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Purification of Acyl CoA:1-acyl-*sn*-glycero-3-phosphorylcholine Acyltransferase

V.C. Gavino[1] and D.W. Deamer[1]

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Abstract

Acyl coenzyme A:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase (EC 2.3.1.23) is capable of forming lipid bilayer vesicles from its soluble substrates lysophosphatidylcholine (LPC) and oleoyl CoA. This suggested a purification method in which rat liver microsomes are first washed with deoxycholate to increase specific activity of the endogenous acyltransferase approximately fivefold, then solubilized by the detergent effect of excess LPC and oleoyl CoA in 1:1 stoichiometric ratios. As the LPC is converted to phosphatidylcholine by acyl group transfer, the detergent effect is lost and lipid vesicles containing the enzyme activity are produced. Other microsomal proteins are excluded from the vesicles. The vesicles may be separated by density gradient flotation and are found to contain acyltransferase with a specific activity of 9–10 μmol/mg/min. This reflects a purification of approximately 140-fold, about ten times greater than achieved in previous studies.

Key Words: Acyltransferase; lysophosphatidylcholine; lipid vesicles.

Introduction

Acyl CoenzymeA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase (EC 2.3.1.23), first demonstrated in rat liver microsomes (Lands, 1960), is also found in several subcellular fractions of mammalian tissues (Van den Bosch *et al.*, 1972), other vertebrates (Holub *et al.*, 1976), *Drosophila melanogaster* (Heckman *et al.*, 1977), plant tissue (Devor and Mudd, 1971), fungi (Das and Banerjee, 1977), *Tetrahymena pyriformis* (Okuyama *et al.*, 1977), and *Escherichia coli* (Proulx and Van Deenen, 1966). The activity of this enzyme (hereafter referred to as LPC acyltransferase)[2] as well as those of other

[1]Department of Zoology, University of California, Davis, California 95616.

[2]Abbreviations: LPC, 1-acyl-*sn*-glycero-3-phosphorylcholine; DOC, sodium deoxycholate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TLC, thin-layer chromatography; DTT, dithiothreitol; PC, phosphatidylcholine; RLM, rat liver microsomes.

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glycerolipid acyltransferase systems provide a mechanism for the net synthesis of membranes (Heckman *et al.*, 1977; Higgins, 1976; Baker and Chang, 1981; Szamel *et al.*, 1981) and lung surfactant (Van Heusden and Van den Bosch, 1981). The combined action of phospholipases and glycerolipid acyltransferases results in the turnover of specific phosphoglycerides and esterified fatty acids (Van den Bosch *et al.*, 1972; Kroner *et al.*, 1981) and permits modulation of the lipid composition of membranes (Piekarski, 1979). Previous studies in this laboratory have demonstrated that LPC acyltransferase can drive the reconstitution of microsomal membrane components that have been solubilized with oleoyl CoA and LPC (Deamer and Boatman, 1980).

The specificity of LPC acyltransferase toward various acyl donors and acceptors has been tested in subcellular fractions (Holub *et al.*, 1979; Holub *et al.*, 1980; Colard *et al.*, 1980; Wise *et al.*, 1980) and with partially purified enzyme preparations (Okuyama *et al.*, 1975; Hasegawa-Sasaki and Ohno, 1980). The results of these experiments suggest that the enzyme is partly responsible for the control of phospholipid composition of membranes (Holub *et al.*, 1980; Okuyama *et al.*, 1975). There are, however, other types of glycerolipid acyltransferases with different specificities that are also present in subcellular fractions (Higgins, 1976; Okuyama *et al.*, 1975; Sonoki and Ikezawa, 1976; Bell *et al.*, 1979; Holub, 1980). If several acyltransferase activities are present in a given membrane preparation, kinetic studies of a given enzyme will be difficult to interpret. We have therefore directed our attention to a method that may permit purification of individual acyltransferases.

