total volume of 1 mL in 250 mM sucrose, 0.5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0, containing 10 mg of fatty acid free bovine serum albumin (fraction V, Miles Laboratories, Elkhart, IN). Microsomes were incubated in the presence or absence of phospholipid transfer protein and in the presence of unilamellar vesicles (2-fold molar excess of phospholipid) of various phospholipid compositions. After 15–120 min, lipid transfer was stopped by adding 2 mL of ice-cold buffer and placing the samples on ice. In one experiment, Glc-6-P phosphohydrolase activity was measured directly on aliquots of this solution. In other experiments, unilamellar vesicles were separated from microsomes by centrifugation for 30 min at 100000*g*. The microsomal pellet was homogenized by hand with a Dounce type homogenizer in 3 mL of either 250 mM sucrose or 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0. Aliquots of the homogenate were analyzed for protein and Glc-6-P phosphohydrolase activity. The remainder was extracted for determination of phospholipid content, cholesterol content, phospholipid composition, and phospholipid transfer.

[1] Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; DPPC, dipalmitoylphosphatidylcholine; PS, phosphatidylserine; diPG, diphosphatidylglycerol; Man-6-P, mannose 6-phosphate; Glc-6-P, glucose 6-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hyroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table I: Glucose-6-P Phosphohydrolase Activity of Microsomes Incubated with Unilamellar Vesicles and Nonspecific Transfer Protein[a]

time (min)	NS-TP (units)	SUV	phospholipid transferred (nmol/mg) egg PC[b]	phospholipid transferred (nmol/mg) DPPC	Glc-6-Pase (%),[c] [nmol/(min·mg)]	microsomal (nmol/mg) PL	microsomal (nmol/mg) CHOL
15	0				411	635	90
60	0				418	629	92
15	0	A	4	5	377	642	83
15	1.25	A	52	83	361 (96)	635	65
60	0	A	10	13	374	643	70
60	1.25	A	60	122	294 (78)	661	64
15	0	B	10		387	644	90
15	1.25	B	198		368 (96)	678	86
60	0	B	37		383	643	89
60	1.25	B	261		321 (83)	708	92

[a] Microsomes (5.5 mg of protein, 2900 nmol of phospholipid) were incubated at 37 °C in 1 mL of 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0, containing 10 mg of fatty acid free bovine serum albumin and 0 or 1.25 units of nonspecific transfer protein. Each incubation contained either 0 or 5000 nmol of PC in the form of small unilamellar vesicles of the following compositions: (A) DPPC/egg PC/diPG/cholesterol (7.5:2.5:1:1 mol/mol) or (B) egg PC/diPG/cholesterol (10:1:1 mol/mol). ^{32}P-Labeled rat liver PC was used as a marker for PC transfer, [^{14}C]DPPC was used as a marker for DPPC transfer, and glycerol [^{3}H]trioleate was used as a nontransferable marker. After 15 or 60 min, the reaction was stopped by addition of 2 mL of cold buffer and by placing the mixture on ice. The microsomes were sedimented by centrifugation for 30 min at 100000*g*, the supernatant was decanted, and the pellet was resuspended in 3 mL of 250 mM sucrose. Aliquots were analyzed for protein, cholesterol, and phospholipid content. PC and DPPC transfers from unilamellar vesicles to microsomes were calculated from the radioactivity present in an aliquot of the extracted microsomal lipid, correcting for adhesion of vesicles to microsomes as determined from glycerol [^{3}H]trioleate, and expressed as nanomoles transferred per milligram of microsomal protein. Glc-6-P phosphohydrolase activity was assayed at 30 °C in the presence and absence of 0.4% taurocholate and is expressed as nanomoles of P_i released per minute per milligram of microsomal protein. Only the results of assays without taurocholate are presented. Each value is the average of duplicate determinations which agreed within 4%. Abbreviations used: NS-TP, nonspecific transfer protein; SUV, small unilamellar vesicles; PL, phospholipid; Glc-6-Pase, glucose-6-phosphate phosphohydrolase; CHOL, cholesterol. [b] Rat liver [^{32}P]PC was used as a marker for egg PC transfer, though the fatty acid compositions are not identical (Getz et al., 1961; Hanahan et al., 1960). Compared to rat liver, egg PC contains less arachidonate and somewhat more oleate; the percentages of palmitate and stearate are quite similar for these PCs. It is unlikely that egg PC transfer calculated from the transfer of rat liver [^{32}P]PC marker is in error by more than 10–15%, because transfer of [^{14}C]DPPC is only 36% slower than that for ^{32}P-labeled rat liver PC when both are present in the same vesicle. [c] Percent of control. The control in each case is identical with the experimental incubation except that it lacks transfer protein.

