Solubilization, Partial Purification, and Reconstitution in Phosphatidylcholine–Cholesterol Liposomes of Acyl-CoA:Cholesterol Acyltransferase†

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ABSTRACT: Acyl-CoA:cholesterol acyltransferase (ACAT) was solubilized from pig liver microsomes with a combination of 1:1% deoxycholate and 1 M potassium chloride. This solubilized activity was then reconstituted in lipid vesicles by diluting the extract into a solution of phosphatidylcholine, cholesterol, and sodium cholate, followed by dialysis. The reconstituted activity was shown to be dependent upon cholesterol in the reconstitution mixture and also shown to vary with changes in the phospholipid headgroup: phosphatidylethanolamine was most active, phosphatidylcholine was next, and phosphatidylserine or phosphatidylinositol was inhibitory. The reconstituted activity showed a migration pattern of ficoll gradients that was distinct from that of the unreconstituted enzyme and similar to that of phospholipid–cholesterol liposomes. These methods provide a technique to assay the ACAT activity in defined lipid environment. The solubilized ACAT fraction was further purified by ammonium acetate fractionation and Sepharose 4B column chromatography. The entire purification procedure yielded a 150-fold increase in ACAT specific activity with 40% of the original activity recovered.

The coordinate control of cholesterol biosynthesis, cholesterol uptake via low density lipoprotein (LDL) receptor, and cholesterol esterification has been established to be the means by which many cell types determine their intracellular cholesterol content [for a review, see Goldstein & Brown (1977)]. Acyl-CoA:cholesterol acyltransferase (ACAT),[1] a microsomal enzyme, is believed to be responsible for the bulk of intracellular cholesterol esterifying activity. Due to its involvement in the above regulatory scheme and the awareness that the enzyme has an important role in the accumulation of cholesterol esters in atherogenesis (St. Clair, 1975; Hashimoto & Dayton, 1977), the elucidation of the mechanism of its regulation has gained considerable attention. Studies with cultured fibroblasts have shown that the enzyme's activity can be stimulated up to 100-fold when the cells are incubated with cholesterol, low density lipoprotein, or certain oxygenated cholesterol analogues (Goldstein et al., 1978). ACAT activation by cholesterol, LDL, or the oxygenated sterol analogues can take place in the presence or absence of cyclohexamide, suggesting that protein synthesis is not involved (Drevon et al., 1980; Goldstein & Brown, 1977). There have also been reported several inhibitory steroids that affect ACAT activity in vitro as well as in vivo (Goldstein et al., 1978; Flint et al., 1973). Brenneman et al. (1977) and Mitropoulos et al. (1980) have reported the regulation of ACAT activity due to changes in saturation of dietary fat; Arbogast et al. (1976) have studied the effect of different serum lipoproteins on ACAT activity. All of these studies suggest that the regulation of ACAT activity is an important cellular function. It is difficult to determine the molecular mechanism(s) of ACAT activation or inhibition by using crude cell extracts as the enzyme source, since the enzyme remains associated with the lipids which are potential modulators of the enzyme. This association of enzyme and lipids prohibits one from determining whether the activation involves a covalent modification to the enzyme molecule, some specific allosteric effect, or a simple increase in substrate availability to the enzyme. So that this obstacle can be overcome, the enzyme should be assayed in a state in which its activity is independent of that of endogenous lipids as well as other possible protein activators. In this paper we report the solubilization, partial purification, and reconstruction in liposomes of pig liver ACAT. These results should allow for further purification of the enzyme and the determination of enzyme activity independent of its native lipid environment.

Experimental Procedures

Materials

All radioactive chemicals were purchased from New England Nuclear. [^{3}H]Oleoyl-coenzyme A was synthesized and purified in this laboratory according to Al-Arif & Blecher (1969). Nanograde chloroform and methanol were from Mallincrodt. All other organic solvents, potassium chloride, and Tris were from Fisher. Fraction V fatty acid free bovine serum albumin was from Miles. Bovine phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine were from Supelco; bovine phosphatidylinositol was from Applied Science. The purities of these lipids were found to be at least 98% (single spot on thin-layer chromatography, detected by charring with 50% sulfuric acid–dichromate spray). Sometimes, phospholipids of egg origin from Sigma Chemical Co. were used and gave essentially identical results. Asolectin was from Associated Concentrates. All other reagents were from Sigma. Unisil was from Clarkson Chemical Co., Inc. Preformed silica gel G thin-layer plates were from Analtech; OV-17 gas–liquid chromatography column was from Supelco. Pig liver was obtained fresh from a local slaughterhouse.