In previous work, LPC acyltransferase was partially purified by detergent and substrate solubilization, gel permeation chromatography, and sucrose density gradient centrifugation of solubilized microsomes (Hasegawa-Sasaki and Ohno, 1980; Yamashita *et al.*, 1981; Weltzien *et al.*, 1979). We have modified the Hasegawa-Sasaki and Ohno method to take advantage of the ability of the enzyme to synthesize lipid bilayer vesicles, given its substrates. The vesicles can be separated by flotation, and this step provides a tenfold enhancement of specific activity.

Materials and Methods

Chemicals

Lysophosphatidylcholine (LPC), Coenzyme A, sodium deoxycholate (DOC), and Tris were purchased from Sigma. Oleoyl chloride was from Nu Chek Prep (Elysian, Minnesota). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was from PL Biochemicals (Milwaukee, Wisconsin). Oleoyl CoA

was synthesized and checked for purity as described previously (Deamer and Boatman, 1980). All other chemicals were reagent grade.

Preparation of Microsomes

DOC-washed rat liver microsomes were prepared according to a previous report (Hasegawa-Sasaki and Ohno, 1980). Male Sprague–Dawley rats weighing 300 to 400 g were used. The 225,000 × *g* pellet obtained from the microsomal suspension solubilized with 0.33% DOC was used for the experiments. The pellet was resuspended in 20 mM Tris-HCl (pH 7.4) containing 0.2 mM EDTA and 50% glycerol and stored at −70°C. The acyltransferase activity was stable for several months under these conditions.

Enzyme Assays

LPC acyltransferase activity was routinely measured spectrophotometrically by following the reduction of DTNB by free CoA released during the enzyme-catalyzed reaction. Absorbancies were measured at 412 nm using a Gilford Model 2400 spectrophotometer. All assays were carried out at 25°C. The assay mixture contained 50 μM each of DTNB and oleoyl CoA in a total volume of 2.0 ml of 0.1 M Tris-HCl at pH 7.4; 100 nmol of LPC were added to start the reaction. DOC-washed microsomes contain an acyl CoA hydrolase activity that contributed to the background rate. This was subtracted from the total rate to calculate for the activity of LPC acyltransferase.

Enzyme-Catalyzed Synthesis of Phosphatidylcholine (PC)

DOC-washed microsomes were solubilized with LPC at a lipid-to-protein ratio of 10 (by weight). The suspension was diluted with 20 mM Tris-HCl (pH 7.4) to a final LPC concentration of 15 mM and then placed in a bath sonicator for 30 sec. To this was added enough 10 mM oleoyl CoA (in 20 mM Tris-HCl, pH 7.4) to obtain an equimolar ratio between LPC and oleoyl CoA. The mixture was placed in a bath sonicator for an additional 30 sec and then allowed to react at room temperature for 1 hr. Increase in light scattering was followed at 500 nm with a Gilford 2400 spectrophotometer. Control experiments were performed using heat-inactivated DOC-washed microsomes. Heat inactivation was carried out by incubating the microsomes at 75°C in a water bath for 3 min.

Thin-Layer Chromatography (TLC)

Lipids were extracted from the reaction mixture (Kates, 1972) and the extract analyzed by TLC on silica-impregnated glass rods (Chromarod-S, Iatron Laboratories, Japan). The rods were developed with chloroform:

methanol: water: acetic acid (60:50:5:1, v/v) and the lipids detected using an Iatroscan Model TH-10 (Ancal, Los Osos, California) equipped with a flame ionization detector. Under these conditions, the relative mobilities (R_f values) of LPC and PC were 0.14 and 0.25, respectively.