Glucose-6-phosphate Phosphohydrolase Activity. Glc-6-P phosphohydrolase activity was measured by a modification of the method of Zakim & Vessey (1973). Microsomes (0.05–0.1 mg of protein) were incubated in a total volume of 0.5 mL in 100 mM sodium acetate and 20 mM Glc-6-P (monosodium salt, Sigma Chemical Co.), pH 6.5, containing 10 mg/mL fatty acid free bovine serum albumin with or without 0.4% sodium taurocholate. Taurocholate has been shown to remove the permeability barrier of the microsomal membrane to glucose 6-phosphate (Arion et al., 1972). Samples were kept on ice for 30 min prior to the addition of Glc-6-P and the initiation of the reaction. Samples were then transferred to a water bath at 30 or 37 °C. The reaction was stopped after 15 min by chilling on ice and adding 0.5 mL of 10% trichloroacetic acid. Phosphohydrolase activity was measured from the P_i released. Standards and blanks were analyzed under identical conditions except that no microsomes were included. In experiments measuring latency of the phosphohydrolase to Man-6-P, Glc-6-P was replaced by 2 mM Man-6-P (Sigma Chemical Co.).

Results

Inhibition of Glucose-6-phosphate Phosphohydrolase by Phospholipid Vesicles. Incubation of unilamellar vesicles composed of PC (of different fatty acid compositions) and cholesterol (1:0.1 mol/mol) with microsomes and phospholipid transfer protein resulted in 16–30% loss of vesicles to the microsomal pellet after centrifugation. Inclusion of 10 mol % diPG significantly decreased this loss of unilamellar vesicles and slightly increased the transfer of PC. Generally, when diPG was included, greater than 90% of the vesicles were recovered, as determined from the nontransferable marker, glycerol [^{3}H]trioleate, in the supernatant after centrifugation.

Microsomes were incubated with DPPC unilamellar vesicles[2] (2-fold molar excess of vesicle phospholipid) and with 0 or 0.85 unit of nonspecific phospholipid transfer protein. Cholesterol (10 mol %) was included in the unilamellar vesicles to minimize cholesterol depletion from the microsomes. After 1 h, approximately 20% of the microsomal phospholipid or 30% of the total microsomal PC was replaced with DPPC, and a greater than 30% inhibition of Glc-6-P phosphohydrolase activity was observed. No inhibition was produced when microsomes were incubated alone with either unilamellar vesicles or nonspecific transfer protein.

For investigation of the effect of phospholipid fatty acid composition, Glc-6-P phosphohydrolase activity was measured at 30 °C after incubations with either DPPC or egg PC unilamellar vesicles.[2,3] A slight inhibition of activity occurred when microsomes were incubated for 15 or 60 min with unilamellar vesicles (Table I). When nonspecific transfer protein was also present, a time-dependent inhibition occurred, reaching approximately 20% after 1 h (Table I). The same inhibition of Glc-6-P phosphohydrolase by phospholipid vesicles was observed when assayed in the presence of taurocholate (data not shown) which removes the permeability barrier of the microsomal membrane to glucose 6-phosphate (Nilsson et al., 1978). Thus the inhibition of phosphohydrolase activity by phospholipid treatment of microsomes was not affected by detergent disruption of the permeability barrier. After incubation of intact microsomes for 1 h with DPPC unilamellar

[2] Unilamellar vesicles composed of DPPC/egg PC/diPG (7.5:2.5:1.0 mol/mol) are referred to as DPPC unilamellar vesicles. Cholesterol content and exact compositions are given in the footnotes to the tables.

