papers and notes on methodology

A simple method for reconstitution of CHO cell and human fibroblast acyl coenzyme A:cholesterol acyltransferase activity into liposomes

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Abstract A new method for reconstituting acyl coenzyme A: cholesterol acyltransferase (ACAT) activity from either Chinese hamster ovary (CHO) or human fibroblast cell extracts into cholesterol-phosphatidylcholine liposomes is described. The method is rapid (< 60 min) and easy to perform. The procedure involves solubilizing the cell extracts with deoxycholate followed by dilution into preformed liposomes. Ficoll gradient analysis demonstrated that, after reconstitution, almost all of the detectable ACAT activity co-migrated with the liposomes. Exogenous cholesterol in the liposomes was absolutely necessary for providing ACAT activity, but not for incorporation of the ACAT enzyme into the vesicle bilayer. Human fibroblast cell extracts prepared from cells grown in medium containing 10% fetal calf serum were found to contain a 10-fold higher microsomal ACAT activity compared to extracts from cells grown in 10% delipidated fetal calf serum. In contrast, when the ACAT activity from these extracts was measured using the reconstitution assay, there was no difference in the specific activities. These results support our previous work (Doolittle, G. M., and T. Y. Chang. 1982. *Biochim. Biophys. Acta.* **713:** 529–537; and Chang, C. C. Y., et al. 1986. *Biochemistry.* **25:** 1693–1699), and suggest that cholesterol regulates ACAT activity in CHO cells and human fibroblasts by mechanism(s) other than modulation of the amount of enzyme. —**Cadigan, K. M., and T. Y. Chang.** A simple method for reconstitution of CHO cell and human fibroblast acyl coenzyme A:cholesterol acyltransferase activity into liposomes. *J. Lipid Res.* 1988. **29:** 1683–1692.

Supplementary key words deoxycholate • cholate dilution • membrane protein reconstitution

Acyl coenzyme A:cholesterol acyltransferase (ACAT) is an integral membrane protein that catalyzes the conversion of intracellular cholesterol and fatty acyl coenzyme A to cholesteryl ester (for reviews see ref. 1 and 2). The enzyme can be assayed indirectly by incubating tissue culture cells with radiolabeled oleic acid and measuring the rate of incorporation of radiolabel into cellular cholesteryl ester (3). Alternatively, ACAT activity in microsomal membranes can be determined using the assay system originally developed by Goodman, Deykin, and Shiratori (4), in which radioactive acyl-coenzyme A is used, and the conversion of radiolabel to cholesteryl ester is measured. In both assays, the concentration of the cholesterol substrate utilized by ACAT is an unknown variable. The above-mentioned assays can be viewed as a combined measurement of the amount of cholesterol available to the enzyme as well as the amount and absolute activity of ACAT. In addition, phospholipids of various headgroups or fatty acid compositions have been shown to have profound effects on ACAT activities in reconstituted liposomes (5–8) and microsomal membranes (9). The unknown status of microsomal phospholipid composition in the vicinity of the ACAT enzyme presents another difficulty in interpreting results obtained using the [^{3}H]oleate pulse and the microsomal ACAT assay.

Different methods that attempt to circumvent the problem of unknown lipid concentration have been developed. ACAT activity in microsomal membranes can be activated by incubation with a source of exogenous cholesterol, either a cholesterol-Triton WR-1339 dispersion (10) or cholesterol-phospholipid vesicles (7). Another approach involves solubilizing the enzyme activity with detergent and then reconstituting ACAT activity by removing the detergent and adding exogenous lipids. ACAT activity has been solubilized and reconstituted from Ehrlich ascites cell microsomes (5, 6) and rat liver microsomes (7) using Triton X-100 for the initial solubilization. Our laboratory has previously reported reconstitution of the pig liver and Chinese hamster ovary (CHO) cell ACAT enzyme using cholate or deoxycholate (DOC) followed by dialysis in the presence of exogenous lipids (8, 11). Recon-

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; CHO, Chinese hamster ovary; CMC, critical micelle concentration; De-S, delipidated fetal calf serum; DOC, deoxycholate; FCS, fetal calf serum; LDL, low density lipoprotein; PC, phosphatidylcholine; TLC, thin-layer chromatography.

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stitution offers the advantage of assaying the enzyme in a defined cholesterol and phospholipid environment. However, the above-mentioned reconstitution procedures were time-consuming since they involved either multiple ultracentrifugations and/or overnight dialysis.

We now report a simple and rapid reconstitution procedure that can be performed within 1 hr and avoids any dialysis or ultracentrifugation steps. It is a modification of the cholate-dilution method described by Racker, Chien, and Kandrach (12). The new method utilizes DOC to solubilize the enzyme and then the detergent concentration is reduced by dilution into a large excess of preformed vesicles of a defined cholesterol and phosphatidylcholine (PC) composition. The applicability of this method is demonstrated by reconstituting ACAT activity from human fibroblasts, wild type CHO cells, and a CHO cell line (25-RA) isolated in this laboratory (13) in which the low density lipoprotein (LDL) receptor and various cholesterogenic enzyme activities have been found to be resistant to suppression by sterol present in the growth medium (13, 14). Due to its simplicity, this method may be of general use for the reconstitution of membrane-bound proteins in general. This method has been previously described in brief form (14).

