

## AN ENZYMATICALLY DRIVEN MEMBRANE RECONSTITUTION FROM SOLUBILIZED COMPONENTS

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### ABSTRACT

Acyltransferase activity is present in a variety of membrane species, including liver microsomes. The substrates of this enzyme are lysophosphatides and acyl CoA derivatives. We have found that the detergent effect of these substrates can be used to solubilize rat liver microsomes. If the solubilized fraction is incubated, the acyltransferase acylates the lysophosphatide and thereby degrades the detergent effect so that vesicular membranes re-form. Gel electrophoresis patterns show that the reconstituted membranes contain all of the major protein components of the original microsomes. A marker enzyme for liver microsomes, NADPH-cytochrome *c* reductase, was present in the reconstituted membranes at 70% of the specific activity in the original microsomes, and freeze-fracture electron microscopy showed intramembrane particles on all fracture faces. This system may provide a useful model for studies of certain membrane biogenesis reactions that utilize acyltransferase *in vivo*.

**KEY WORDS** acyltransferase · membrane reconstitution · liver microsomes

Acylation cycles involving the action of phospholipases A<sub>1</sub> and A<sub>2</sub>, the resultant lysophosphatides, and selective acyltransferases play important roles in modulating membrane liquid composition. These include the biosynthesis and turnover of specific phosphoglycerides (37) as well as net phospholipid synthesis during membrane biogenesis (16, 18, 27) and production of lung surfactant (35, 36). Acylation cycles may also modulate membrane fluidity after stimulation of lymphocyte proliferation (10–12) or phagocytosis by leucocytes and macrophages (8). Acyl coenzyme A (CoA): monoacylphosphoglyceride acyltransferase (EC 2.31.23) acylates lysophosphoglycerides to form diacyl derivatives, as originally demonstrated in rat liver microsomes by Lands (7, 26). This enzyme activity has subsequently been found in several subcellular fractions of mammalian tissues (37), tissues of other vertebrates (20), plant tissue (6), fungi (4), and *Escherichia coli* (30).

The acyltransferase rate in rat liver microsomes

is sufficient to double the lipid content of the membrane in 30 min, and this suggested to us that the system might have potential as a model for the study of membrane biogenesis *in vitro*. In this report, we describe a method in which the detergent properties of two acyltransferase substrates, lysophosphatidylcholine and oleoyl coA, were utilized to solubilize rat liver microsomal membranes as mixed micelles. We found that the subsequent synthesis of phosphatidylcholine by the acyltransferase activity removed the detergent effect such that reconstituted membrane structures appeared.

### MATERIALS AND METHODS

#### *Materials*

ATP, reduced nicotinamide adenine dinucleotide phosphate (NADPH), lithium CoA, oleoyl chloride, Trizma buffer (Tris), and equine cytochrome *c* were obtained from Sigma Chemical Co., St. Louis Mo. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Lysophosphatidylcholine (LPC) and phospholipid standards were purchased from Avanti Biochemicals, Inc., Birmingham, Ala. Thin-layer chromatography plates (Silica Gel 60, 0.25 mm), were obtained from EM Laboratories, Inc., Elmsford, N. Y.

## Isolation of Rat Liver Microsomes

Male laboratory rats (180–200 g) were fasted for 18–24 h, sacrificed by decapitation, and their livers were perfused with 0.9% NaCl, 1 mM EDTA, at 0°C (7). The livers were weighed, washed with several changes of the perfusate, minced, and homogenized in buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.3) using a motor driven Potter-Elvehjem Teflon pestle (5). The homogenate was centrifuged twice at 10,000 g for 20 min, and the pellets were discarded. The supernate was centrifuged at 105,000 g for 60 min. The pellet was resuspended in 0.15 M Tris, pH 8.0, and centrifuged again for 60 min. The resulting pellet was resuspended to ~25 mg protein/ml in 0.25 M sucrose, 10 mM Tris, pH 7.3, divided into convenient aliquots, frozen in liquid nitrogen, and stored at –70°C until used.