[3] Unilamellar vesicles composed of egg PC/diPG/cholesterol (10:1:1 mol/mol), rat liver PC/diPG/cholesterol (10:1:1 mol/mol), and rat liver PC/egg PE/diPG/cholesterol (7.5:2.5:1:1 mol/mol) are referred to as egg PC, rat liver PC, and egg PE unilamellar vesicles, respectively.

Table II: Glucose-6-phosphate Phosphohydrolase Activity of Microsomes Incubated with PC Unilamellar Vesicles of Varied Fatty Acid Composition[a]

NS-TP (units)	SUV	phospholipid transferred (nmol/mg) PC[b]	DPPC	Glc-6-Pase (%)[c] [nmol/(min·mg)]	microsomal PL (nmol/mg)
				336	524
1.25				363 (108)	512
	A	15	14	343	530
1.25	A	60	84	264 (77)	511
	B	24		337	542
1.25	B	215		271 (80)	593
	C	22		322	595
1.25	C	234		261 (81)	643

[a] Microsomes (6 mg of protein, 2700 nmol of phospholipid) were incubated as described in Table I with either 0 or 6000 nmol of PC in the form of unilamellar vesicles of the following compositions: (A) DPPC/egg PC/diPG/cholesterol (7.5:2.5:1:2 mol/mol), (B) egg PC/diPG/cholesterol (10:1:1 mol/mol), and (C) rat liver PC/diPG/cholesterol (10:1:1 mol/mol). Labeled lipid was included as described in Table I. After 60 min, the reaction was stopped, and the microsomes were sedimented and resuspended in 3 mL of 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0. Further analyses were performed as described in Table I. Only the results of Glc-6-P phosphohydrolase activities assayed at 30 °C in the presence of taurocholate are included, but the findings were the same without taurocholate. Each value is the average of duplicate determinations which agreed within 10%. Abbreviations used: see Table I. [b] See Table I. [c] Percent of control as in Table I.

Table III: Effect of Transfer Protein Specificity and Unilamellar Vesicle Composition on Glc-6-P Phosphohydrolase Activity[a]

PL-TP	SUV	Glc-6-Pase (%)[b] [nmol/(min·mg)] t = 60 min	t = 120 min
		450	430
	A	430	450
PC-TP	A	400 (93)	420 (93)
NS-TP	A	320 (74)	300 (67)
	B	430	450
PC-TP	B	420 (98)	430 (96)
NS-TP	B	350 (81)	320 (71)

[a] Microsomes (2 mg of protein, 1000 nmol of phospholipid) were incubated as described in Table I with either 1.25 units of PC-specific transfer protein or 1.25 units of nonspecific transfer protein and either 0 or 2000 nmol of PC in the form of unilamellar vesicles of the following compositions: (A) DPPC/egg PC/diPG/cholesterol (7.5:2.5:1:1.5 mol/mol) or (B) rat liver PC/diPG/cholesterol (10:1:1 mol/mol). No radioactive markers were included. After 60 or 120 min 50 μL (0.1 mg of microsomal protein) was removed and assayed for Glc-6-P phosphohydrolase activity at 37 °C in the absence of taurocholate as described under Materials and Methods. Each value is the average of duplicate determinations which agreed within 5%. These results are representative of two separate experiments. Abbreviations are the same as in Table I except the following: PC-TP, PC-specific transfer protein; PL-TP, phospholipid transfer protein. [b] Percent of control as in Table I.

vesicles[2] and nonspecific transfer protein, 20–25% of the total microsomal phospholipid was replaced by DPPC. A 30% decrease in cholesterol content of microsomes was observed after incubation with DPPC-containing unilamellar vesicles[2] (with or without transfer protein), in spite of the presence of 10 mol % cholesterol in the vesicles. No change in cholesterol content was observed when microsomes were incubated with egg PC containing unilamellar vesicles[3] in the presence or absence of nonspecific transfer protein (Table I). The fact that the Glc-6-P phosphohydrolase activity is identical for microsomes incubated for 60 min with either DPPC or egg PC unilamellar vesicles in the absence of transfer protein would indicate that 30% depletion of microsomal cholesterol has very little effect on phosphohydrolase activity.