Methods

ACAT Assay. The ACAT assay is a modification of that of Goodman et al. (1963). The optimum conditions for activity

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[1] Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; BSA, bovine serum albumin; GLC, gas–liquid chromatography; CoA, coenzyme A.

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were obtained by using 0.5 mg of fatty acid free BSA, between 3 and 40 μg of protein sample, 10 nmol of [9,10(N)-$^3H_2$]-oleoyl-coenzyme A (2.3 μCi/μmol) in a final volume of 0.2 mL in 50 mM Tris–1 mM EDTA at pH 7.7. We found the presence of sulfhydryl protecting agents (mercaptoethanol and dithiothreitol) unnecessary. The reaction was started with the addition of the [^{3}H]oleoyl-coenzyme A and incubated for 30 min at 30 °C. Activity was linear with time and protein in this range. The reaction was stopped by the addition of 3 mL of chloroform–methanol (2:1) with the subsequent addition of 1 mL of water by using the extraction procedure of Folch et al. (1957). The chloroform layer was then evaporated under nitrogen and then resuspended in 50 μL of ethyl acetate containing 25 μg of cholesterol oleate and applied to a thin-layer chromatography plate (silica gel G). The plates were then developed in petroleum ether–anhydrous ether (9:1). The cholesterol ester spot was localized by iodine staining and removed to a scintillation vial for counting. To assay the enzyme after solubilization, it was necessary to dilute the enzyme–deoxycholate solution to a deoxycholate concentration below 0.5 mg/mL in buffer A for 4 h at 4 °C before assaying. Control experiments have indicated that the microsomal ACAT activity is near maximal if only endogenous cholesterol is used as the substrate, since addition of exogenous cholesterol in Tween-80 (Gaylor, 1964) results in only a 50% increase in activity, consistent with similar measurements by others (Goldstein et al., 1978; Drevon et al., 1980).

Enzyme Solubilization Procedure. Pig liver microsomes were prepared at 4 °C from livers obtained on the day of slaughter. The livers were cut into 1-in. cubes and homogenized in a Waring blender at 500 g of liver in 1 L of Tris–1 mM EDTA, pH 7.7 (buffer A). The homogenate was then centrifuged for 30 min at 10000*g*, and the supernatant was then centrifuged at 30000 rpm for 60 min in a Beckman rotor, 30. The microsomal pellet formed was resuspended at 75 mg/mL protein in buffer A and was then stored at −70 °C before use. The ACAT activity is stable in these microsomes for over 8 months. Prior to extraction of the ACAT activity from these microsomes, the membranes were given a low detergent extraction to remove other proteins. This was done by making the microsomes 36 mg/mL in protein and 1 M potassium chloride and 5 mg/mL in deoxycholate (the latter is added last while slowly stirring) in buffer A. The mixture was then centrifuged for 60 min at 48000 rpm at 4 °C in a Beckman 50 Ti rotor (all subsequent centrifugations were performed identically to this one unless otherwise indicated). The supernatant was then discarded, and the loose pellet was washed once in an equal volume of buffer A by resuspending and recentrifuging. The loose pellet was then made 30 mg/mL in protein and 1 M in potassium chloride again, and deoxycholate was added to a concentration of 11 mg/mL. This solution is then centrifuged again, and the supernatant is diluted 1 in 2 in 10 mg/mL sodium cholate in buffer A (this supernatant will be referred to as solubilized enzyme). This preparation contains 6.0 mg/mL protein, 5.0 mg/mL cholate, 5.5 mg/mL deoxycholate, and 0.5 M potassium chloride in buffer A; the ACAT activity in this preparation remains soluble and stable for at least 18 h at 4 °C.

Lipid–Cholate Mixture. Phospholipid–cholesterol–cholate mixtures were prepared for reconstitution by first mixing the phosphatidylcholine and cholesterol at a ratio of 5 to 1 (by weight) in hexane. Hexane was then evaporated under nitrogen, and enough 20 mg/mL sodium cholate in buffer A was added to give a final concentration of 10 mg/mL phosphatidylcholine and 2 mg/mL cholesterol. This solution (usually 2 mL) was then flushed with argon, sealed, and sonicated in a Bausch & Lomb Balsonic bath sonicator until clear (approximately 5 min). Tetradecane was then added at a final concentration of 0.075 g/mL, and the mixture was sonicated for approximately 5 min under argon until a white emulsion was formed. The mixture was then centrifuged in a SS34 rotor at 12000 rpm at 4 °C in a Sorvall RC-2B centrifuge to form a clear solution with a cloudy white layer on the surface. The clear solution is used for reconstitution and is referred to as the phosphatidylcholine–cholesterol–cholate mixture. Control experiments have indicated that there is no selective loss of either phospholipid, cholesterol, or cholate in the final mixture as determined by phospholipid assay (Kagawa & Racker, 1966) and isotopically labeled cholesterol and cholate, respectively. Preformed vesicles are made by dialyzing this mixture for the indicated amount of time, depending on the experiment, against a 100-fold excess of buffer A at 4 °C.