Glycerol Gradient Centrifugation

DOC-washed microsomes (400 μg protein) were allowed to react with 8 μmol each of LPC and oleoyl CoA in 2.0 ml of 0.1 M Tris-HCl at pH 7.4. The reaction mixture was placed in a bath sonicator for 30 sec. The reaction was allowed to proceed at room temperature for 1 hr after which it was again placed in the bath sonicator for 30 sec and then diluted with an equal volume of glycerol. The suspension was divided equally into three aliquots and each aliquot placed in a centrifuge tube; 1.0 ml each of 30, 15, and 0% glycerol in 20 mM Tris-HCl, 0.1 mM dithiothreitol (DTT), and 1 mM EDTA (pH 7.4) was sequentially layered on top of the reaction mixture in each centrifuge tube. Centrifugation was carried out at 25,000 rpm for 18 hr in a Beckman SW 39 rotor. Light scattering bands were detected by tungsten illumination at right angles to the centrifuge tubes. These light scattering bands were collected and tested for LPC acyltransferase activity. Control experiments were performed using heat-inactivated DOC-washed microsomes as described previously in this section. The presence of 0.1 mM dithiothreitol in the gradient was essential for enzyme stability. To obtain maximal LPC acyltransferase activity, the enzyme–substrate mixture was bath sonicated for 30 sec before oleoyl CoA was added to start the reaction.

Other Procedures

Protein was assayed by the Lowry method (Lowry *et al.*, 1951) using crystalline bovine serum albumin as standard. A modification of the Lowry procedure was used for protein concentrations of less than 10 μg/ml (Bensadoun and Weinstein, 1976). Negative staining for electron microscopy was carried out as described previously (Deamer and Boatman, 1980). Specimen preparation for freeze-fracture electron microscopy was according to Fisher and Branton (1974) using 25% glycerol as a cryoprotectant.

Results

Both LPC and oleoyl CoA at certain concentration ratios were found to be inhibitory to LPC acyltransferase (Table I). For example, 0.5 mM oleoyl CoA was inhibitory when the LPC concentration was 0.05 mM, but was no longer inhibitory when the LPC concentration was increased to 0.5 mM.

Table I. The Effect of Substrate Concentration on LPC Acyltransferase Activity[a]

	Oleoyl CoA (mM)		
LPC (mM)	0.05	0.5	2.5
0.05	6.4 ± 0.5[a,b] ($n = 6$)	2.7 ± 0.2 ($n = 3$)	0
0.5	6.2	6.6 ± 0.3 ($n = 3$)	
2.5	7.1		3.5
5.0	4.4		

[a]The assay mixture contained 18 μg protein, 0.05 mM DTNB, and the indicated amounts of LPC and oleoyl CoA in a total volume of 2.0 ml 0.1 M Tris-HCl at pH 7.4. LPC acyltransferase activity is expressed as nanomoles free CoA released per minute.

[b]Data reported as mean ± SEM (n = number of separate experiments; other data points without standard errors are single determinations.)

Furthermore, 2.5 mM oleoyl CoA completely inhibited LPC acyltransferase activity when the LPC concentration was 0.05 mM, but was only partially inhibitory when the LPC concentration was also raised to 2.5 mM (Table I). At the high concentrations of LPC and oleoyl CoA used in the experiments described in Figs. 1 and 2, LPC acyltransferase activity was maintained if microsomes were solubilized with the LPC before the addition of oleoyl CoA. When oleoyl CoA was added at high concentration to the microsomes before the LPC, enzyme activity was lost (data not shown).

DOC-washed microsomes were solubilized with LPC at a lipid-to-protein ratio of 10 (by weight). The solubilized microsomes synthesized PC when treated with oleoyl CoA at a concentration equimolar to the LPC (Fig. 1). Fig. 1A shows the lipid profile of the reaction mixture 5 min after the addition of oleoyl CoA. At this point, only a small amount of phosphatidylcholine (PC) was present relative to LPC. Figures 1B and 1C are the lipid profiles of the reaction mixture at 20 and 60 min incubation times, respectively. In Fig. 1B, the peak area ratio between PC and LPC increased from 0.15 (at 5 min) to 3.0 at 20 min. At 60 min, the PC-to-LPC peak area ratio further increased to 22. The specific activity of the microsomes used in this experiment was 330 nmol/min/mg and the substrate concentrations were 20 μmol each of LPC and oleoyl CoA per milligram microsomal protein. Under these conditions, the enzyme reaction should take at least 60 min to go to completion. Figure 1C shows that the reaction was almost complete at 60 min and that the equilibrium position favors the formation of PC from LPC and oleoyl CoA. No attempt was made to identify the material running close to the solvent front in Figs. 1A–1C. Part of this material might be free fatty acid liberated