For further investigation of the effect of phospholipid fatty acid composition, intact microsomes were incubated with DPPC, egg PC, and rat liver PC unilamellar vesicles[2,3] for 1 h at 37 °C in the presence or absence of 1.25 units of nonspecific transfer protein. The cholesterol content of the DPPC unilamellar vesicles was increased to 20% in an attempt to hold the microsomal cholesterol content constant during incubation. Glc-6-P phosphohydrolase, assayed at 30 °C in the presence (Table II) or absence of taurocholate, was inhibited to about the same extent after incubation of intact microsomes with the three types of unilamellar vesicles, suggesting that the inhibition is not primarily due to changes in phospholipid fatty acid composition.

The inhibition of Glc-6-P phosphohydrolase activity by incubation of microsomes with nonspecific transfer protein and PC unilamellar vesicles of various fatty acid compositions could be caused by one of several effects on the microsomal membrane. For differentiation of the effects caused by exchange of microsomal PC for vesicle PC from effects caused by the net transfer of PC to the microsome, experiments were done comparing the inhibition in the presence of the nonspecific transfer protein with inhibition in the presence of the PC-specific transfer protein. Intact microsomes were incubated for 60 and 120 min at 37 °C with DPPC or rat liver PC unilamellar vesicles[2,3] in the presence of either 1.25 units of nonspecific transfer protein or 1.25 units of PC-specific transfer protein. Very little inhibition of Glc-6-P phosphohydrolase activity, assayed at 37 °C in the absence of taurocholate, resulted from incubation of microsomes with the PC-specific transfer protein and either of the unilamellar vesicles (Table III). However, as shown before, incubation of microsomes for 120 min with either of the unilamellar vesicles and nonspecific transfer protein caused approximately 30% inhibition of Glc-6-P phosphohydrolase activity. Microsomes incubated for 60 min with DPPC vesicles[2] and transfer protein showed quantitatively similar levels of inhibition when assayed at 37, 30, and 21 °C either in the presence or in the absence of taurocholate (data not shown).

Effect of Lipid Modification of the Microsomal Membrane on Glucose-6-phosphate Phosphohydrolase Activity. The difference observed in the previous experiment probably results from the properties of the purified transfer proteins. The PC-specific transfer protein accelerates the exchange of microsomal PC for lipid vesicle PC whereas the nonspecific transfer proteins are capable of accelerating heteroexchange[4] of phospholipid as well as catalyzing net phospholipid transfer (Crain & Zilversmit, 1980b). The observed inhibition may therefore result from a net increase in the microsomal PC content, from a net decrease in the PE and/or PI content, or from a change in total phospholipid content. These possibilities were studied by incubating microsomes for 60 min at 37 °C with either DPPC, rat liver PC, or egg PE unilamellar vesicles[2,3] in the presence of either PC-specific transfer protein or nonspecific transfer protein. No change in phospholipid head-group composition occurred in incubations containing either PC-specific transfer protein or no transfer protein (Table IV). No inhibition of Glc-6-P phosphohydrolase activity, assayed at 30 °C in the presence or absence of taurocholate, was observed for these samples with the exception of the incubations containing DPPC unilamellar vesicles and the PC-

[4] Heteroexchange, protein-catalyzed exchange of one phospholipid class for another.

Table IV: Effect of the Change in Microsomal Phospholipid Composition on Glucose-6-phosphate Phosphohydrolase Activity[a]

PL-TP	SUV	phospholipid transferred (nmol/mg) DPPC	phospholipid transferred (nmol/mg) PC[b]	microsomal phospholipid composition (nmol/mg) DPPC	total PC	PE	PI	Glc-6-Pase [nmol/(min·mg)]
					350	150	72	292
	A	3	8	3	340	150	74	285
NS-TP	A	103	65	103	360	110	49	180
PC-TP	A	156	68	156	360	160	78	250
	B		28		370	150	74	313
NS-TP	B		306		510	110	56	217
PC-TP	B		190		360	150	71	299
	C		19		350	150	70	327
NS-TP	C		192		410	170	54	313
PC-TP	C		150		370	160	72	355