MATERIALS AND METHODS

Materials

PC (Type XI), cholesterol, and Ficoll (Type 400) were from Sigma. Sodium DOC was from Behring Diagnostics. Charcoal-purified cholic acid was a gift from Dr. Bernard Trumpower (Dartmouth Medical School); a 20% sodium cholate solution was made by titration to pH 7.5 with NaOH. Cholestyramine was from Bristol-Myers Co. (Evansville, IN). Unlabeled oleyl coenzyme A was synthesized as described by Goldman and Vagelos (15) and [^{3}H]oleyl coenzyme A was synthesized by another method (16). Quantitation of oleyl coenzyme A was made assuming an extinction coefficient at 260 nm of 15.4 $mM^{-1}cm^{-1}$ (17). Purity was judged as > 98% by TLC analysis in a solvent system of butanol–acetic acid–water 5:2:3 and by measuring the A_{232}/A_{260} ratio (18).

Cell culture

A primary culture of human fibroblasts was obtained from the foreskin of a healthy newborn. The tissue was dissociated using bacterial collagenase and trypsin as described by Dayer et al. (19) and the fibroblasts were used between the 7th and 15th passage. Both CHO cells and the fibroblasts were grown as monolayers in Costar flasks, fibroblasts in MEM (Gibco), supplemented with 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate, and CHO cells in F-12 medium (Sigma) plus antibiotics as previously described (14). Both media contained 10% fetal calf serum (FCS; from Sigma) or where indicated, 10% delipidated fetal calf serum (De-S). Delipidated serum was prepared according to a published procedure (20), as modified by Chin and Chang (21). Fibroblasts were passed using 0.05% trypsin–0.02% EDTA for cell detachment and a 0.002% trypsin solution was used for CHO cells. Stock cultures were stored at −70°C in medium containing 20% FCS plus 10% dimethyl sulfoxide for CHO cells or 5% dimethyl sulfoxide–5% glycerol for fibroblasts.

For the experiments described in this report, except where otherwise noted, both 25-RA and wild type CHO cell lines were plated at a density of 3×10^6 cells/150-cm^2 flask and grown for 66–70 hr, with a medium change at 48 hr and another medium change 2 hr prior to harvest. Human fibroblasts were plated at 7×10^5 cells/150-cm^2 and grown for 7 days with medium changes on the 4th and 6th day, and 2 hr prior to harvest. All three cell types were harvested by the hypotonic shock and scraping method (22) and then stored in a Tris-HCl (50 mM)–EDTA (1 mM) buffer (pH 7.7) at 4°C for immediate reconstitution, or frozen at −70°C for later use.

Originally, all cell extracts were frozen and stored for up to 3 weeks before thawing for reconstitution (14). These frozen preparations consistently produced high specific activity upon reconstitution. However, during the experiments reported in this study, a variable and inconsistent decrease in reconstituted ACAT activity was observed when frozen extracts were used, especially when they were stored for more than 1 week. The reason for this is not clear but it appears not to be inactivation of the ACAT enzyme, since unreconstituted microsomal ACAT activity was not decreased. Therefore, fresh cell extracts were used for all experiments in this study except for those reported in Fig. 5. When key aspects of this experiment were repeated using fresh cell extracts, the results were identical to those shown.

Reconstitution assay

The reconstitution procedure was as follows: All solutions were in a 50 mM Tris-HCl, 1 mM EDTA buffer (pH 7.7). A DOC/PC solution was added to cell homogenates to obtain a final concentration of 20 mg/ml DOC, 4 mg/ml PC, and approximately 2 mg/ml cell protein. The mixture was incubated at 4°C. Final ACAT specific activity did not differ when the incubation at 4°C was varied between 5 and 60 min. A 20-min incubation was used for all experiments in this study. Aliquots of solubilized cell extract (15 μl for CHO cells and 30 μl for fibroblasts unless stated otherwise) were diluted 16-fold (v/v) into cholesterol–PC liposomes (225 μl of preformed liposomes for the CHO cells and 450 μl for the human fibroblasts) prepared by the cholestyramine method (23). The vesicles were composed of approximately 1.5 mg/ml cholesterol and 10 mg/ml PC (cholesterol/PC molar ratio = 0.3). After an

incubation of the solubilized cell extract and preformed liposomes for 10–20 min at 4°C (incubations of up to 2 hr did not affect ACAT activity), either 60 μl (for CHO cell assay) or 120 μl (for fibroblast) of [^{3}H]oleyl coenzyme A (250 μM + 12.5 mg/ml fatty acid-free bovine serum albumin; 8 × 10^6cpm/ml) was added to start the ACAT assay. This final concentration of 50 μM oleyl coenzyme A was found to be at a saturating level for both the microsomal and reconstituted ACAT assays of all three cell types examined. After incubation for 10 min at 37°C, the reaction was stopped by the addition of $CHCl_3$-CH_3OH 2:1, and ACAT activity was determined as previously described (11, 24). Control experiments using [^{14}C]cholesteryl oleate as an internal standard showed a recovery of radiolabel after lipid extraction and TLC analysis of 70–77%, which was taken into account when calculating specific activity. Protein was determined by a modification (25) of the Lowry et al. (26) procedure. For the Ficoll gradient analysis, PC content was analyzed using the Bartlett method (27).