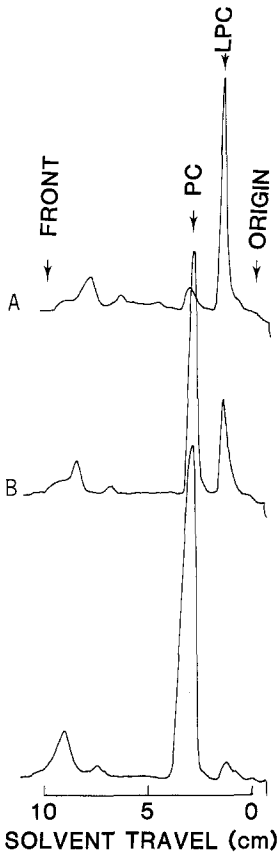

Fig. 1. Lipid analysis of an acylation reaction mixture. 400 μg of DOC-washed microsomes were initially solubilized with 8 μmol each of LPC and oleoyl CoA in 2.0 ml of 0.1 M Tris-HCl (pH 7.4). The reaction mixture was sampled at specified time intervals (A, 5 min; B, 20 min; C, 60 min) and lipids extracted and analyzed by TLC on silica-impregnated glass rods (see text).

from oleoyl CoA by an acyl hydrolase activity present in microsomal preparations (Deamer and Boatman, 1980). Under the conditions of the experiment in Fig. 1, this acyl hydrolase activity was apparently not very active since lipid analysis showed that the PC-to-LPC peak area ratio was 22 after a 60-min incubation (Fig. 1C) even though no attempt was made to

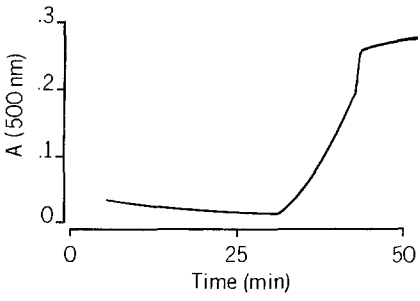

Fig. 2. Increase in light scattering as a result of the action of LPC acyltransferase on DOC-washed microsomes solubilized with LPC and oleoyl CoA. The reaction mixture contained 400 μg of protein and 8 μmol each of LPC and oleoyl CoA in 2.0 ml of 0.1 M Tris-HCl at pH 7.4. The specific activity of the protein used in this reaction was 330 μmol/min/mg.

introduce an acyl CoA-regenerating system as described previously (Deamer and Boatman, 1980).

Figure 2 shows the increase in light scattering as a result of PC synthesis under the conditions described for the acylation reaction in Fig. 1. PC synthesis catalyzed by LPC acyltransferase in crude preparations of microsomes solubilized with LPC and oleoyl CoA resulted in an increase in light scattering as reported previously by this laboratory (Deamer and Boatman, 1980). That observation was confirmed in the present report using partially purified DOC-washed microsomes. The increase in light scattering reached a plateau after 45 to 50 min. Although light scattering may not be an appropriate assay for acyltransferase activity, it is interesting to note that maximum turbidity in the reaction mixture was reached at an incubation time that closely matched the results of the experiment in Fig. 1. DOC-washed microsomes that were inactivated either by heat or by a high concentration of oleoyl CoA showed no LPC acyltransferase activity by the spectrophotometric DTNB assay, nor was increased light scattering observed upon incubation.

The acylation reaction mixture described in Fig. 2 was mixed with an equal volume of glycerol and floated through a step gradient consisting of 30, 15, and 0% glycerol. A turbid band collected at the interface of the 15 and 0% glycerol solutions (Fig. 3) and will be referred to as the D-15 band. Turbid bands were not obtained from mixtures containing heat-inactivated or oleoyl CoA-inactivated DOC-washed microsomes (Fig. 3). A turbid band also collected at the interface of the 30 and 15% glycerol solutions (hereafter referred to as the D-30 band) but did not do so consistently (data not shown). A brown pellet always appeared at the bottom of the centrifuge tubes and was devoid of LPC acyltransferase activity.