[a] Microsomes (5 mg of protein, 2800 nmol of phospholipid) were incubated as described in Table III with either 0 or 5000 nmol of PC + PE in the form of unilamellar vesicles composed of (A) DPPC/rat liver PC/diPG/cholesterol (7.5:2.5:1:1.5 mol/mol), (B) rat liver PC/diPG/cholesterol (10:1:1 mol/mol), or (C) rat liver PC/egg PE/diPG/cholesterol (7.5:2.5:1:1 mol/mol). Labeled lipid was included as described in Table I. After 60 min, the reaction was stopped, and the microsomes were sedimented and resuspended in 3 mL of 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0. Protein and phospholipid content, Glc-6-P phosphohydrolase activity, and DPPC transferred were determined as described in Table II. Only the results of phosphohydrolase assayed in the absence of taurocholate are included. Phospholipid composition was measured as described under Materials and Methods and expressed as nanomoles of phospholipid per milligram of microsomal protein. PI may be contaminated with PS, though previous reports indicate PS is a minor component of rat liver microsomal membranes (Colbeau et al., 1971). Each value is the average of duplicate determinations which agreed within 5%. These results are representative of two separate experiments. Abbreviations used: see Tables I and III. [b] See Table I.

specific transfer protein where about 88% of the control activity was measured. This small inhibition is probably due to the replacement of 43% of the microsomal PC by DPPC (Table IV). However, in incubations containing nonspecific transfer protein, microsomes showed substantial changes in phospholipid composition. Microsomes incubated with DPPC-containing lipid vesicles[2] incurred a slight increase in PC content, a 27% decrease in PE content, and a 34% decrease in PI content (Table IV). Correlated with this was a 37% inhibition of Glc-6-P phosphohydrolase activity. Microsomes incubated with rat liver PC containing lipid vesicles[3] also demonstrated a 27% decrease in PE and 24% decrease in PI content but incurred a 38% increase in PC content (Table IV). A 31% inhibition of enzyme activity was measured in these microsomes. Microsomes incubated with nonspecific transfer protein and PE-containing lipid vesicles[3] showed a 23% loss of PI, a slight increase in PE, and a 17% increase in PC content (Table IV). These microsomes showed essentially no change in Glc-6-P phosphohydrolase activity. Quantitatively similar effects were observed for the same microsomes assayed in the presence of taurocholate (data not shown).

The role of PI in Glc-6-P phosphohydrolase activity was further investigated with the PI-specific phospholipase C from *Staphylococcus aureus*. Incubation of microsomes for 1 h at 37 °C with 10 μg of phospholipase C caused hydrolysis of over 50% of the PI (Table V). Almost no change in Glc-6-P phosphohydrolase activity occurred as a result of this (Table V). This is in agreement with the experimental findings described above, indicating that PI is not directly involved in the enzyme's activity.

Table V: Effect of PI Hydrolysis on Glucose-6-phosphate Phosphohydrolase Activity[a]

phospholipase C (μg)	microsomal PL composition (nmol/mg) PC	PE	PI	Glc-6-Pase [nmol/(mg·min)]
0	346	138	78	280
1.3	332	138	49	288
5.0	342	131	41	275
10.0	350	137	35	263

[a] Microsomes (4 mg of protein, 2300 nmol of phospholipid) were incubated at 37 °C with phosphatidylinositol-specific phospholipase C(0–10 μg). After 1 h, the microsomes were sedimented by centrifugation and resuspended in 250 mM sucrose, 5 mM Hepes, 1 mM dithiothreitol, and 0.5 mM EGTA, pH 7.0. Glc-6-P phosphohydrolase activity was assayed as described in Table II in the absence of taurocholate. Phospholipid composition was analyzed as described in Table IV. Each value is the average of duplicate determinations which agreed within 5%. These results are representative of two separate experiments. Abbreviations used: see Table I.