Reconstitution Procedure. The reconstitution procedure is a modification of the cholate dialysis procedure (Kagawa & Racker, 1971). Solubilized enzyme is diluted 1 to 10 in the above phosphatidylcholine–cholesterol–cholate mixture in a total volume of 0.4 mL and placed in a small dialysis bag with Fisher 667B dialysis tubing and Spectrum Medical Industries dialysis tubing closures. The mixture is then dialyzed against a 100-fold excess of buffer A at 4 °C for 8 h. The dialyzed enzyme is then diluted 1 in 5 into the ACAT assay mixture to determine ACAT activity.

Ficoll Gradients. Linear gradients were formed in Beckman 5-mL cellulose tubes by using a Hoeffer SG102 gradient maker with a 0.3-mL 50% sucrose in buffer A cushion, 2 mL of 10% ficoll in buffer A as the heavy phase, and 2 mL of buffer A for the light phase. Samples were either loaded on top of the gradients or diluted 1.5-fold with 50% sucrose in buffer A and loaded on the botton of the gradients at 4 °C. The gradients were centrifuged for 6 h in a Beckman SW56 or a SW60 rotor at 50000 rpm at 4 °C. Fifteen 0.3-mL fractions were collected from each tube and assayed for ACAT activity and phospholipid content.

ACAT Purification by Ammonium Acetate Fractionation and Sepharose 4B Column Chromatography. Solubilized enzyme was fractionated at 4 °C by ammonium acetate precipitation. A solution of ammonium acetate saturated at 4 °C in buffer A was made up and stored at 4 °C. By use of approximately 20 mL of solubilized enzyme, saturated ammonium acetate was added slowly while stirring up to a concentration of 18% saturation. This produced a brownish white precipitate; this suspension was stirred for 3 min and centrifuged at 9000 rpm in an SS 34 rotor of a RC-2 Sorvall centrifuge. The clear red supernatant was removed by pouring. More saturated ammonium acetate was slowly added to the supernatant to bring the final concentration to 27% saturation and to produce a red precipitate. This suspension was again stirred for 3 min and centrifuged as above. The pellet in this case was much softer than the above, and the supernatant must be carefully removed by using a pipet. This pellet was then resuspended in 50 mM triethanolamine–1 mM EDTA, pH 7.7 (buffer B), at a volume $^1/_8$ that of the starting solubilized enzyme to form a clear red-orange solution, generally near 10 mg/mL protein. This solution was then layered on top of a Sepharose 4B column. Usually 0.5 mL of sample was run on a 1.5 × 33 cm column preequilibrated in 0.2% deoxycholate in buffer A or B. Buffer B is preferred because DOC tends to precipitate after 24 h in buffer A at 4 °C and does not precipitate even over long periods of time in buffer B. A column of these dimensions was run at 20–25 mL/h, and

Table I: Solubilization and Partial Purification of ACAT

enzyme preparation	total protein (mg)	sp act.[a] (nmol min^{-1} mg^{-1})	recovery[b] of act. (%)	x-fold[b] purifcn
(1) microsome	1667	0.09	100	1
(2) pellet after 5 mg/mL DOC extraction	300	0.50	100	5.5
(3) supernatant after 11 mg/mL DOC extraction	130	1.01	86	11
(4) solubilized enzyme	130	1.00	84	11
(5) ammonium acetate fractionation	23	3.7	55	37
(6) Sepharose 4B column chromatography	4.1	15.0	40	150

[a] For enzyme preparations 1-4, the activity was assayed after dilution; for enzyme preparations 5-6, the activity was assayed after reconstitution; both procedures were described under Methods. [b] Recovery of activity is calculated by using microsomal activity as 100%, and purification is based on the increase in specific activity.

fractions of 2–4 mL were collected. The ACAT fractions would come out between 55 and 65 mL of eluant, just ahead of the major protein peak and red color.

Once the enzyme was fractionated by Sepharose chromatography, it became necessary to assay all fractions by the reconstitution procedure described above to recover activity.