In one experiment where both D-15 and D-30 bands were observed, 11% of the total LPC acyltransferase activity was recovered from the D-15 fraction (Table II). In another experiment where only a D-15 band was observed (Fig. 3), the band contained 9.6% of the total enzyme activity (Table II). In both experiments, the protein content of the D-15 bands was barely detectable by the micro-Lowry procedure (Bensadoun and Weinstein, 1976). Our best estimates for the protein content of these bands range from 5 to 20 μg/ml with an estimated specific activity of 9–10 μmol/min/mg protein. There was no detectable acyl hydrolase activity in the bands (data not shown).

The light scattering data shown in Fig. 2 were correlated with the appearance of vesicular structures in microsomes solubilized and incubated with LPC and oleoyl CoA for 1 hr. Typical vesicular structures of the original microsomes are shown in Fig. 4A. These structures disappeared when the microsomes were solubilized with LPC and oleoyl CoA and were replaced by 10 nm micellar particles. After an incubation period of 1 hr, detergent depletion and PC synthesis resulted in the formation of membranous vesicular structures that ranged from 50 to 200 nm in diameter (Fig. 4C). The vesicles

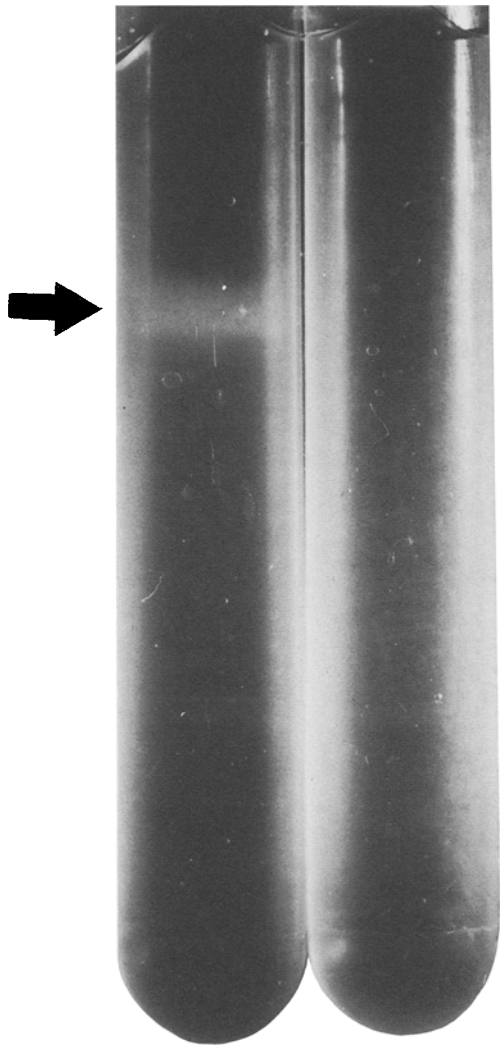

Fig. 3. Discontinuous density gradient centrifugation of an acylation reaction mixture. 400 μg of DOC-washed microsomes were initially solubilized with 8 μmol each of LPC and oleoyl CoA in 2.0 ml 0.1 M Tris-HCl (pH 7.4) and incubated at 25°C for 1 hr. The light-scattering band in the tube on the left is at the interface of the 15 and 0% glycerol solutions. The tube on the right had inactive enzyme (see text).

