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Membrane Structure: Lipid-Protein Interactions in Microsomal Membranes*

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Abstract. The relationships of phospholipid to membrane structure and function were examined in hepatic microsomes. Findings indicate that normal microsomal membrane structure is dependent on lipid-protein interactions and that it correlates closely with glucose-6-phosphatase activity. Modification of most phospholipid with phospholipase-C is associated with widening of the membrane which can be reversed following readdition of phospholipid.

Introduction. Treatment of rat liver microsomes with phospholipase-C results in inactivation of glucose-6-phosphatase.[1] This inactivation can be reversed by addition of a total microsomal lipid fraction, a microsomal phospholipid fraction, mixtures of phospholipid such as Asolectin, or individual phospholipids.[1] In the present study, we have investigated the relationship of morphologic change in the microsomal membrane to lipid content and to activity of glucose-6-phosphatase, using conditions previously described to show the lipid requirement of this enzyme.[1] The results indicate a close correlation between normal membrane ultrastructure and glucose-6-phosphatase activity, demonstrate reversible morphologic changes after treatment of microsomal membranes with phospholipase-C, and show the importance of lipid-protein interactions in membrane structure. These data were recently presented in preliminary form.[2]

Materials and Methods. Microsomes were prepared from livers of 150 to 200-gm male Holtzman rats which were fasted with free access to water for 48 hr prior to sacrifice. At the time of killing, the livers were immediately removed and immersed in 0.25 *M* sucrose (0°–4°C) containing 0.01 *M* Tris (pH 8.0) and 1 mM EDTA. The livers were weighed and homogenized with a Teflon-glass homogenizer in the sucrose-Tris-EDTA solution. Microsomes were isolated at $3.2 \times 10^6 g \times \text{min}^1$ and the final pellet was resuspended in this solution that, in addition, contained 1 mM dithiothreitol.

Glucose-6-phosphatase activity was measured by phosphate release.[1] Phospholipase-C treatment of microsomes was performed with a commercial preparation (Sigma) derived from *C. welchii.* The enzyme was dissolved in 0.01 *M* Tris buffer, pH 7.5–8.0, at a concentration of 1 to 3 mg protein/ml. Incubation of microsomes with phospholipase-C was performed in the presence of 2.5 mM calcium chloride and 125 mM Tris (pH 8.0) at 20°C for 30 min. Acetone treatment of microsomes was performed with 10% water in acetone.[3]

Microsomal lipids were extracted by the method of Folch *et al.*[4] and phospholipid was separated from neutral lipid by elution on a silicic acid column.[1] Micellar preparations of phospholipids were prepared by sonic oscillation using a model S-75 Branson Sonifier for 1 to 3 min in 20 mM Tris (pH 8.0) containing 1 mM EDTA.

Reconstitution was studied by incubating treated microsomes with a two- to threefold

excess of phospholipid; either microsomal phospholipid or Asolectin (a mixture of soybean phosphatides). In some instances, reconstitution was performed in the presence of 1 mM dithiothreitol.

For electron microscopy, control and treated microsomes were fixed in suspension by the addition of a sodium cacodylate-buffered (0.1 *M*) mixture of 4% glutaraldehyde and 4% formaldehyde[5] to the microsomal suspension. After fixation in suspension for 30 min at 20°C, the microsomes were collected on Millipore filters (pore size 0.10 μm) by using vacuum filtration. The Millipore filters were fixed for an additional 60 min and then were washed for 1 to 3 hr in 67 mM sodium cacodylate buffer (pH 7.4), containing 7.5% sucrose, postfixed in 1% *s*-collidine-buffered osmium tetroxide (pH 7.4), dehydrated in a series of ethanols, and flat embedded in Epon. The specimens were stained *en bloc* with uranyl acetate and the thin sections were double stained with uranyl acetate and lead citrate. Fragments of the embedded sheets were re-embedded in slots in Epon blocks and sectioned at approximately 300 Å in a plane normal to the surface of the Millipore filter. Suspensions of Asolectin or microsomal phospholipid micelles were prepared for electron microscopy in an identical fashion. Glucose-6-phosphatase was demonstrated cytochemically as described in the legend of Figure 3*c*.

Micrographs were taken using an Hitachi HU-11 microscope at a primary magnification of 30,000. Magnification was calibrated with a replica or a diffraction grating, and all lens currents were standardized. The treated microsomes from each experiment were photographed immediately after photography of corresponding control microsomes and without changing the lens parameters. Positive projection prints were made on lantern slide plates at constant magnification. Measurements of membrane thickness were made with a Joyce-Loebl microdensitometer. These were taken in regions showing the trilaminar structure. This trilaminar structure is observed when the section is normal to the membrane surface. Varying the angle of section would be expected to affect the breadth of each peak but not the peak-to-peak measurement.