Other Analytical Determinations. Protein was determined according to Lowry et al. (1951); phospholipid was determined according to Kagawa & Racker (1966). Tetradecane content of the reconstitution mixture was determined by extracting 200 μL of the dialyzed reconstitution mixture according to Folch et al. (1957) with nanograde solvents. The tetradecane was separated from the phospholipid on a small Unisil column and then quantitated by GLC with an OV-17 column at 100 °C by using dodecane as a standard.

Results

Solubilization and Partial Purification Using Sequential Detergent Extraction. In preliminary experiments, attempting to extract ACAT from the pig liver microsomes with numerous nonionic or ionic detergents or with high concentrations of salt alone, we found that very little active enzyme could be extracted. We therefore utilized the combination of high salt and deoxycholate (DOC). This combination has been used by MacLennan (1970) to potentiate the effect of the detergent, yielding excellent solubilization and recovery of activity of the adenosinetriphosphatase from sarcoplasmic reticulum. As seen in Figure 1, at 10 mg/mL deoxycholate and 1 M potassium chloride, all of the ACAT activity and 82% of the protein are found in the 100000*g* supernatant fraction. Above 15 mg/mL deoxycholate the supernatant activity begins to decrease, which may be due to partial enzyme denaturation by detergent. The fact that very little ACAT activity is extracted at 5.0 mg/mL deoxycholate while the bulk of the microsomal protein is removed (Figure 1) suggested the use of a two-step extraction procedure to solubilize ACAT activity. As can be seen in Table I, the sequential extraction procedure yields a 5.5-fold increase in specific activity after the first extraction (using 5 mg/mL DOC), and an additional 2.0-fold increase after the second extraction (using 11 mg/mL DOC) with 85% of the original ACAT activity solubilized. After this second extraction, the enzyme is diluted by 2 volumes of 1.0% cholate which we found to be necessary to maintain ACAT in solution if the salt is removed by dialysis.

Further Purification of Solubilized ACAT. The supernatant from the second DOC extraction (Table I) is fractionated by using ammonium acetate precipitation. The first fraction (0–18% cut) contains approximately 7% of the total ACAT activity and 20% of total protein and is usually discarded. The second fraction (18–27% cut) contains approximately 70% of the total ACAT activity; this fraction coprecipitates enough detergent (cholate and deoxycholate) such that it readily resolubilizes and contains approximately 20 mg/mL detergent

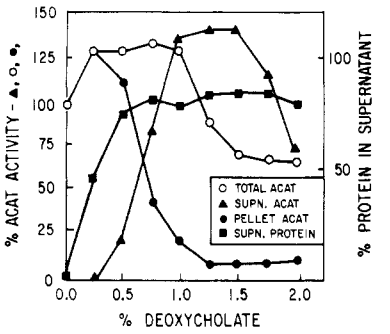

FIGURE 1: Effect of deoxycholate concentration on enzyme extraction. Microsomal protein and potassium chloride were mixed so as to yield a final concentration of 36 mg/mL and 1 M, respectively, in 50 mM Tris–1 mM EDTA, pH 7.7 (buffer A). Sodium deoxycholate (by using a 10% stock solution in H_2O) was slowly added while stirring to give the concentration indicated. An aliquot of this mixture was then diluted and assayed for ACAT activity to determine total activity. The mixture was then centrifuged at 48 000 rpm for 1 h at 4 °C in a Beckman 50 Ti rotor. The supernatant and the pellet were then diluted 1/50 from the original volume and assayed for ACAT activity, and the supernatant was assayed for protein. ACAT activities were determined as described under Methods. Each point represents duplicate assays, none of which vary more than 6% from the mean. Symbols: total activity (○); supernatant activity (▲); pellet activity (●); supernatant protein (■).

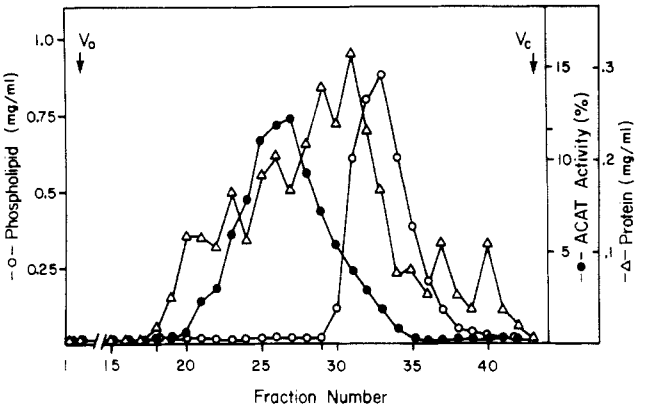

FIGURE 2: Chromatography of solubilized ACAT on the Sepharose 4B column. 0.5 mL of the ammonium acetate fraction (18–27% cut) (see Methods) was loaded on top of a 1.5 × 33 cm column of Sepharose 4B preequilibrium with 0.2% NaDOC in buffer A. The column flow rate was 25 mL/h and 2 mL-fractions were collected. Each fraction was assayed for ACAT activity after reconstitution as described under Methods. Fractions were also assayed for protein and phospholipid content according to Methods. The void volume (V_0) and column volume (V_c), indicated by arrows, were determined by 850-Å latex particles and β-mercaptoethanol content, respectively. Symbols: (●) ACAT activity; (△) protein; (○) phospholipid.