Table II. Purification of Acyl CoA:1-acyl-*sn*-glycero-3-phosphorylcholine Acyltransferase

Fraction	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Recovery (%)
RLM[a]	70	15,000	
DOC–RLM[b]	340 ± 18[c] ($n = 3$)	6600 ± 220 ($n = 3$)	44 ± 15
D–15[d]	10,000, 9000	1700, 1400[e]	11, 9.6

[a]Salt-washed microsomes.
[b]DOC-washed microsomes.
[c]Mean ± SEM (n = number of samples).
[d]Light-scattering band obtained after density gradient centrifugation.
[e]Only small aliquots (330 and 130 nmol/min, respectively) of DOC–RLM were subjected to density gradient centrifugation, and these numbers represent values multiplied by the appropriate scaling factor.

isolated by density gradient centrifugation into a turbid band (Fig. 3) are shown in Fig. 4D. These contain most of the enzyme activity and newly synthesized phosphatidylcholine. Samples of microsomes and vesicles from the turbid band were also examined by the freeze-fracture method. Rat liver microsomes typically display numerous intramembranous particles which are more densely distributed on the P fracture face (Fig. 5A). As expected, these particles are largely absent from the vesicles produced by acyltransferase activity (Fig. 5B).

Discussion

In the purification procedure reported here, we take advantage of the fact that the product of acyltransferase activity can be phosphatidylcholine in the form of lipid bilayer vesicles (Deamer and Boatman, 1980). Our rationale was to solubilize DOC-washed rat liver microsomes with a large excess of LPC and oleoyl CoA so that the original protein and lipid components would be diluted into mixed micelles composed of the two detergent-like substrates of the enzyme. At a 20:1 ratio of micromoles lipid/mg protein, and assuming an average molecular weight of 50,000 for microsomal proteins, the mole ratio of lipid to protein is in the range of 1000:1. Ideally, each of the resulting micelles would contain at most one protein molecule. If that protein has acyltransferase activity, it would catalyze the formation of phosphatidylcholine, both from the substrate in that micelle and from neighboring micelles that do not contain the enzyme. When a sufficient amount of phosphatidylcholine has accumulated, lipid bilayer vesicles would form. Assuming that little or no fusion with extraneous protein occurs, these vesicles could then be separated from the remaining protein by virtue of their decreased density.