Results. As shown in Table 1, treatment with phospholipase-C resulted in loss of 83% of the original glucose-6-phosphatase activity. This treatment also resulted in the hydrolysis of approximately 70% of microsomal phospholipids that were extracted and analyzed as described.[1] Incubation of treated microsomes with excess Asolectin or microsomal phospholipids resulted in complete restoration of glucose-6-phosphatase activity; reactivation with phospholipid in the presence of dithiothreitol gave similar results. In a previous publication[1] it was shown that essentially all microsomal lecithin and sphingomyelin and approximately 60% of phosphatidylethanolamine were cleaved by phospholipase-C treatment. It was also shown that the products of phospholipase-C action themselves have no effect on the observed inactivation or on the reactivation after addition of phospholipid. Acetone-treated microsomes lost 98% of glucose-6-phosphatase activity with a concomitant loss of 90% of the microsomal phospholipid. Postincubation with Asolectin failed to restore control levels (Table 1).

Morphologic studies of untreated microsome fractions revealed a population of vesicular profiles (Fig. 1*d* and *e*). The vesicles contained a rather sparse flocculent material and occasionally smaller internal vesicles. Contaminating mitochondria, lysosomes, and microbodies were absent. The membrane which limited the vesicles showed a trilaminar appearance. Occasionally, suggestions of striations perpendicular to the laminae were observed. Microdensitometer tracings across the membrane in areas cut normal to the membrane surface yielded two distinct peaks corresponding to the dense laminae separated by a trough (Fig. 1*a*). A frequency-distribution plot of peak-to-peak thickness of

TABLE 1. *Effect of lipid depletion and reconstitution on the glucose-6-phosphatase activity of hepatic microsomes.*

Condition	Glucose-6-phosphatase activity (per cent untreated)
(A) Untreated microsomes	(100)*
(B) Phospholipase C-treated microsomes	17
(C) (B) + Asolectin	119
(D) (B) + microsomal phospholipid	147
(E) (B) + dithiothreitol	19
(F) Acetone-treated microsomes	2
(G) (F) + Asolectin	7

The samples were kept in ice prior to the activity determination for the same interval of time as that which elapsed between preparation of the various samples and fixation.
* 0.697 μmole P_i released/10 min/ml incubation mixture.

untreated microsomal membranes is shown in Figure 2. The mean thickness was 50 Å, however, there was a small secondary population with a mean thickness of 75 Å. Electron micrographs of microsomes incubated for demonstration of glucose-6-phosphatase activity revealed accumulations of lead phosphate reaction product along the dense inner and outer laminae of the membrane (Fig. 3*c*); the central clear area was free of reaction product.

Phospholipase-C-treated microsomes showed general preservation of the vesicular structure (Fig. 1*f*), although ribosomes appeared fewer in number. The trilaminar structure of the membranes was well preserved (Fig. 1*f*) and in fact appeared more distinct than in control preparations. In addition, small homogeneous densities were often seen eccentrically applied to the surface of the microsomes (Figs. 1*f* and 3*a*). These densities appeared to be continuous with the space between the dense outer and inner laminae, producing a signet ring appearance (Figs. 1*f* and 3*a*). These droplets are assumed to represent the released diglyceride possibly mixed with other components. Additional flocculent material was located between the microsomes. Measurements of the peak-to-peak thickness of phospholipase-C-treated microsomes (Fig. 1*b*) revealed a single population with a mean value approximately 1.5 times that of the control preparation (Fig. 2); this population differed significantly ($p < 0.01$) from the control population.

In order to evaluate the possibility that the increase in center-to-center spacing was due to an asymmetric decrease in thickness of the dense layers, individual tracings of the dense layers were measured. It was found that these layers increased rather than decreased in thickness thus precluding the possibility that the increase in center-to-center spacing was related only to a decrease in the dense layers.