and 10 mg/mL protein when resuspended at a volume $^1/_8$ that of the starting material. The remaining supernatant contains nearly half of the original protein and no ACAT activity. The ACAT specific activity of the 18–27% ammonium acetate

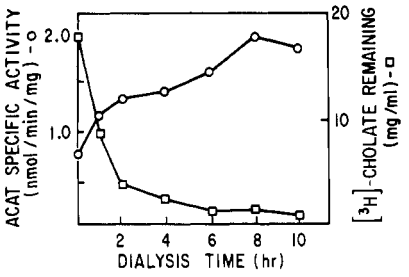

FIGURE 3: Effect of dialysis on ACAT activity and cholate concentration. Solubilized enzyme was diluted 1/10 into the phosphatidylcholine–cholesterol–cholate mixture (see Methods). In this case the sodium cholate was isotopically labeled [2,4(N)-^{3}H]cholate (25000 dpm/mg). This mixture was then dialyzed at 4 °C against a 100-fold excess of buffer A for the indicated amount of time. At each time point, an aliquot was diluted 1/5 and assayed for ACAT (O), and an aliquot was counted in a scintillation counter to determine the amount of [^{3}H]cholate (□).

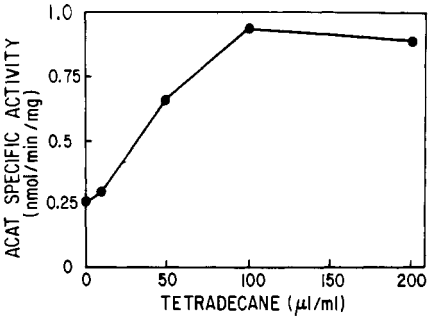

FIGURE 4: Effect of tetradecane on reconstitution. Phosphatidylcholine–cholesterol–cholate solution was prepared as indicated under Methods with the exception that the amount of tetradecane was varied by using 0, 1, 10, 50, and 100 μL of tetradecane/mL instead of the standard 100 μL/mL as described under Methods. Solubilized enzyme was diluted 1/10 into the above mixture of varying tetradecane content and then reconstituted and assayed for ACAT activities as described under Methods.

fraction is found to be between 3 and 4 nmol min^{-1} (mg of protein)$^{-1}$. This material is then further purified by Sepharose 4B chromatography in the presence of 0.2% sodium deoxycholate as described under Methods. Figure 2 shows the chromatogram. After chromatography by the Sepharose 4B column, we find that it is necessary to reconstitute the ACAT-containing fractions in lipid vesicles (see below) before any ACAT activity is recovered. This can be rationalized by the finding that the enzyme activity is separated from the bulk of the phospholipid fraction during column fractionation (Figure 2). We also find that this procedure efficiently separates the bulk of cholesterol from the ACAT-containing fractions. After Sepharose chromatography, the pooled fraction enriched in ACAT activity contains only approximately 7% of the phospholipid and cholesterol originally associated with the microsomal proteins. [A total of 0.81 mg of phospholipid and 0.028 mg of cholesterol per mg of protein were found in the microsome; these values remained constant in the solubilized enzyme preparation. In the pooled Sepharose fraction enriched in ACAT activity, these values were found to be drastically reduced to 0.067 and 0.002, respectively (Doolittle, 1981).] Table I summarizes the entire purification procedure which routinely provides a 150–190-fold increase in ACAT specific activity, with 40% of the original activity recovered.

Reconstitution of Solubilized ACAT in Phospholipid Vesicles. As a means of reconstitution, we applied the cholate dialysis procedure (Kagawa & Racker, 1971), modified as described under Methods. Preliminary experiments have indicated that it was necessary to add cholesterol to the reconstitution mixture of phospholipid and detergent to act as the substrate for the enzyme reaction. The routine reconstitution procedure consists of diluting the solubilized enzyme (6.5 mg/mL protein) 1 to 10 into a solution of 10 mg/mL phosphatidylcholine, 2 mg/mL cholesterol, and 20 mg/mL sodium cholate, followed by dialysis at 4 °C. As seen in Figure 3, the ACAT activity of this mixture increases with the time of dialysis for up to 10 h; this increase in activity correlates with the time course of cholate removal from the mixture.