Discussion

The microsomal glucose-6-phosphatase system is comprised of two activities, a glucose-6-phosphate carrier and a phosphohydrolase located on the lumenal membrane surface (Nilsson et al., 1978; Arion et al., 1980). In 1968, Duttera and co-workers found that microsomes treated with a nonspecific phospholipase C lost 80–90% of their glucose-6-phosphatase activity. Activity was restored by incubation with lysolecithin or PE but not with PC. They also reported inhibition of activity caused by phospholipase A or acetone treatment and partial reactivation by phospholipids. Since then, a number of investigators have used these and similar techniques to study the lipid dependence of glucose-6-phosphatase and often have come to conflicting conclusions [for a review, see Duck-Chong (1976)]. Another line of evidence for the lipid dependence of glucose-6-phosphatase was provided by Eletr et al. (1973) who found abrupt changes in Arrhenius plots of enzyme activity at about 19 °C, corresponding to a break in the temperature dependence of the ESR spectra of membranes containing lipophilic spin-labeled probes. Both the breaks in the temperature dependence of the ESR spectra and the enzyme activity were abolished by treatment with phospholipase A. In spite of the effort which has gone into studying the lipid dependence of glucose-6-phosphatase, Duck-Chong (1976) concluded that due to limitations of the methodology, the specific involvement of phospholipid in either the regulation or expression of glucose-6-phosphatase was not conclusively established.

In some membranes, cholesterol affects enzyme activity (Owen & McIntyre, 1978; Lad et al., 1979). Therefore, since uncatalyzed movement of cholesterol can occur between lipid bilayers and its movement is facilitated by nonspecific transfer proteins (Crain & Zilversmit, 1980a), cholesterol was included in the lipid vesicles at the phospholipid/cholesterol ratio of the microsomal membrane. Even so, a 20–30% depletion of

cholesterol occurred in microsomes incubated with DPPC unilamellar vesicles[3] in the presence or absence of nonspecific transfer protein. This is consistent with cholesterol's high "affinity" for DPPC unilamellar vesicles reported by Nakagawa et al. (1979), and therefore, the cholesterol/phospholipid ratio was increased for these vesicles. Inhibition of enzyme activity, however, was not correlated with cholesterol depletion. Near-maximal depletion of microsomal cholesterol (30%) by incubation of membranes for 15 min with DPPC vesicles and transfer protein did not result in significant inhibition of enzyme activity. Furthermore, the Glc-6-P phosphohydrolase activities of microsomes incubated for 60 min with DPPC or with egg PC unilamellar vesicles[2,3] in the absence of transfer protein were the same, although the DPPC-exposed microsomes contained 30% less cholesterol but the same amounts of phospholipids as the microsomes exposed to egg PC. In separate experiments, it was found that varying the cholesterol level of the lipid vesicles used in the incubations had no effect on glucose-6-phosphate phosphohydrolase activity (unpublished results).

Incubation of microsomes with nonspecific transfer protein and PC-containing lipid vesicles resulted in a time-dependent inhibition (4–50%) of Glc-6-P phosphohydrolase activity. In each experiment, microsomal phospholipid was replaced by vesicle phospholipid by incubation with transfer protein. The microsomes were separated from lipid vesicles by centrifugation and resuspended in buffer. One aliquot of this suspension was subjected to detergent disruption for 30 min at 4 °C whereas a second aliquot was incubated in buffer for 30 min at 4 °C. Glc-6-P phosphohydrolase activities of these aliquots were compared to their respective base-line values (assays on microsomes incubated with lipid vesicles but no transfer protein). The fact that the Glc-6-P phosphohydrolase activity was inhibited to the same extent for phospholipid-replaced microsomes which were then either disrupted with taurocholate or not treated with detergent demonstrates that the effect of the phospholipid replacement is on the lumenal phosphohydrolase activity and not on the Glc-6-P carrier. Furthermore, similar inhibition was noted with vesicles composed principally of rat liver, egg, or dipalmitoyl-PC, suggesting that PC fatty acid saturation is not of primary importance. Incubation of microsomes with PC-specific transfer protein and vesicles composed of egg or rat liver PC under identical conditions resulted in no inhibition whereas incubation with vesicles containing DPPC caused a slight inhibition of activity. Little or no inhibition (less than 10%) occurred when loss of PE from the microsomes was prevented by incubating microsomes with PE-containing unilamellar vesicles in the presence of either transfer protein or when over 50% of the PI was hydrolyzed by the PI-specific phospholipase C. It is therefore concluded that Glc-6-P phosphohydrolase activity depends on the concentration of PE in the membrane. The membrane's cholesterol content, PI content, and PC fatty acid saturation have less effect on phosphohydrolase activity.