## Preparation of Oleoyl Coenzyme A

Oleoyl CoA was prepared from oleoyl chloride and lithium CoA by a modification of the method of Reitz et al. (31). The CoA was dissolved in 20 ml of stabilized tetrahydrofuran-water (7:3 vol/vol). The reaction mixture was continuously bubbled with nitrogen and the pH was maintained at 8 with additions of 0.1 N NaOH. The oleoyl chloride was added in 50- $\mu$ l aliquots over a period of 2 h until no CoA was detected by reaction with DTNB. The pH was adjusted to 4 with 10% HClO<sub>4</sub>. The residual tetrahydrofuran was evaporated by a nitrogen stream, and 1.5 ml of 10% HClO<sub>4</sub> was added to the remaining 10 ml of solution. An equal volume of ether was added, and the reaction mixture was centrifuged. The insoluble oleoyl CoA collected at the interface and was retrieved by pipetting. The oleoyl CoA was washed several times with acetone followed by ether. The dry powder was weighed, assayed for purity, and stored in a light-proof container under argon in the freezer. Typical yields from 50 mg of CoA were 30–50 mg of oleoyl CoA of 85–89% purity. The purity was determined by absorption at 260 nm (33) and by DTNB reaction with the CoA released by acyltransferase activity (7).

## Enzyme Activity Measurements

Acyltransferase activity was monitored three ways: by the reaction of CoA with DTNB, by the increase in turbidity at 500 nm, and by quantitative lipid analysis.

The acyltransferase activity of the isolated microsomes was measured by continuous recording of CoA reaction with DTNB at 412 nm (7) using a Beckman DBG spectrophotometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) with chart recorder attachment. The reaction mixture contained 0.25–0.5 mg of microsomal protein, 100 nmol of lysophosphatidylcholine (LPC), 100 nmol of oleoyl CoA, 1  $\mu$ mol of DTNB, in a total final volume of 2 ml of 0.1 M Tris, pH 7.5. Acyltransferase activities of the microsomes ranged from 43 to 50 nmol/mg·min.

In other experiments, higher concentrations of microsomes and substrates were used to produce relatively large samples for turbidity measurements at 500 nm, lipid analyses, gel electrophoresis, marker enzyme measurements, and electron microscopy. These reaction mixtures typically contained 1.0 mg/ml of microsomal protein, LPC ranging from 0.5 to 2.0  $\mu$ mol/mg protein in different experiments, 0.1 mM CoA, 5 mM Mg-ATP, in 0.1 M Tris-Cl buffer, pH 7.5. After addition of the LPC to the membrane suspension in Tris buffer, the solution was briefly sonicated to break up aggregates (10 s, 40 W, Biosonik III, [Bronwill Scientific, Rochester, N. Y.] 1-cm probe). An amount of oleoyl CoA equimolar to the LPC was added, and the suspension was

immediately filtered through a 0.22- $\mu$ m Millipore filter (Millipore Corp., Bedford, Mass.), followed by incubation at 23°C. A complicating factor in using rat liver microsomes for these studies is that a thioesterase activity is also present which catalyses hydrolysis of acyl CoA derivatives. Over a long incubation period, the esterase could produce extensive breakdown of oleoyl CoA so that the equimolar stoichiometry with LPC would be lost. For this reason, ATP and CoA were components of the reaction mixture, and permitted an endogenous acyl CoA synthase activity to recycle free oleic acid into oleoyl CoA. Preliminary experiments demonstrated that this condition did in fact inhibit net hydrolysis of oleoyl CoA during the incubation period.

For quantitative lipid analyses, samples were prepared as described above and then incubated either on ice (controls) or at 23°C (experimental) for 2 h. At the end of the incubation, lipid was extracted with chloroform-methanol as described previously (1, 23). The samples were evaporated under nitrogen, and aliquots of known volumes were spotted on Silica Gel 60 TLC plates and developed in chloroform-methanol-water (65:25:4 vol/vol). After visualization with iodine vapor, the spots were aspirated into glass wool-stoppered Pasteur pipettes. The silica was extracted twice with 5 ml of chloroform-methanol-acetic acid-water (50:50:10:10) followed by two extractions with 2 ml of methanol. The solvents were evaporated under nitrogen, and the lipid phosphorus was determined (29).

NADPH-cytochrome *c* reductase was used as a marker for incorporation of an active enzyme into the reconstituted membranes. The activity was monitored by the method of Fujita et al. (14).