RESULTS AND DISCUSSION

Optimization of the ACAT reconstitution

The rationale of the reconstitution assay described in this paper is as follows. First, the ACAT enzyme is solubilized from the microsomal membrane by DOC. After solubilization, the enzyme preparation is diluted into a large excess of preformed cholesterol-PC liposomes. The DOC concentration is effectively reduced by dilution and by interaction with PC in the preformed vesicles. The solubilized ACAT enzyme then becomes associated with the cholesterol-PC liposomes, presumably by incorporation into the vesicle bilayer.

Fig. 1 shows the effect of increasing the DOC concentration during the solubilization step on the final reconstituted ACAT activity. A concentration of 15 to 30 mg/ml DOC was found to give maximal ACAT activity for all three cell types, 25-RA, wild type CHO cells, and human fibroblasts. It was found that the addition of a small amount of PC in the detergent mixture gave slightly higher ACAT activities (data not shown), perhaps by protecting the enzyme from inactivation during the solubilization. In all experiments reported in this paper, a concentration of 20 mg/ml DOC and 4 mg/ml PC was used.

The effect of increasing amounts of solubilized cell extract diluted into cholesterol-PC vesicles on the final ACAT activity was examined (**Fig. 2**). The data are plotted as ACAT activity versus either the amount of solubilized cell protein added to the preformed vesicles (Fig. 2A) or the final detergent/PC molar ratio present in the mixture (Fig. 2B). The apparent difference between the linearity with respect to protein of CHO cells and human fibroblasts is due to the doubling of the volumes of solubilized cell extracts and preformed liposomes used for the human fibroblast assay to increase its sensitivity (see Fig. 2, legend). When the results are replotted in Fig. 2B, the data suggest that the detergent/PC molar ratio is the critical parameter determining how much solubilized cell extract can be added to vesicles and still remain on the linear part of the curve. The curve appears to remain linear up to a detergent/PC molar ratio of 0.3. In other experiments not shown, it was found that as long as the final detergent/PC molar ratio in the reconstitution mixture was maintained at 0.3 or lower, there was no difference in the specific activities of reconstituted ACAT activity when solubilized cell extracts from the same cell type ranged from 1.0 to 2.5 mg protein/ml. A detergent/PC molar ratio of 0.3 corresponded to a 16-fold dilution (v/v) of the solubilized cell extract into cholesterol-PC vesicles and was used in all subsequent experiments. Under these conditions, ~85% of the detergent was DOC from the solubilized cell extract and the remainder was residual cholate from the preformed liposomes, which were made using the cholestyramine method (23). The residual cholate concentration was ~0.65 mM, a value approximately 10 times lower than its critical micelle concentration (CMC) (28). The final DOC concentration was 3.2 mM, a value similar or higher than some of the CMCs reported for DOC in various aqueous buffers (28, 29). However, the minimum DOC/phospholipid ratio required for membrane disruption has been found to be 0.6 (29). Therefore, it is probable that on the linear portion of the

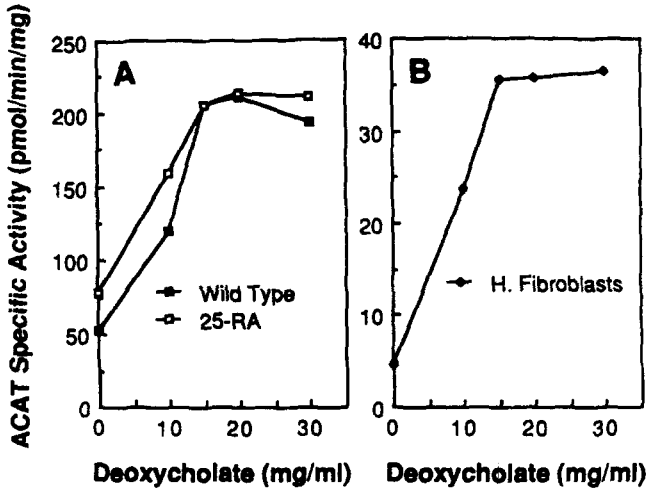

Fig. 1. Effect of increasing the DOC concentration used in the solubilization step on reconstituted ACAT activity of (A), wild type and 25-RA CHO cells and (B), human fibroblasts. Extracts were treated with the indicated amount of DOC plus 4 mg/ml PC except for the zero detergent samples, where PC was not included. Aliquots of the extracts (~20 μg protein) were then diluted 24-fold(v/v) into cholesterol-PC vesicles and assayed for ACAT activity as described in Materials and Methods. The values are the average of duplicate assays from the same detergent-treated extracts and ranged within 7% of the mean.

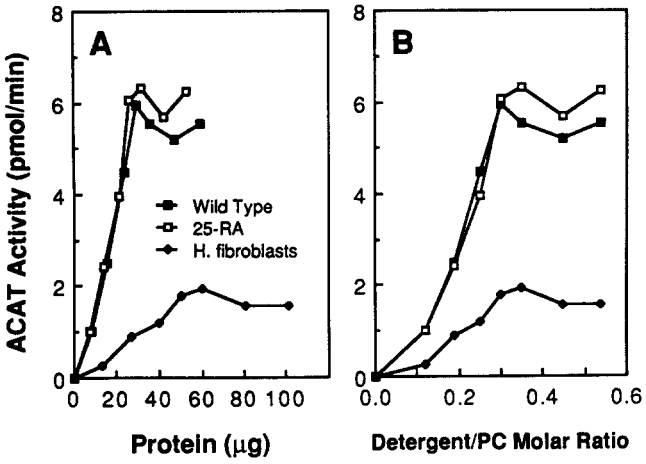

Fig. 2. Effect of increasing amounts of solubilized extract (4–30 μl for CHO cell extracts, 8–60 μl for fibroblast extracts) diluted into preformed cholesterol-PC liposomes (210 μl for CHO cell assay and 420 μl for fibroblasts) on reconstituted ACAT activity expressed as (A), the amount of cell protein present in the mixture and (B), the detergent/PC molar ratio of the mixture. The values are the average of duplicate ACAT assays and ranged within 7% of the mean.