Examination of microsomes reconstituted with microsomal phospholipid or with Asolectin again revealed a vesicular appearance with preservation of the trilaminar structure (Fig. 1*g*). Vesicles and the spaces between them appeared relatively empty as compared with the other preparations; the dense eccentric droplets seen in phospholipase-C-treated microsomes were absent. In addition to the microsomes there were complex lamellar membranous profiles (Fig. 3*b*) identical with those in preparations of purified micelles (Fig. 3*d*) and, accordingly, these were assumed to represent excess phospholipid which had not been

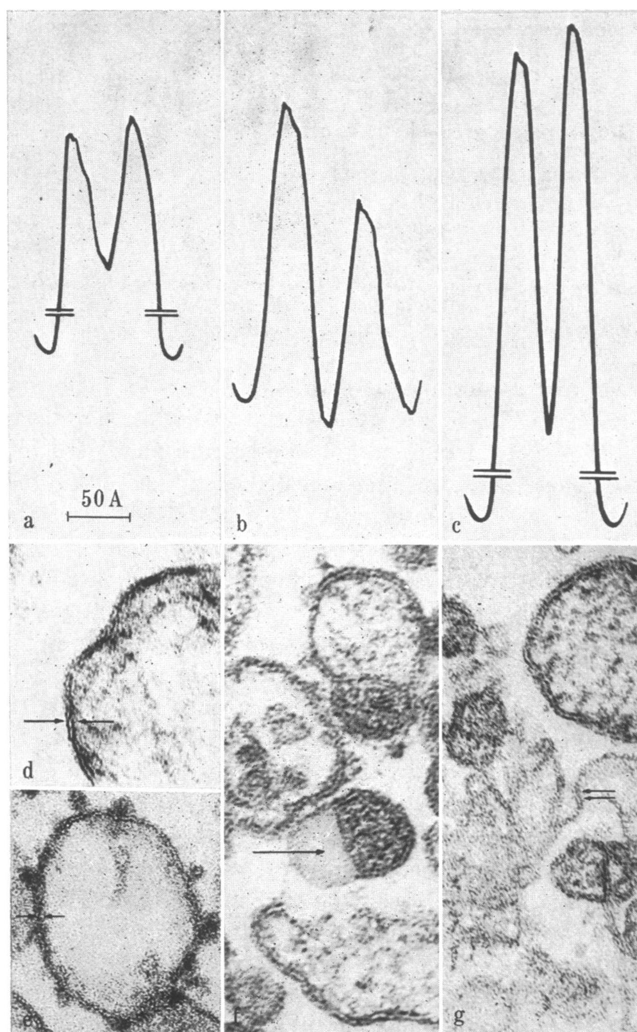

FIG. 1.—Electron micrographs and microdensitometer tracings of control and treated microsomal membranes. (*a*)–(*c*) represent microdensitometer traces across typical membranes in control, phospholipase-treated, and reconstituted microsomes, respectively. The peaks in these tracings represent the dense areas seen within the membrane in electron micrographs as shown in (*d*)–(*g*). (*a*)–(*c*) are shown at the same magnification; the bar shows 50 Å at this magnification.

(*d*) and (*e*) are micrographs of control microsomes. Note the typical trilaminar appearance of the membrane as indicated by the free arrows in (*d*) and note the suggestion of periodicity in the membrane structure shown by the apposing arrows in (*e*). (*f*) is a micrograph of microsomes treated with phospholipase-C. These membranes are thicker as shown in Fig. (*b*) and often show the accumulation of homogeneous areas of intermediate density between the inner and outer dense layers (Fig. 1*f*, *arrow*). These are interpreted as accumulations of diglycerides. (*g*) shows microsomes reconstituted by addition of Asolectin. The double arrow indicates a lamellar profile which probably represents a section of a lipid micelle.

All electron micrographs were taken at identical magnification without changing lens parameters. Magnification in (*d*), (*f*), and (*g*) is ×195,000. Magnification in (*e*) is ×210,000.

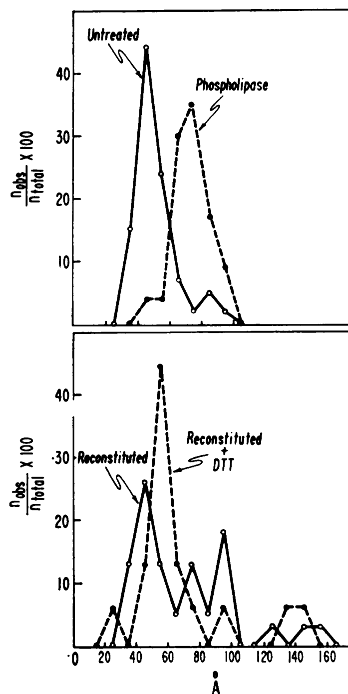

FIG. 2.—Frequency-distribution plot of membrane thicknesses in untreated microsomes, phospholipase-C-treated microsomes, and microsomes reconstituted with Asolectin in the presence or absence of dithiothreitol. The statistical significance of these distributions was analyzed by the method of Kolmogorov and Smirnov.[20] Phospholipase-C-treated microsomes were significantly different from untreated microsomes ($p < 0.01$), whereas microsomes reconstituted in the presence of dithiothreitol did not significantly differ from control values. Microsomes reconstituted with Asolectin alone showed evidence of secondary larger populations, suggesting that all membranes did not return to normal. These measurements are based on the pooled results of four different experiments with the following total measurements in each group: untreated, 42; phospholipase-C-treated, 23; reconstituted, 39; and reconstituted with dithiothreitol, 16.