## Protein Determination and Gel Electrophoresis

Five ml of reconstituted membranes or native membranes containing 5 mg of total protein were centrifuged for 1 h at 100,000 g in an SW39 rotor (Beckman). Protein in the pellet was determined by the method of Lowry et al. (28). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to Laemmli (25) using the higher ionic strength running gel buffer described by Glabe (15). The proteins were resolved on a 10% running gel with a 3% stacking gel. The gels were stained with Coomassie blue (9). A Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) with linear transport accessories and chart recorder was used to scan the stained gels.

## Electron Microscopy

Aliquots (10  $\mu$ l) were taken from the incubation medium after varying time intervals and negatively stained by addition of 10  $\mu$ l of 3% ammonium molybdate, pH 7.4, followed by draining and drying the Formvar-coated grids. Stained samples were examined at magnifications of  $\times 20,000$  and  $\times 39,000$  in a Hitachi HU 11 E electron microscope. The micrographs shown here are typical results from three replicate experiments.

Freeze-fracture electron microscopy was also performed. Centrifugal pellets (105,000 g, 1 h) were obtained from the reconstitution system after the standard 2-h incubation, and from control microsomes similarly treated, but omitting LPC and oleoyl CoA. The resulting pellets were infiltrated with 30% glycerol, and aliquots were transferred to specimen planchettes and frozen in liquid nitrogen-cooled Freon. Freeze-fracture was carried out in a Balzers apparatus (Balzers Corp., Nashua, N. H.) by standard methods (13).

## RESULTS

To establish that most of the combined detergent effect of the lysophosphatidylcholine and oleoyl CoA was degraded by the acyltransferase system, it was necessary to measure changes in lipid content during the 2-h incubation period (Table I). Lipid analyses of untreated microsomes have been included for comparison. We found that the reaction progressed nearly to completion, so that most of the LPC in the experimental series was converted to phosphatidylcholine (PC). The PC content of the reconstituted membranes increased to three times the value found in controls and to four times the amount found in untreated microsomes.

If synthesis of phosphatidylcholine through acylation of LPC does reconstitute membranes from the solubilized components, it would be expected that the newly formed membranes would increasingly scatter light, and that the kinetics of the reconstitution process could thereby be followed by turbidity changes. Fig. 1 charts turbidity of the reaction mixture over the 2-h incubation period. Turbidity increased from a nearly clear original filtrate to a visibly turbid suspension. In other experiments (data not presented), increases in turbidity were found to correlate with the evolution of CoA released during acylation. Under oil immersion phase contrast microscopy, samples at the beginning of incubation were devoid of particles. Toward the end of the incubation period, numerous particles and vesicular structures could be seen at the limit of resolution.

Fig. 2*a-f*, shows negative stains of samples taken at timepoints during incubation. The untreated microsomes had a normal vesicular appearance (Fig. 2*A*). After the solubilizing steps at time zero, essentially no membranous structures were visible (Fig. 2*B*). Instead, fields were filled

with dispersed particles ranging around 5 nm in diameter, together with occasional 5 × 30-nm rods. During the first 10–30 min of incubation, loosely aggregated rods and disks were seen (Fig. 2*C* and *D*). More clearly defined membranes appeared to grow out of the aggregated material (Fig. 2*E* and *F*) and, after 1–2 h, most of the membranous structures were in the form of multilamellar vesicles.

Fig. 3 shows densitometric scans of SDS-polyacrylamide gels of rat liver microsomes and the reconstituted membranes. No major differences in protein composition were found. However, it should be noted that only a fraction (10–40%) of the original protein appeared in the centrifugal pellets of reconstituted membranes, suggesting that the major fraction of protein was in a form that did not sediment after 1 h at 105,000 g.

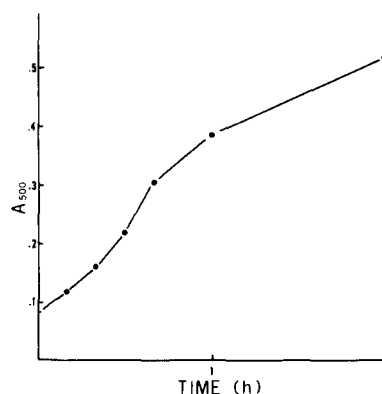


FIGURE 1 Turbidity changes during the acylation reaction. Turbidity was measured during a 2-h incubation period by absorbance changes at 500 nm. No turbidity changes occurred in controls which were kept on ice during the incubation period, or if the microsomes were heated to 100°C for 1 min before solubilization in the reaction mixture. See Table I for conditions.