Our original source of phosphatidylcholine was pepared in tetradecane solution, and later we found that the tetradecane had some stimulatory effect on ACAT activity and also made the amount of activity reconstituted highly reproducible. Figure 4 shows the increase of activity recovered with increasing amounts of tetradecane included in the reconstitution mixture. When the amount of tetradecane was quantitated by GLC, it was found to be 0.05 mg/mg of phospholipid of the reconstituted vesicle. In three separate experiments, we

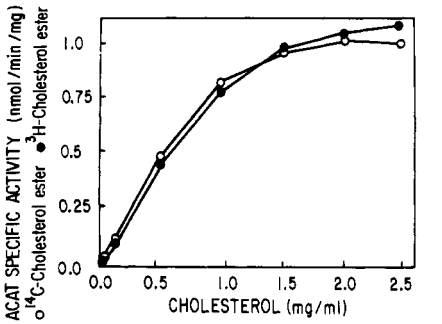

FIGURE 5: Effect of cholesterol content on reconstituted ACAT activity. Solubilized enzyme (initial specific activity of 1.02 nmol min^{-1} mg^{-1}) was diluted 1/10 into phosphatidylcholine (10 mg/mL), sodium cholate (20 mg/mL), and the indicated amount of [4-^{14}C]-cholesterol (50000 dpm/nmol); the mixture was otherwise prepared identically to the reconstitution mixture described under Methods. The mixtures were then reconstituted and diluted 1/5 for measurement of ACAT activity of (●) [^{3}H]cholesterol ester formed or (O) [^{14}C]cholesterol ester formed. Each point represents the average of duplicate assays, none of which vary more than 5% from the mean.

found that the specific activity of the solubilized enzyme preparation as determined directly by dilution or by the optimized reconstitution procedure described above differs by less than 8%, indicating that the recovery of ACAT activity after reconstitution is nearly 100% (Doolittle, 1981).

Effect of Substrate Concentration on Reconstituted ACAT Activity. We next reconstituted the enzyme in varying amounts of [^{14}C]cholesterol with a constant amount of phospholipid and then measured ACAT activity. The specific activity was determined by counting the amount of [^{14}C]-cholesterol ester formed from [^{14}C]cholesterol and the amount of [^{3}H]cholesterol ester formed from [^{3}H]oleoyl-CoA. As seen in Figure 5, these two activities agree very closely and show the same saturation point as well as the complete lack of activity when cholesterol is absent from the reconstitution mixture. These results strongly suggest that the endogenous lipids have been replaced by the exogenously added lipids. Figure 6 shows a separate experiment where the oleoyl-CoA substrate saturation curve was determined by the cholesterol ester formed using the two isotopically labeled substrates. The two activities are also identical, again indicating that the exogenously added lipids have completely replaced the endogenous lipids in the reconstituted enzyme. We have also found that the optimum oleoyl-CoA saturation curve is obtained if the oleoyl-CoA and fatty acid free BSA are kept at a constant ratio (see methods), confirming the results of a recent paper

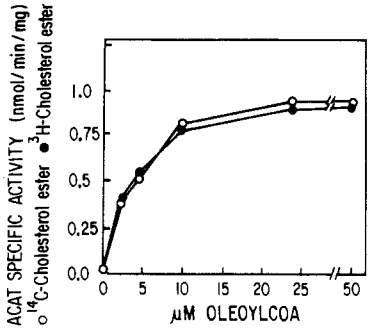

FIGURE 6: Effect of oleoyl-CoA concentration of reconstituted enzyme activity. Solubilized enzyme (initial specific activity of 0.96 nmol min^{-1} mg^{-1}) was diluted 1/10 into the reconstitution mixture containing [4-^{14}C]cholesterol at 5000 dpm/nmol and reconstituted according to Methods. The reconstituted enzyme was then diluted 1/5 and assayed for ACAT activity with the indicated amount of [9,10 (N)^{3}H]oleoyl-CoA. The fatty acid free BSA added to the enzyme assay and oleoyl-CoA were kept at constant ratio of 1 mg of BSA to 20 nmol of oleoyl-CoA. (○) ACAT activity using [^{14}C]cholesterol ester; (●) ACAT activity using [^{3}H]cholesterol ester. Each point represents the average of duplicate determinations, none of which vary more than 10% from the mean.