DOC/PC curve, the vesicles are essentially intact, although the bilayer must be perturbed to a significant extent by the detergent. As seen in Fig. 1A, under optimal conditions the specific activity of CHO cell ACAT, either from wild type or 25-RA is approximately 200 pmol/min per mg cell protein. This is comparable to or slightly higher than the recent values reported from this laboratory (24) using the dialysis method for reconstituting ACAT activity. Thus, it appears that the detergent left after dilution does not interfere with ACAT activity.

Of the phospholipid in the reconstitution assay, 97% is from the cholesterol-PC vesicles, ~2.7% from the exogenous PC added during the solubilization, and ~0.3% from the cell membranes (assuming a concentration of 0.3 μmol phospholipid/mg cell protein) (14) indicating that endogenous phospholipid is diluted approximately 300-fold during the reconstitution.

Fig. 3 shows a time course of cholesteryl ester formed in the mock-reconstituted (cell extracts without DOC solubilization diluted into cholesterol-PC vesicles) and reconstituted ACAT assays of cell extracts from wild type CHO cells (Fig. 3A) and human fibroblasts (Fig. 3B). Results similar to those of wild type were obtained with 25-RA (data not shown). The reconstitution assay was linear for at least 20 min for both cell types. All the experiments in this paper use an assay time of 10 min, although 20 min or longer may prove to be beneficial for increasing the sensitivity of the assay.

The primary culture of human fibroblasts has a low ACAT activity compared to CHO cells (Figs. 1–3). For the microsomal ACAT assay or the mock-reconstitution assay, the average specific activities are 8.9 and 7.7 pmol/min per mg protein. These activities are similar to those obtained using an [^{3}H]oleate pulse to assay the enzyme in intact cells (data not shown). The average specific activity of reconstituted fibroblast ACAT activity is 45.9 pmol/min per mg protein. This five-fold activation of reconstituted over microsomal ACAT specific activity is similar to the fourfold activation of the wild type CHO enzyme activity seen in Fig. 1A. These results are consistent with the observation that ACAT is not saturated with its substrate cholesterol in the microsomal membranes (reviewed in refs. 1 and 2). However, we cannot rule out the possibility that reconstitution produces a conformational change in the ACAT enzyme that results in higher activity without an increased supply of cholesterol. It is clear from the data in Figs. 1 and 3 that the reconstituted ACAT activity is highly elevated compared to the mock-reconstituted ACAT activity, suggesting that solubilization of the membrane-bound enzyme with detergent is necessary for insertion of ACAT into the vesicle bilayer to occur upon dilution.

Table 1 shows the solubilization of ACAT activity from cell extracts after treatment with either a DOC/PC solution or a buffer control. All of the recovered ACAT activity in the buffer-treated extracts was found in the pellet after ultracentrifugation, confirming earlier work from this laboratory (11). In the DOC/PC-treated cell extracts, a shift in ACAT activity was seen, with the majority (78 to 93%) of the recovered activity found in the supernatant. The pellet from the detergent-treated extracts had little ACAT activity, but after a second DOC/PC treatment followed by dilution into vesicles, a 4- to 11-fold increase in activity was observed. These elevated activities are reported in Table 1 and suggest that the remaining

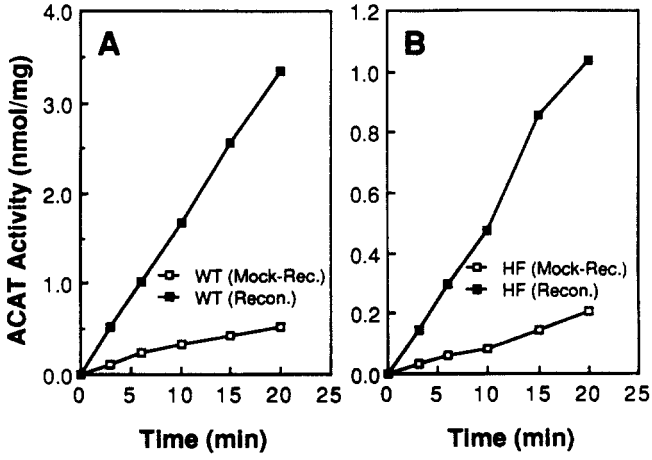

Fig. 3. Time course of cholesteryl oleate formation after cell extracts treated with buffer only (mock-reconstituted) or 20 mg/ml DOC and 4 mg/ml PC (reconstituted) are diluted into cholesterol-PC vesicles and assayed for ACAT activity; (A), wild type CHO cells and (B), human fibroblasts. Values are the average of duplicate assays and ranged within 10% of the mean.