TABLE I  
*Acylation of Lysophosphatidylcholine by Rat Liver Microsomes*

	Controls	Experimental	Native membranes
Total lipid phosphate	1.71 ± 0.02 (4)	1.76 ± 0.12 (4)	0.38 ± 0.01 (3)
Lysophosphatidylcholine	0.96 ± 0.05 (6)	0.04 ± 0.01 (6)	Trace
Phosphatidylcholine	0.52 ± 0.02 (6)	1.41 ± 0.03 (6)	0.30 ± 0.01 (3)

Samples containing 0.8–1.0 mg/ml microsomal protein, 1.0 μmol/mg protein lysophosphatidylcholine, 0.1 mM CoA, 5 mM Mg-ATP, 0.1 M Tris buffer, pH 7.5, and either 1.0 μmol/mg protein oleoyl CoA (experimental) or an equivalent volume of buffer, were sonicated, filtered, incubated, and the lipids were extracted and analyzed as described in Materials and Methods. The lipids of untreated membranes were analyzed as well. The results are presented as μmol of lipid phosphate/mg protein (mean ± SEM), and the number of determinations is shown in parentheses.

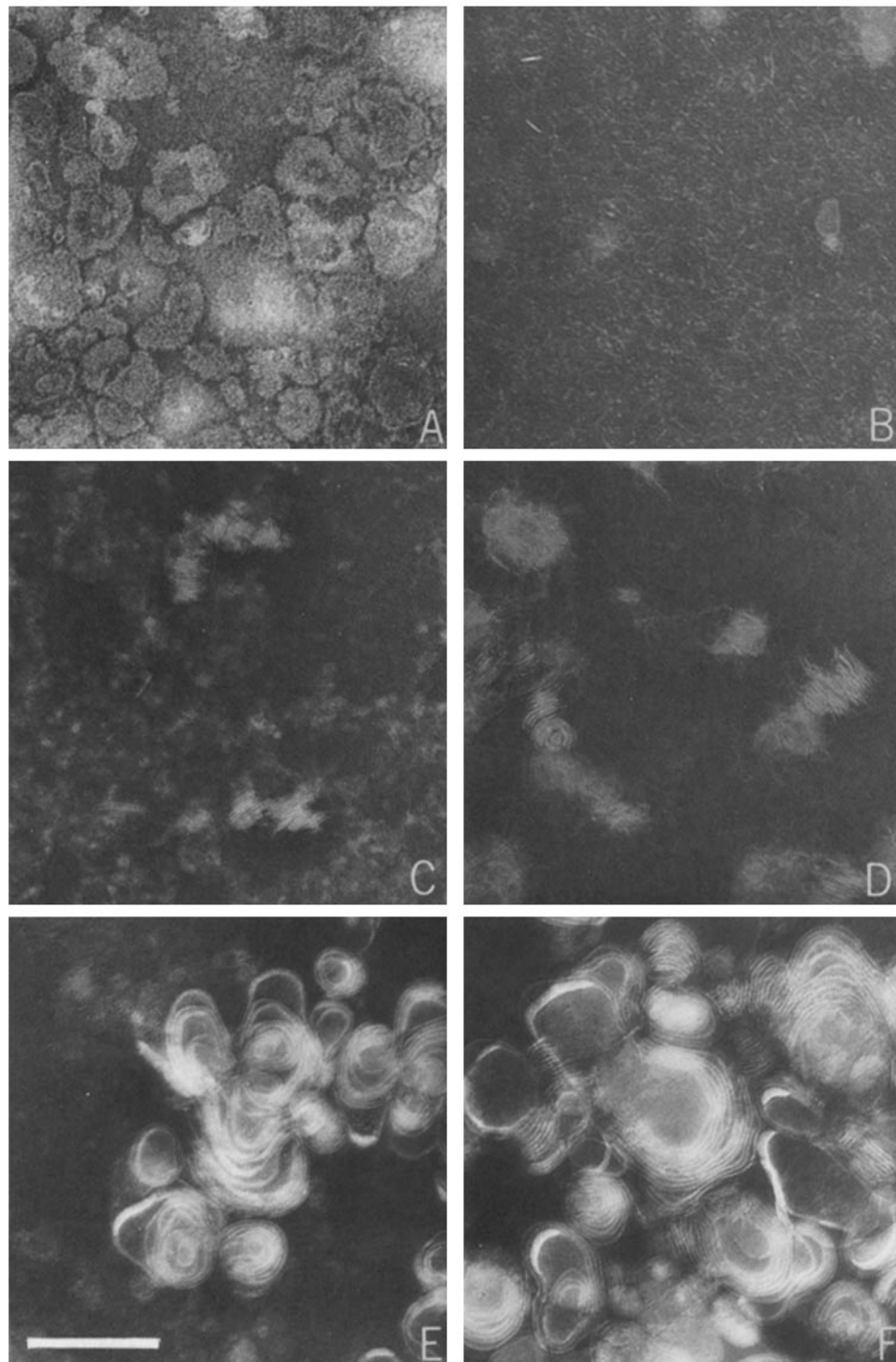


FIGURE 2 Negative stains of membrane formation during the acylation reaction. Aliquots were stained with ammonium molybdate as described in the text, and typical fields are shown. Fig. 3 *A* shows the appearance of the original microsomes. Other samples were taken after the solubilized reaction mixture was filtered through a 0.22  $\mu\text{m}$  Millipore filter at time zero. Those shown here were taken at zero (*B*), 10 (*C*), 30 (*D*), 60 (*E*), and 120 (*F*) min. See Table I for conditions. Bar, 0.2  $\mu\text{m}$ .  $\times 92,000$ .