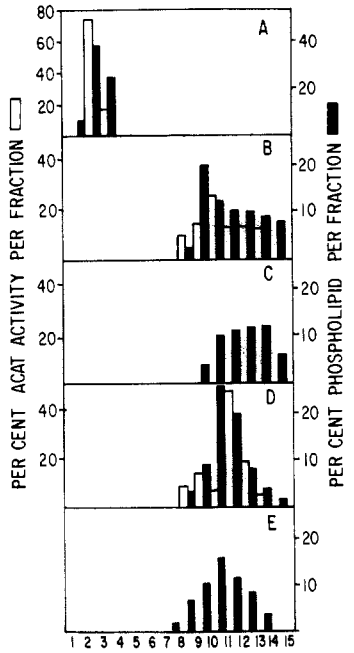

FIGURE 7: Migration of enzyme on ficoll gradients. The sample was loaded on a 0–10% ficoll gradient (see Methods) and centrifuged for 8 h at 50 000 rpm in a Beckman SW56 or a SW60 rotor. Fifteen fractions of 0.3 mL were collected and assayed for ACAT activity and phospholipid content. (A) 0.2 mL of solubilized enzyme was dialyzed identically to reconstituted enzyme except without the lipid–cholate reconstitution mixture and layered on top of the ficoll gradient. (B) 0.2 mL of reconstituted enzyme was layered on top of a ficoll gradient. (C) 0.2 mL of preformed vesicles (see Methods) was layered on top of a gradient. (D) 0.120 mL of reconstituted enzyme was mixed with 0.08 mL of 50% sucrose in buffer A and then layered on the bottom of the ficoll gradient. (E) 0.12 mL of preformed vesicles was mixed with 0.08 mL of 50% sucrose in buffer A and then layered on the bottom of a gradient. Solid bars represent the percent of phospholipid recovered in each fraction and open bars represent the percent of ACAT activity in each fraction by using the amount in the sample before centrifugation as 100% in each case.

(Lichtenstein & Brecher, 1980).

Characterization of Reconstituted ACAT Activity on Density Gradients. Although the above data strongly suggest that the enzyme has now been placed in a determined lipid environment, we have further characterized the reconstituted activity on ficoll gradients (Goldin & Rhoden, 1978). Using these gradients, one can separate the nonreconstituted enzyme from the reconstituted enzyme on the basis of buoyant density. In our case one would expect the reconstituted enzyme to greatly decrease in density due to the increased proportion of associated lipids. In Figure 7, panel A shows the migration of nonreconstituted enzyme after it has been dialyzed in a manner identical with that of the reconstituted enzyme. Due to its high protein to lipid ratio (1:1 by weight), the enzyme migrates to the bottom of the gradient. After reconstitution, the protein to lipid ratio becomes very low (1:16), and as can be seen in panel B, the migration pattern of the reconstituted enzyme is distinct from that of the nonreconstituted enzyme, is identical with the bulk of the phospholipid, and is very similar to the migration pattern of pure phospholipid–cholesterol vesicles (panel C). If the reconstituted enzyme is loaded at the bottom of the gradient (panel D), its migration pattern is very similar to that of the top loaded aboved reconstituted enzyme (panel B), again parallels the bulk of the phospholipid, and is identical with the migration pattern of phospholipid–cholesterol vesicles loaded the same way (panel E). These experiments indicate that the reconstituted ACAT activity has been incorporated into the phospholipid vesicles. Similar experiments using a more purified ACAT fraction (with specific activity of 20 nmol min^{-1} mg^{-1}) as the enzyme source has yielded identical results (data not shown).

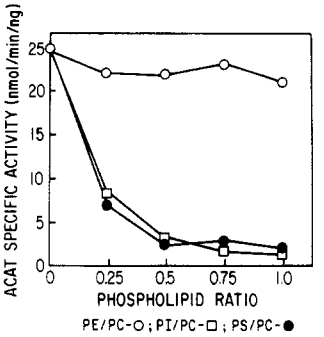

FIGURE 8: Effect of phospholipid headgroup on reconstituted, partially purified ACAT activity. The pooled ACAT fraction of a Sepharose 4B column was used as the source of ACAT activity for reconstitution (see Methods). Phospholipids indicated in the graph were used to replace the appropriate amount of phosphatidylcholine in each case. The following stock solutions were used to make the appropriate lipid–cholate reconstitution mixture: (1) 10 mg/mL phosphatidylcholine (PC) with 3.0 mg/mL cholesterol and 20 mg/mL cholate; (2) 10 mg/mL phosphatidylinositol (PI) in 20 mg/mL cholate; (3) 10 mg/mL phosphatidylserine (PS) in 20 mg/mL cholate; (4) 10 mg/mL phosphatidylethanolamine (PE) in 20 mg/mL cholate; (5) 10 mg/mL phosphatidylcholine in 20 mg/mL cholate. These stocks were then mixed to give the appropriate ratio of phospholipid and used as described under Methods for reconstitution at the following final concentration: 10 mg/mL phospholipid, 1.5 mg/mL cholesterol, and 20 mg/mL cholate. Symbols: (○) phosphatidylethanolamine/phosphatidylcholine; (□) phosphatidylinositol/phosphatidylcholine; (●) phosphatidylserine/phosphatidylcholine. In each case, tetradecane was included in the reconstitution mixture. All points represent the mean of duplicate assays, none of which varies more than 6% from the mean.