TABLE 1. Fractionation by ultracentrifugation of ACAT activity from cell extracts incubated with either buffer or a detergent/PC solution[a]

Cell Type	Detergent	Total	Supernatant		Pellet	
			pmol/min			
Human fibroblasts	–	11.5	0.0	(0.0)	6.6	(57.6)
	+	66.9	54.3	(81.2)	8.4	(12.6)
Wild type CHO	–	68.7	0.0	(0.0)	36.4	(53.0)
	+	431.3	315.7	(73.2)	25.4	(5.9)
25-RA CHO	–	188.4	0.6	(0.3)	109.4	(58.1)
	+	473.3	298.6	(63.1)	85.2	(18.0)

After incubation of the cell extracts with buffer or detergent, aliquots were diluted into cholesterol-PC vesicles as described in Materials and Methods and assayed to determine total ACAT activity. The rest of the cell extracts were centrifuged at 45,000 rpm for 45 min in a Beckmann 70.1 Ti rotor at 4°C. The supernatants were collected and aliquoted into cholesterol-PC vesicles and assayed for ACAT activity. The pellets were washed by gently resuspending in buffer and recentrifuging at 45,000 rpm for 45 min at 4°C. These washed pellets were then resuspended in buffer by Dounce homogenization at 4°C. The pellets from the extracts originally treated with buffer were directly assayed for ACAT activity and the pellets of the detergent-treated extracts were treated with 20 mg/ml DOC and 4 mg/ml PC in a manner identical to the procedure for solubilization of cell extracts described in Materials and Methods. The DOC/PC-treated pellets were then diluted into cholesterol-PC vesicles before assaying for ACAT activity. Percent recoveries are shown in parentheses. Each value is the average of duplicates and ranged within 10% of the mean.

[a]Twenty mg/ml DOC and 4 mg/ml PC.

microsomal membranes may still contain some ACAT enzyme but some factor necessary for activity, perhaps phospholipid and/or cholesterol, has been removed. In total, the data in Table 1 suggest that at least 70–90% of the enzyme is solubilized during the initial detergent/PC treatment.

Fig. 4 shows the migration profile of microsomal or reconstituted ACAT activity from cell extracts of 25-RA and human fibroblasts loaded on two-step Ficoll gradients. Results similar to those obtained with 25-RA were found when wild type cell extracts were used (data not shown). The results demonstrate that the cholesterol-PC liposomes migrate to the top of the gradient (Fig. 4A, C and E). When solubilized cell extracts are diluted into these liposomes, most or all of the detectable ACAT activity co-migrates with the phospholipid (Fig. 4C and E). In untreated cell extracts, all of the recovered ACAT activity is found at the bottom of the gradient (Fig. 4B and D). These results are qualitatively consistent with the results of Doolittle and Chang (11), who reconstituted wild type CHO cell ACAT activity using dialysis to remove DOC. However, under present conditions, full recovery of the microsomal ACAT activity was only obtained when the collected fractions were first incubated with cholesterol-PC vesicles overnight. When this incubation was not included, the recovery of microsomal ACAT activity was only 20–30% (data not shown). This loss of activity is perhaps due to separation of the ACAT enzyme from cholesterol during the ultracentrifugation. The total recovery of reconstituted ACAT activity that co-migrated with the cholesterol-PC vesicles was not dependent on any additional treatment. While the recoveries of CHO ACAT activity ranged from 67 to 109%, the recovery of human fibroblast activity was 157–192% in three separate experiments. The mechanism of this activation is not clear; it may be due to inherent differences between the ACAT enzyme from hamster and human cells. In summary, these data are consistent with the ultracentrifugation data of Table 1, and we conclude that the majority of the ACAT activity is solubilized and becomes associated with the cholesterol-PC liposomes, probably by insertion into the vesicle bilayer.

Dependence of the reconstituted assay on exogenous cholesterol

As shown in **Fig. 5**, reconstituted ACAT activity of all three cell types is entirely dependent on the cholesterol present in the liposomes. No activity (the lowest possible detection is 2 pmol/min per mg for the reconstitution assay) was ever seen when cell extracts were reconstituted into PC vesicles containing no cholesterol. The response to increasing amounts of cholesterol in the vesicles was a slightly sigmoidal curve that leveled off at between 0.2 to 0.3 mol cholesterol per mole of PC. This curve is almost identical to the one published earlier from our laboratory (11) using dialysis to remove the detergent. Since there is no detectable DOC left after reconstitution by dialysis (11), we conclude that the relatively large amount of DOC remaining after dilution (see Fig. 2) does not interfere with the effect of cholesterol on reconstituted ACAT activity. The small decrease in activity at cholesterol/PC ratios above 0.3 is reproducible and may be similar to the observation previously made by other investigators (5, 7) who suggested that the decrease in activity was due to cho-