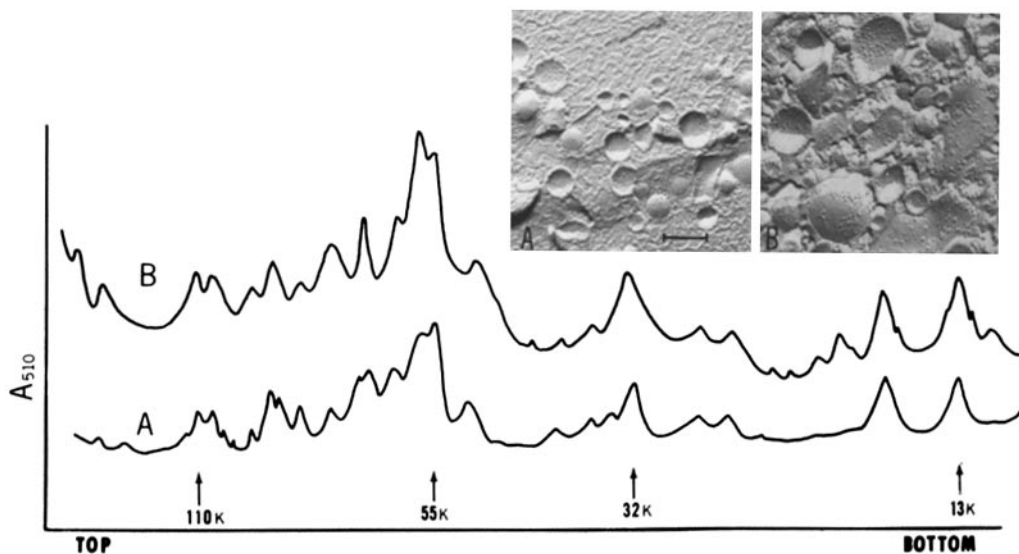


FIGURE 3 Gel scanning patterns in control (A) and reconstituted (B) membranes. The reconstituted membranes did not differ significantly from the controls in their protein composition. Molecular weights of several prominent bands are taken from a mol wt/ $R_f$  graph prepared with mol wt standards run on the same gel. Absorbance is in arbitrary units. The insets show freeze-fracture images of control (A) and reconstituted (B) membranes prepared under the same conditions. The fracture faces of both membranes displayed prominent intramembrane particles. Bar, 0.2  $\mu\text{m}$ .

The insets of Fig. 3 show freeze-fracture images of the original microsomes (Fig. 3 A) and reconstituted membranes (Fig. 3 B). As expected from the negative-stain results, the reconstituted membrane structures had considerably larger diameters. Prominent particulate structures were distributed on both concave and convex fracture faces. The multilamellar structures appearing in negative stains were only occasionally observed in freeze-fracture patterns, probably because cross-fracture of entire vesicles is a relatively rare event.