integrated into the microsomes. Measurements of reconstituted microsomes (Fig. 1*c*) showed a large population similar to untreated preparations (Fig. 2); there were, however, additional populations which represented thicker membranes suggesting a bi- or triphasic population and indicating that some membranes did not return to control thickness. If one looks at the fraction of the population that has a dimension comparable to the major peak in the untreated sample, it appears that reconstitution in the presence of dithiothreitol leads to a more effective reconstitution, although the distribution of values did not differ statistically from values on membranes reconstituted without dithiothreitol. Examination of acetone-treated microsomes revealed complete loss of typical microsomal vesicles. Numerous fine filamentous profiles represented the only remnants of microsomal membranes (Fig. 3*e*). These dense irregular profiles are assumed to represent mostly residual protein and were often observed to enclose clear spaces which formed a regular pattern along the residual membrane material.

The widening of the membrane seen after modification of most phospholipids suggests that changes in protein conformation occur in this condition. We suggest that the membrane widens because hydrophobic bonding with lipids is important for normal protein conformation in microsomal membranes and, when most of the phospholipids are modified, the proteins change in a way which influences the protein-protein interactions. This is in agreement with the concept that membrane proteins have a genetically determined sequence of predominantly hydrophobic amino acids which are dependent on a hydrophobic environment for selection of a final three-dimensional structure.[8] These results

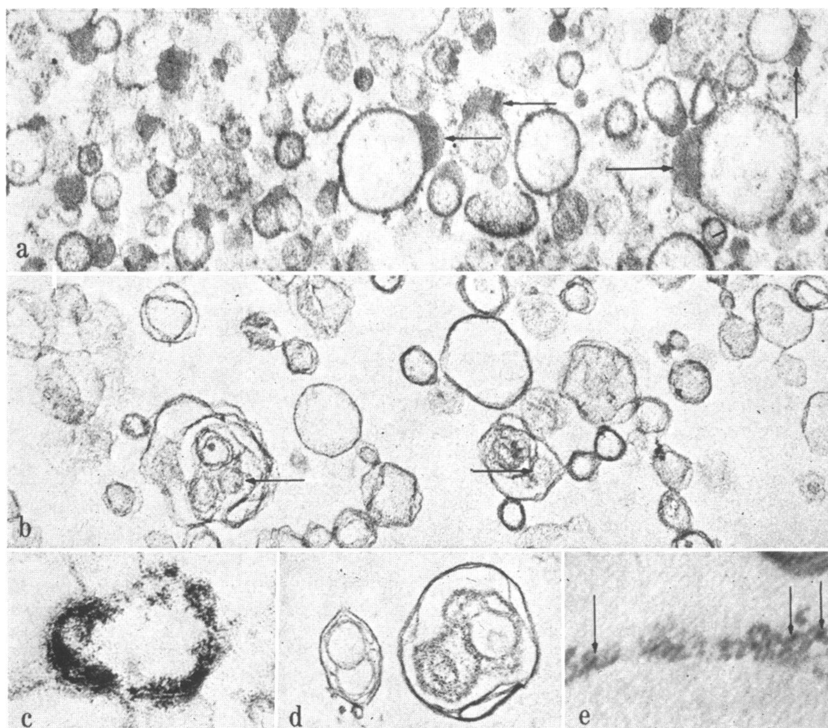

FIG. 3.—(*a*) Electron micrograph showing microsomes treated with phospholipase-C. Note the numerous circular areas of intermediate density which represent accumulations of diglyceride released by the phospholipase treatment. In several areas (*free arrows*) these densities are seen eccentrically located along the margin of a microsome producing a "signet ring" appearance. In high resolution pictures (Fig. 1*f*), these accumulations are seen to be located between the outer and inner dense layers of the microsomal membrane. ×48,000.

(*b*) Electron micrograph showing microsomes after reconstitution with Asolectin. Note the micelles of added Asolectin (*free arrows*) which consist of clusters of concentrically-arranged dense lamellae. ×44,000.

(*c*) Control microsomal preparations showing histochemical localization of glucose-6-phosphatase activity. Note the accumulation of the dense lead phosphate reaction product along the inner and outer surfaces of the membrane. Glucose-6-phosphatase was demonstrated as follows. Microsomes were fixed in 4% sodium cacodylate-buffered paraformaldehyde for 8 min; the microsomes were collected on Millipore filters, washed 30 min in buffer, and were incubated for 10 min in the medium described by Wachstein and Meisel.[21] The microsomes were then postfixed in osmium tetroxide as described in the text. ×31,500.