These conclusions are at variance with those of Dyatlovitskaya et al. (1979), who used a similar technique to alter the phospholipid composition of hepatoma and liver microsomal membranes. In contrast to this study, they reported that incubation of microsomes with sonicated PC vesicles and rat liver transfer protein in the presence or absence of deoxycholate resulted in no change in Glc-6-Pase activity. However, in accordance with our conclusions, they observed only small decreases in microsomal PE content (1–10%) due to their use of rat liver postmicrosomal supernatant containing predominantly PC transfer activity (10 mg contains roughly 0.1–0.2 unit of nonspecific transfer protein and 1–2 units of PC transfer protein). Furthermore, they reported that incubation of microsomes with sonicated PE vesicles and lipid transfer protein fraction stabilized the Glc-6-Pase activity during incubation. No such stabilization occurred when microsomes disrupted with deoxycholate were incubated. Finally, they found that incubation of rat hepatoma 27 microsomes, containing low PC content and abnormally low Glc-6-Pase activities with PC vesicles (except DPPC) and lipid transfer fraction in the presence of deoxycholate, stimulated this enzyme 2–3-fold. However, no effect resulted from incubation of hepatoma microsomes with PC vesicles and the lipid transfer fraction in the absence of deoxycholate, though the resulting microsomal lipid composition was identical. On the basis of the difference in the effect of PC vesicles and transfer protein on intact vs. detergent-disrupted hepatoma microsomes and the difference in the effect of PE vesicles and transfer protein on intact vs. detergent-disrupted liver microsomes, they concluded that the glucose 6-phosphate transport unit is dependent on PE, whereas the phosphohydrolase is dependent on PC. These conclusions were based on two assumptions. First, that PE and PC are asymmetrically arranged (Nilsson & Dallner, 1977), though these findings have not been confirmed by others, also using phospholipase degradation (Sundler et al., 1977; Higgins & Dawson, 1977). Second, Dyatlovitskaya et al. (1979) assume the transbilayer movement of phospholipid at 22 °C is slow. Zilversmit & Hughes (1977) found rapid transbilayer movement of phospholipid at 30 °C in rat liver microsomes, in agreement with van den Besselaar et al. (1978) who measured PC transbilayer movement at 37 and 25 °C.

The conclusions of our study are consistent with other recent studies of microsomal glucose-6-phosphatase activity. A correlation between the decline in glucose-6-phosphatase and a decreased PE content during carcinogenesis was observed by Burlakova et al. (1976), though in some hepatomas activity is low whereas the PE to PC ratio is higher than normal (Bergelson et al., 1970; Waite et al., 1977). When microsomes were modified with nonspecific phospholipase C or toluene-2,4-diisocyanate, a reversible inhibition of activity was correlated with PE hydrolysis or modification (Schulze & Speth, 1980). A correlation was also found by Burlakova et al. (1979) between the extent of modification of PE by phospholipid effectors (antioxidants and delipidation) and glucose-6-phosphatase activity. Lastly, PE has been found to be effective in the reactivation of glucose-6-phosphatase after inhibition caused by nonspecific phospholipase C treatment (Duttera et al., 1968; Cater et al., 1974).

Phospholipid transfer proteins are potentially powerful tools in the elucidation of the phospholipid dependence of enzyme activity. In this study, they have been used to alter the phospholipid composition of the rat liver microsomal membrane without the destructive side effects of phospholipases or of disintegration–reconstitution procedures. Substitution of PC for PE appears to decrease Glc-6-P phosphohydrolase activity.

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