The results of our experiments generally support this scheme. We found

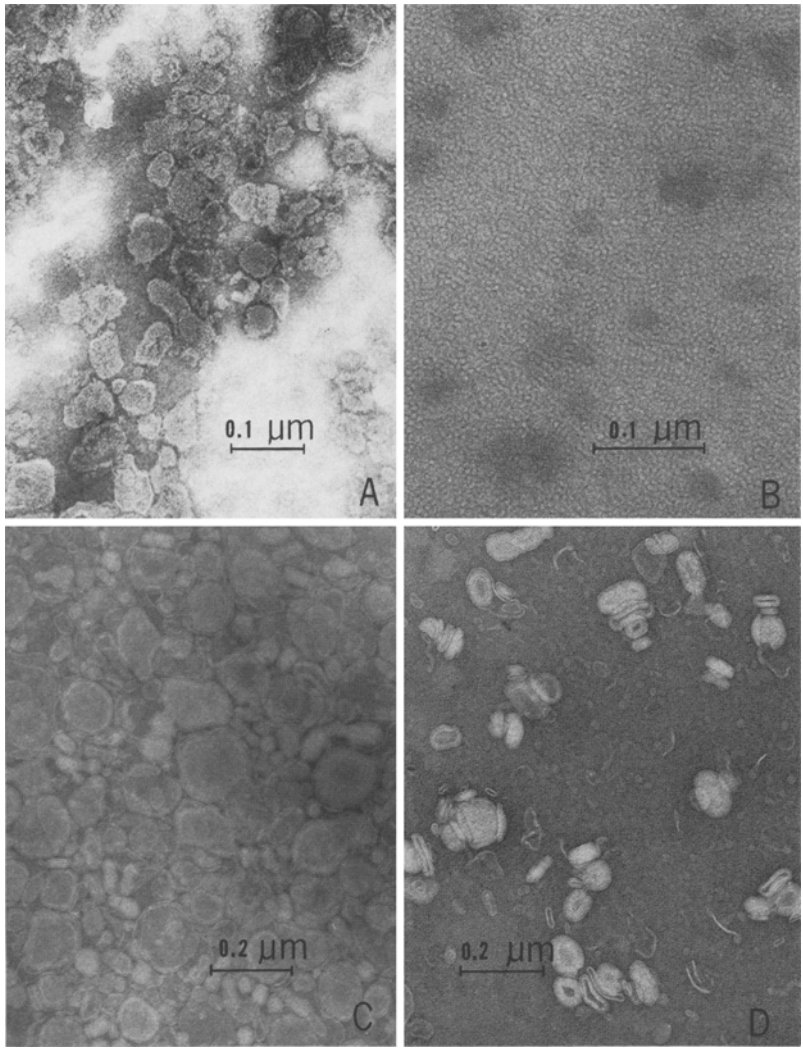

Fig. 4. Negative stains of membranes formed during the LPC acyltransferase-catalyzed acylation reaction. Aliquots were stained with ammonium molybdate as described previously (Deamer and Boatman, 1980), and typical fields are shown (A, original microsomes; B, DOC-washed microsomes initially solubilized with LPC and oleoyl CoA; C, reaction mixture after 1 hr incubation; D, light-scattering band obtained after density gradient centrifugation of reaction mixture).

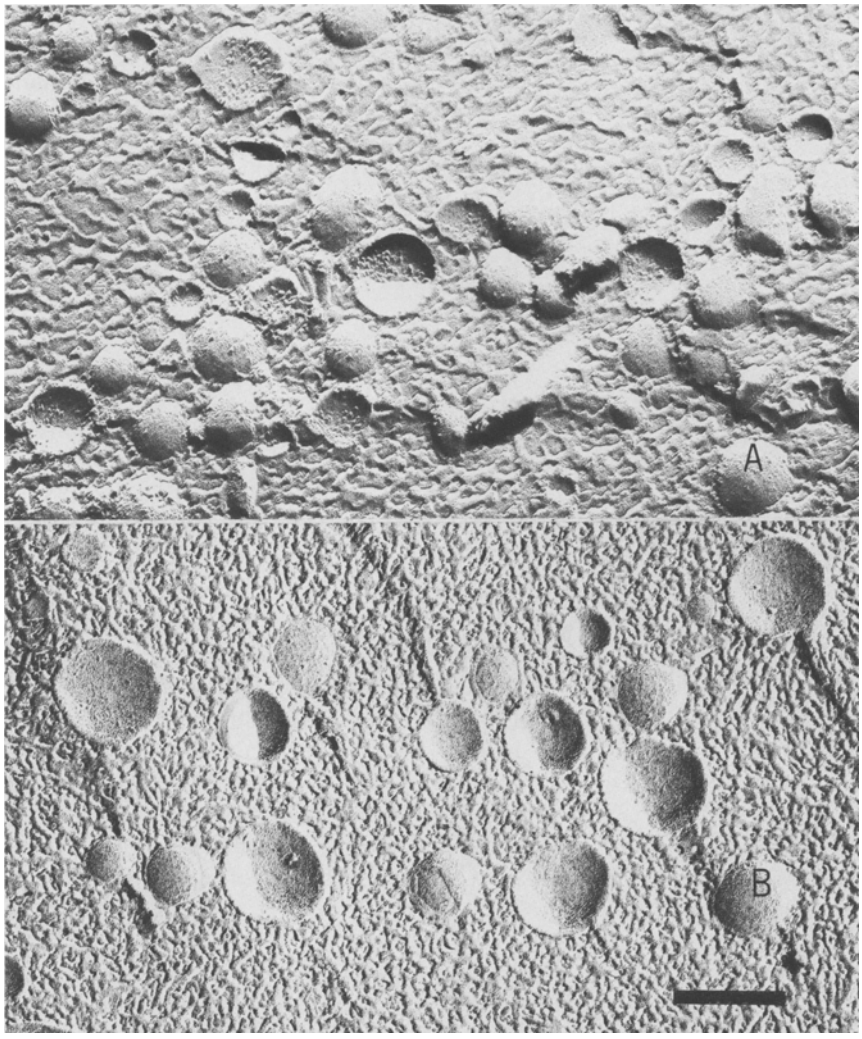

Fig. 5. Freeze-fracture images of rat liver microsomes and lipid vesicles produced by LPC acyltransferase activity. (A) The microsomes show intramembranous particles typical of such membranes. (B) The lipid vesicles are relatively devoid of particles. Bar shows 0.2 μm.