(*d*) Electron micrograph of Asolectin preparations showing the typical micelles. ×38,500.

(*e*) High magnification micrograph of microsomal membrane after treatment with acetone-water. Observe that the typical trilaminar structure is not visible; membrane structure has broken down. The membrane is represented by irregular dense profiles (*arrows*) which are believed to represent residual protein. ×137,500.

are in contrast to the study of Lenard and Singer on erythrocyte membranes after treatment with phospholipase C.[9] These authors observed no changes in protein conformation as measured by circular dichroism after removal of phospholipids. It should be pointed out, however, that erythrocyte membranes have a higher protein-to-lipid ratio than endoplasmic reticulum and that, in the case of erythrocyte membranes, hydrophobic interactions with lipid may be less important for maintenance of final membrane structure.[7]

It was striking to observe that the trilaminar structure of the microsomal membrane was completely maintained with most phospholipid absent. This is compatible with the assertion that osmium concentration in membranes occurs at hydrophilic interfaces.[7] The dense lines in electron micrographs therefore probably reflect interaction with the hydrophilic end of proteins as much as interaction with hydrophilic ends of phospholipids on the unsaturated fatty acids. The results also emphasize the importance of protein for the structural integrity of the microsomal membrane although at the same time they demonstrate the importance of lipid-protein interactions; if lipids are removed with 10% water in acetone, the membrane structure breaks down. This loss of structure after acetone-water treatment is in contrast to results with the inner membrane of mitochondria[10] and, once again, the protein-lipid ratios are significantly higher in mitochondrial inner membranes than they are in endoplasmic reticulum.[7]

Another striking finding in our experiments was the accumulation of diglyceride within the membrane after treatment with phospholipase-C. Finean and Martonosi noted the appearance of diglyceride droplets in electron micrographs of phospholipase-C-treated muscle microsomes[11] but did not comment on their ultrastructural location. This accumulation of intramembranous, relatively apolar, lipid suggests, in view of the considerations mentioned above, the presence of two layers of protein. It is presumed that after release of diglyceride, accumulation occurs within the hydrophobic interior of the membrane. This observation gives experimental support to the theoretical model of apolar lipid accumulation within a bilayer recently proposed by Danielli.[12] When excess phospholipid is added, the accumulated diglyceride evidently escapes from the membrane since it does not appear after reconstitution.

These studies, along with others,[13, 14] emphasize the need to reexamine the bimolecular leaflet hypothesis of membrane structure and emphasize that different structural factors may be important in different types of cellular membranes. In the case of microsomal membranes, our studies emphasize the importance of both lipid and protein to maintain normal fine structure. To be consistent with our data, a macromolecular model for the microsomal membrane must explain the following: (1) phospholipid molecules are readily accessible to or reach the membrane surfaces; (2) the dense lines observed in membranes after postfixation in osmium are present after modification of most phospholipid; (3) the membrane must be capable of reversible changes in dimension related to phospholipid-protein interactions; (4) the apparent accumulation of diglyceride in the interior of the membrane; (5) the presence of dense lines presumably representing protein on each side of the diglyceride; and (6) the trilaminar structure is present when most phospholipid is modified, although in this state the membrane conformation differs from normal. Such a model might involve a mosaic of repeating protein and lipid subunits,[15] but it is not clear from these results whether the protein mosaic is necessarily continuous. A certain proportion of bilayer might well occur. The important role that changes, in even a single membrane "protomer," can have in determining the over-all architecture of the membrane is emphasized in the hypothesis of Changeux *et al.*[16] Their hy-

pothesis also assists in explaining the observations that various morphologic classes of membranes exist.[17-19]

These results may also have meaning for studies of membrane synthesis and turnover. They indicate that the potential for normal structure and function can be maintained after phospholipid modifications and expressed when phospholipid is added. The facilitation of reconstitution of structure by dithiothreitol would also suggest a potential role for disulfide interchange, but the *in vivo* role of these potentialities is unknown.

Finally, these results emphasize the possibility that attempts to isolate individual components of membrane systems will be handicapped by the interdependence of membrane components for their normal structure and function. A given component may exhibit its normal structure and specificity irrespective of its environment, but the importance of phospholipid for membrane dimension is a reminder that the typical component in a membrane system, such as the endoplasmic reticulum, would appear to be dependent on direct or indirect interaction with the other components of the membrane.

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