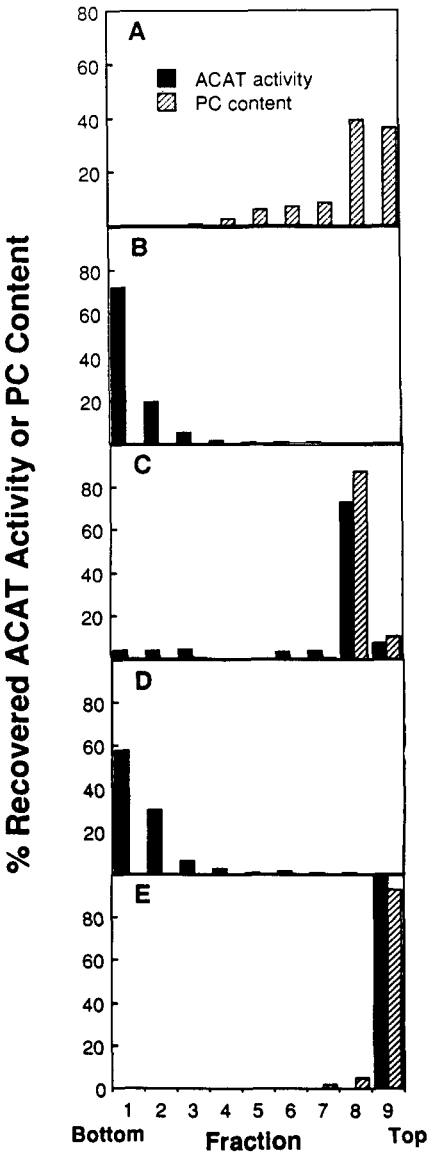

Fig. 4. ACAT activity and PC profiles on a two-step Ficoll gradient consisting of two discontinuous steps of 10% Ficoll (0.5 ml; approximate buoyant density = 1.07 g/ml) and 6% Ficoll (2.5 ml; approximate buoyant density = 1.03 g/ml) layered on top of a 50% sucrose cushion (0.27 ml). The samples (0.24 ml) analyzed were: (A), untreated cholesterol-PC vesicles; (B), untreated 25-RA cell extract; (C), DOC solubilized 25-RA cell extract diluted into cholesterol-PC vesicles; (D), untreated human fibroblast cell extract; (E), human fibroblast extract treated as in (C). (B) and (D) contained approximately 600 μg cell protein and (C) and (E) approximately 30 μg protein. (A), (C) and (E) contained 3 μmol PC and 0.9 μmol cholesterol in the form of liposomes and were mixed with 0.12 ml 50% sucrose and loaded at the bottom of the gradient at the 50% sucrose/10% Ficoll interface. Remaining samples were mixed with buffer and loaded at the top of the gradient. After centrifugation at 50,000 rpm in a Beckman SW60 rotor for 6 hr, nine 0.35 ml fractions were collected and assayed for ACAT activity and PC content. Aliquots (0.16 ml) of each fraction were mixed with 0.08 ml of cholesterol-PC vesicles and incubated overnight at 4°C before the ACAT assay was performed. No phospholipid analysis was performed on samples (B) or (D). The results are reported as % of recovered ACAT activity or PC content. The absolute recovery of PC ranged from 60 to 85%. The recoveries of ACAT activity were for each sample: (B), 109%; (C), 70%; (D), 178%; and (E), 192%.

lesterol causing changes in the physical properties of the liposome bilayer.

The data in Fig. 5 suggest that ACAT enzyme reconstituted into liposomes containing no cholesterol does not have activity, and that increasing the amount of cholesterol in the liposomes leads to a corresponding increase in ACAT activity. An alternative explanation is that cholesterol in the liposomes is necessary for insertion of the enzyme into the bilayer. This has been observed for the insertion of certain integral membrane proteins into bilayers in the absence of detergent (30). To rule out this possibility in our reconstitution assay, ACAT was reconstituted into PC vesicles containing no cholesterol and then reactivated by the addition of exogenous cholesterol in the form of cholesterol-PC liposomes. The addition of DOC to the mixture was required to observe activation of the ACAT activity. The results of one such experiment are shown in **Fig. 6**. Solubilized cell extracts were reconstituted into PC liposomes and no ACAT activity was detectable in these preparations. Upon addition of cholesterol-PC liposomes, measurable activity becomes apparent after a 4-hr incubation at 4°C. A more dramatic activation is seen when DOC is added to the vesicle solution followed by incubation at 4°C. Although there is some variation in absolute ACAT activities at different detergent/PC ratios in various experiments, a detergent/PC molar ratio of 0.3 always gave the greatest activation. No activation of ACAT activity was observed when DOC alone was added to the extracts reconstituted into PC liposomes, or when cholesterol was not included in the vesicles added after reconstitution (data not shown), indicating that the activation is dependent upon both cholesterol and DOC. Bile salts are known to stimulate the rate of spontaneous phospholipid transfer between membranes (31). Whether DOC facilitates transfer of cholesterol to the PC vesicles containing ACAT or stimulates movement of the ACAT enzyme from one vesicle population to another is not known.

For comparison, solubilized cell extracts were also diluted into liposomes containing an optimal molar ratio of cholesterol/PC (0.3) and then treated with cholesterol-PC liposomes and DOC (Fig. 6). Above a detergent/PC ratio of 0.3, the amount of activity in the control vesicles drops sharply. At detergent/PC ratios higher than 0.3, a drastic decrease in the turbidity of the vesicles was observed which may indicate a total disruption of the vesicle bilayer.

The data in Fig. 6 do not rule out the possibility that the ACAT enzyme in the reconstitution assay using vesicles containing no cholesterol was not inserted in the PC bilayer but only became incorporated into a bilayer after the addition of cholesterol-PC liposomes and DOC. To address this possibility, solubilized cell extracts diluted into pure PC vesicles containing no cholesterol were fractionated on a Ficoll gradient, and the fraction containing