NADPH-cytochrome *c* reductase is a marker enzyme for hepatic microsomes (5) and was used in the present study to determine whether an active microsomal enzyme was incorporated into the reconstituted membranes. In four determinations, it was found that the activity in the control microsomes (incubated and centrifuged) was  $80 \pm 14$  nmol cytochrome *c* reduced  $\text{mg}^{-1} \text{min}^{-1}$ , and that that of the reconstituted membranes was  $55 \pm 11$  nmol cytochrome *c* reduced  $\text{mg}^{-1} \text{min}^{-1}$  (mean  $\pm$  SD). We concluded that the acylation procedure had in fact incorporated a significant fraction of the original enzyme into the reconstituted membranes in an active form.

## DISCUSSION

This paper demonstrates the feasibility of an enzymatic reconstitution of membrane structures from rat liver microsomes solubilized by naturally occurring amphiphiles. The disruption and reconstitution of membranes seen in Fig. 2 *a-f* are best explained in terms of detergent effects balancing hydrophobic associations. After solubilization (determined by solution clarification and the absence of closed vesicles), small particles and rods predominated in the negative stain image. The rod shape is characteristic of large micelles formed by amphiphiles with a single hydrocarbon chain, such as lysophosphatidylcholine and oleoyl CoA (34). Similar rodlike structures in negative-stain images have been observed in water-lysophosphatidylcholine phases containing high lipid concentrations (21).

During incubation, associations of rods and small particles formed intermediate species with the appearance of multilaminar rods. At the end of incubation, the background of small particles was virtually absent and only vesicles were seen. These transitions, if the small particles are in fact mixed micelles of lipid containing hydrophobic

proteins, are consistent with phase diagrams of lysophosphatidylcholine, phosphatidylcholine, and water, which predict the coexistence of micelles and bilayers while the LPC/PC ratio is still relatively high (32). The enzymatic addition of the second acyl chain to lysophosphatidylcholine thermodynamically favors the formation of bilayers (34). Changes in the size and organization of the particles seen in the electron micrographs are mirrored by the relative spectrophotometric turbidities recorded at the same timepoints. The enzymatic removal of detergent resulting in the formation of phospholipid and protein-containing vesicles is analogous to the formation of vesicles from dialyzed protein, phospholipid and cholate mixtures (17, 19, 22).

The results reported here permit the conclusion that the membranes produced by the acylation reaction can be characterized as "reconstituted" and are not simply bilayers of newly synthesized phospholipid. Polyacrylamide gel patterns show that the major protein components of the original microsomes are present in centrifugal pellets of the reconstitution system. Secondly, a marker enzyme of hepatic microsomes, NADPH-cytochrome *c* reductase, is present in the reconstituted membranes and has a specific activity ~70% that of the control microsomes. Finally, freeze-fracture images of the reconstituted membranes reveal prominent intramembrane particles, which suggests that the hydrophobic protein components of the original membranes are inserted into the lipid bilayer. However, we cannot conclude that proteins have been incorporated in their original configuration with respect to the inner and outer membrane surfaces.

The procedure described here has several potential applications. First, the addition of known lysophosphatide-acyl CoA pairs would permit variation of the phospholipid content of any membrane that contains the appropriate acyltransferase enzymes. Second, since gel electrophoresis showed that the major protein components of the microsomes were incorporated into the reconstituted membranes, it follows that exogenous proteins might also be incorporated so that hybrid membranes could be produced for experimental purposes. The results in Table I show that even the low level of acyltransferase in liver microsomes can acylate concentrations of lysophosphatidylcholine up to three times the initial phosphatidylcholine content. A purified acyltransferase would permit even more efficient reconstitutions, and

recent successes in isolating acyltransferases and transacylases (2, 24, 38) suggest that purification of the rat liver enzyme is feasible.

Finally, the cell may use similar lipid synthesis pathways during membrane biogenesis and membrane fusion processes (37). For instance, the similarities in appearance and growth of the reconstituted vesicles to lamellar bodies in lung tissue (3) are remarkable. The lamellar bodies are concentric lamellae of dipalmitoylphosphatidylcholine and protein (2, 3). The phosphatidylcholine is considered to be formed by acylation of lysophosphatidylcholine and transacylation pathways (35, 36). Growth of lamellar bodies is postulated to occur through fusion of vesicular structures (2). The experimental system described here may provide a model for investigation of such processes.

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