that when DOC-washed microsomes were solubilized with 20 μmol each of LPC and oleoyl CoA, the acyltransferase activity was sufficient to convert over 95% of the substrates into PC after a reaction time of 1 hr. Increased light scattering (Fig. 1) and electron microscopy (Figs. 4 and 5) indicated that lipid vesicles were forming. Furthermore, when the sonicated reaction mixture was subjected to discontinuous density gradient centrifugation, a turbid band

appeared that contained LPC acyltransferase with a specific activity of 9–10 μmol/min/mg (Table II). Turbid bands did not appear if the enzyme was inhibited by heating. The specific activity in the resulting vesicles was an order of magnitude greater than previously reported for partially purified enzyme (Hasegawa-Sasaki and Ohno, 1980), suggesting that the procedure described here holds considerable promise for the isolation and characterization of this enzyme. Only a few micrograms of protein appeared with the turbid band, and this amount has so far been refractory to analysis by gel electrophoresis. A large-scale preparation is now under way to collect sufficient quantities of the enzyme for further studies.

An interesting result of the present work is the observation that certain concentrations of oleoyl CoA completely inhibited LPC acyltransferase (Table I). This is in contrast to other reports (Hasegawa-Sasaki and Ohno, 1980) that oleoyl CoA was not inhibitory at the concentrations tested. Wise *et al.* (1980) reported similar results. Both of these studies reported some inhibition with LPC, and our results also suggest this effect (Table I). However, Okuyama *et et.* (1977) did not observe LPC inhibition in rat liver microsomes.

Despite these uncertainties, our results indicate that the enzyme is sensitive to its environment and that it will be important to define this lysophosphatide-to-acyl CoA ratio in future kinetic studies. For example, we found that varying the LPC concentration alone was enough to influence the inhibitory effect of oleoyl CoA (Table I). Figures 1, 2, and Table I also suggest that protein concentration has an effect on reaction rate that cannot be explained on the basis of enzyme concentration alone. For example, the acylation reaction seemed to proceed at maximum velocity when microsomes at 200 μg protein/ml were treated with 4 mM concentrations of LPC and oleoyl CoA (Figs. 1 and 2). On the other hand, the same microsomes at 18 μg protein/ml was at maximum reaction velocity when treated with 0.05 or 0.5 mM concentrations of both substrates. Increasing both substrate concentrations to 2.5 mM resulted in a decrease in reaction velocity (Table I). Another observation that suggested that LPC acyltransferase is sensitive to its lipid environment was the need to sonicate the turbid band with substrate LPC in order to obtain maximum reaction velocity. However, an alternative explanation is that the vesicular enzyme is surrounded by large amounts of phosphatidylcholine which often take the form of oligolamellar vesicles. This might make the protein physically inaccessible to its substrates.

There are several potentially significant applications of a procedure that will provide acyltransferase in a purified form. First, such a procedure may lend itself to purification of other metabolic enzymes that have lipid as a product. For instance, several reports in the literature describe other glycerolipid acyltransferases that use substrates other than LPC or oleoyl CoA

(Higgins, 1976; Okuyama *et al.*, 1975; Sonoki and Ikezawa, 1976; Bell *et al.*, 1979; Holub, 1980). It may be possible to selectively enrich such acyltransferases by using appropriate lysophosphatide-acyl CoA pairs for phospholipid synthesis and subsequent fractionation.

Second, a purified enzyme may provide a relatively simple procedure for the synthesis of certain phospholipids, particularly those containing radioactive or fluorescent labels. Finally, the procedure offers an interesting alternative to detergent solubilization and dialysis as a method for reconstituting biological membranes or producing model membranes with specified lipid and protein components. The activity has already been shown in principle to be capable of reconstituting microsomal membranes (Deamer and Boatman, 1980).

Acknowledgments

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