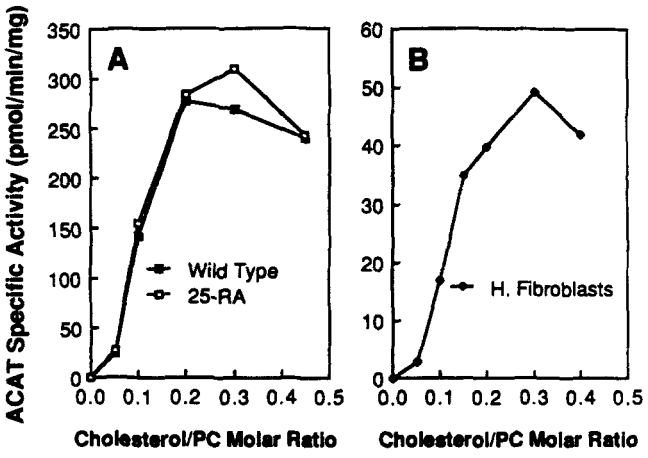

Fig. 5. Effect of increasing the cholesterol/PC molar ratio on reconstituted ACAT activity in (A) 25-RA and wild type CHO cells and (B) human fibroblasts. Aliquots of solubilized cell extracts were diluted into cholesterol-PC vesicles of increasing cholesterol concentration and assayed for ACAT activity. Values are the average of duplicate assays and ranged within 10% of the mean.

the PC vesicles was collected and treated as described in Fig. 6. **Fig. 7** summarizes the data before (Fig. 7A) and after (Fig. 7B) migration of the PC vesicles on Ficoll gradients similar to the ones described in Fig. 4. Even after fractionation on the Ficoll gradient, latent ACAT activity is associated with the PC vesicles, which can be activated by incubation with cholesterol-PC vesicles and DOC. It should be noted that, in unfractionated preparations, the reactivated ACAT activity from PC vesicles is almost never as high as controls reconstituted into cholesterol-PC vesicles and treated identically (Figs. 6 and 7A), even though the cholesterol-PC concentrations in both assays are the same. The latent and control activities are the same in Fig. 7B at one detergent/PC ratio, but this is not always observed and may be due to the relatively low (67%) recovery of ACAT activity from the control gradients in this particular experiment. Although these results cannot determine that the amount of enzyme incorporated into PC liposomes is identical to the amount inserted into cholesterol-PC liposomes, the results do suggest that lack of insertion into the bilayer is not the reason for the lack of ACAT activity in pure PC vesicles. Rather, it appears that a substantial amount of ACAT enzyme is incorporated, but is inactive due to lack of cholesterol.

Comparison of reconstituted ACAT activities from CHO cells and human fibroblasts grown in 10% FCS or 10% De-S

In cultured human fibroblasts and wild type CHO cells grown in 10% FCS, the microsomal ACAT activity from cell extracts is much higher than that found in cells grown in 10% De-S medium (**Table 2**), consistent with previous findings that ACAT activities in cultured cells are highly regulated by sterol present in the growth medium (3, 11, 24, 32). Previously, Doolittle and Chang (11) showed that the reconstituted ACAT activities (using dialysis to remove the DOC) from wild type CHO cell extracts grown in 10% FCS or 10% De-S are essentially the same.

In Table 2 we extend these observations to human fibroblasts and 25-RA cells. When untreated cell extracts were assayed for microsomal ACAT activity, a tenfold difference was seen between human fibroblast cell extracts prepared from cells that had been grown in 10% FCS or 10% De-S. Similar results were obtained with wild type CHO cell extracts. In 25-RA cells, the difference is only twofold. This may be because 20 hr growth in 10% De-S medium is not long enough to deplete 25-RA's large amount of endogenous cholesteryl ester (33). In cholesterol ester-loaded mouse peritoneal macrophages, the cholesteryl ester is continuously being hydrolyzed to form free cholesterol which can then be reesterified and thus retain a relatively high rate of cholesterol esterification (34). Upon reconstitution, the differences in ACAT activity between cells grown in 10% FCS or 10% De-S are abolished. These data are consistent with the hypothesis that modulating the amount of ACAT enzyme is not a major mechanism for sterol-dependent ACAT regulation in cultured cells (11, 24).

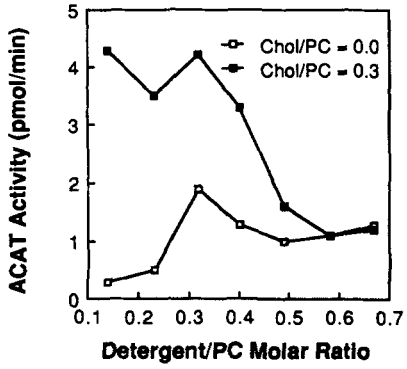

Fig. 6. Reactivation of ACAT activity in PC vesicles upon incubation with cholesterol-rich liposomes and increasing amounts of DOC. Cell extracts were reconstituted into either pure PC liposomes (Chol/PC = 0.0) or cholesterol-PC vesicles (Chol/PC = 0.3). Aliquots of the reconstituted mixtures without cholesterol (0.16 ml containing ~20 μg protein and 1.7 mg PC) were mixed with 0.26 ml cholesterol-PC liposomes (cholesterol/PC molar ratio of 0.47), vortexed briefly then incubated with increasing concentrations of DOC. As controls, reconstituted vesicles containing cholesterol were mixed with vesicles of a cholesterol/PC ratio of 0.3 plus DOC. After incubation at 4°C for 3–4 hr, the samples were assayed for ACAT activity. Values are plotted as pmol of cholesteryl oleate formed per min versus the detergent/PC ratio. There is no detergent/PC value of 0.0 because residual DOC from the solubilized cell extract and residual cholate from the liposome preparations were present before additional DOC was added. Reconstitution mixtures containing no cholesterol had no detectable ACAT activity when incubated with buffer instead of cholesterol-rich vesicles. Another control showed 3.4 pmol/min of cholesteryl oleate formed in reconstitutions using cholesterol-PC liposomes plus incubation with buffer. Duplicates ranged within 10% of the mean.