Effects of Phospholipid Headgroup Variation on the Partially Purified Reconstituted ACAT. ACAT fractions after the Sepharose column chromatography were reconstituted in phospholipids of varying headgroups. As seen in Figure 8, the phospholipids with a net negative charge, phosphatidylserine and phosphatidylinositol are inhibitory, and the net neutral charged headgroup, phosphatidylethanolamine, has a weak stimulatory effect on activity. Identical results are found with the less purified ACAT fractions (Doolittle, 1981) and suggest that during reconstitution, net negatively charged phospholipids somehow inhibit the enzyme activity.

Discussion

The ACAT enzyme was solubilized by using a combination of deoxycholate and 1 M potassium chloride. The high concentration of deoxycholate required for enzyme extraction and the lack of ability of high salt alone to extract the enzyme both suggest that ACAT is an integral membrane protein. We have taken advantage of this fact and have employed a sequential extraction, first low detergent with salt and then higher detergent with salt, and have obtained nearly 100% solubilization and 10-fold purification. The solubilized enzyme retains 100% activity after 18 h at 4 °C and loses activity only slowly afterward. Starting with the solubilized enzyme prepared by this procedure, we have achieved further purification using ammonium acetate fractionation and Sepharose 4B column chromatography. The ACAT-containing fraction after Sepharose column chromatography becomes extensively delipidated and requires lipid reconstitution for activity measurements.

To reconstitute the enzyme in liposomes of known cholesterol and phospholipid content, we have used the cholate dialysis procedure (Kagawa & Racker, 1971). This procedure allows easy manipulation of the phospholipid and cholesterol content of the reconstituted enzyme as shown in Figures 5 and 7. When assayed at optimal conditions, the activity of the reconstituted enzyme is near 100% as indicated in Table I. We have found that the addition of the hydrocarbon tetradecane in the reconstitution mixture has yielded higher activities as well as more reproducible results. We found that tetradecane was also needed for reconstitution when the more extensively purified ACAT fraction (after Sepharose chromatography) was used as the enzyme source. At present we do not know if the effect of the tetradecane is on the catalytic activity of ACAT or if it is in some way needed for the reconstitution procedure itself. Further experiments are needed to resolve this question.

Figure 5 shows the need for cholesterol to be present in the reconstitution mixture. The loss of activity when no cholesterol is added to the reconstitution mixture indicates that the added lipids are displacing the endogenous lipids; i.e., the added phospholipid is diluting the cholesterol that was originally present in the solubilized enzyme to the point where no activity is detectable in the assay. The agreement between the activities measured with the two different substrates also suggests that the enzyme is incorporated into the added lipids. This interpretation is reinforced by the results presented in Figure 6 showing that the amount of cholesterol ester formed from the two different substrates again coincides when the oleoyl-CoA concentration is varied. Linear density gradients of ficoll were used to characterize the physical state of the enzyme, before and after reconstitution. The results obtained (Figure 7) indicate that the reconstitution procedure had actually caused the solubilized enzyme to become incorporated in the phospholipid–cholesterol vesicles.

As has been found with other membrane-bound enzymes (Agnew & Popjak, 1978), ACAT activity is affected by the structure of the phospholipid headgroup used for reconstitution. The data in Figure 8 suggest that the enzyme is most active in phosphatidylethanolamine and phosphatidylcholine and is severely inhibited by phosphatidylserine. These differences may be due to the various net charges possessed by the headgroups among the different phospholipids present in the reconstituted vesicle; however, this can only be regarded as a tentative interpretation, since our current data cannot rule out the possibility that changing the lipids used for reconstitution actually inhibits the incorporation of the enzyme into liposomes. This observation will be further explored in the future.

In conclusion, we have solubilized and partially purified the enzyme in a form where it is stable and amenable to further purification and developed a procedure to reconstitute ACAT in a defined lipid environment that is not affected by the native lipid content of the enzyme. We will use the methods reported here as standard enzyme assays to purify further the enzyme and to study its properties.

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