It appears that in all aspects examined the reconstituted 25-RA and wild type CHO cell ACAT activities are identical within experimental error. In ten separate experiments in which wild type and 25-RA cell extracts were reconstituted at the same time and under identical conditions, the average reconstituted ACAT specific activities were 218.5 ± 30.0 (SD) for wild type and 240.1 ± 24.8 (SD) for 25-RA. Thus, the five to seven times higher cellular cholesteryl ester (14, 33) and the two to three times higher ACAT activity when assayed by an [^{3}H]oleate pulse or unreconstituted assay (14) seen in 25-RA cells compared to wild type CHO cells are not due to a mutation in the ACAT enzyme or an overproduction of ACAT. The elevated ACAT activity in 25-RA is most likely the consequence of increased cholesterol biosynthesis and number of LDL receptors (13, 14), which provides more substrate for the ACAT enzyme.

In conclusion, this report describes a new method for reconstituting ACAT activity from tissue culture cells. The method is simple, rapid and the results obtained are similar to those seen with the longer and more cumbersome dialysis procedure (11). These reconstitution procedures measure ACAT activity independently of the original lipid composition of the microsomal membrane. Assaying the enzyme in a defined cholesterol-phospholipid environment allows a more precise analysis of the effect of cholesterol and other lipid molecules on the ACAT enzyme than other methods where cholesterol is added to the microsomal membranes (7, 10).

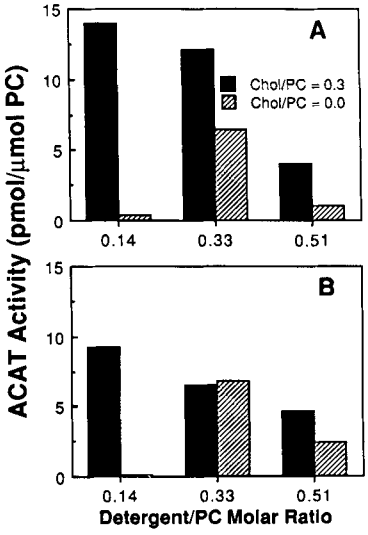

Fig. 7. Activation of ACAT activity reconstituted into pure PC vesicles upon incubation with cholesterol-rich vesicles plus DOC before (A) and after (B) fractionation on a Ficoll gradient. Reconstituted cell extracts were loaded onto Ficoll gradients and centrifuged as described in Fig. 4. The fractions containing the liposomes were collected and incubated with cholesterol-rich vesicles and DOC as described in Fig. 6. To control for dilution of the sample and loss of material during collection of fractions from the Ficoll gradients, the ACAT activity is expressed as pmol cholesteryl oleate formed per μmol of reconstituted vesicle PC present in the assay. Duplicates ranged within 10% of the mean.

TABLE 2. Reconstituted and microsomal ACAT activities in cells grown in 10% fetal calf serum or 10% delipidated fetal calf serum medium

Cell Type	Medium	ACAT Specific Activity	
		Microsomal	Reconstituted
		pmol/min per mg	
Human fibroblasts	10% FCS	11.8	57.8
	10% De-S	1.1	70.1
Wild type CHO	10% FCS	48.9	211.0
	10% De-S	5.3	181.3
25-RA CHO	10% FCS	82.3	223.4
	10% De-S	48.3	227.6

CHO cells and human fibroblasts were grown in 10% FCS medium as described in Materials and Methods. At 20 hr prior to harvesting, the monolayers were washed three times with phosphate-buffered saline and grown in medium supplemented with either 10% FCS or 10% De-S. There was an additional medium change 2 hr before harvesting. The cell extracts were then assayed for microsomal ACAT activity using the method originally developed by Goodman et al. (4) as described by Cadigan et al. (14), or for reconstituted ACAT activity as described in this paper. Values are the average of duplicates that ranged within 10% of the mean.

The earlier reconstitution procedures reported from this laboratory (8, 11) and the present one are distinct from the other ACAT reconstitution assays reported in the literature (5–7) in the amount of exogenous lipid added. In the latter reconstitution procedures, the ratio between the amount of exogenous lipid to endogenous lipid was 2 or 3 to 1. This is probably why these assays were not totally dependent on exogenously added cholesterol. The methods used in our laboratory, adapted from the procedure described by Papazian, Rahamimoff, and Goldin (35), utilize a large excess of exogenous lipid. In the present assay, cellular lipids are diluted approximately 300-fold by the cholesterol-PC liposomes and the assay is entirely dependent upon the cholesterol in the added liposomes.

The major disadvantage of the reconstitution method described in this report is the large amount of detergent remaining after reconstitution. In principle, the amount of detergent could be reduced by diluting smaller aliquots of solubilized extracts into preformed liposomes. This would require a greater sensitivity in the assay to accurately determine human fibroblast activity. This could be accomplished by increasing the assay time and the specific radioactivity of the [^{3}H]oleyl CoA.

Because of its net charge, DOC may not be an optimal detergent for purification of the ACAT enzyme after solubilization. Thus far, attempts to reconstitute ACAT activity using the nonionic detergent NP-40 have not

been successful, although other detergents have not been tried. Further experiments are needed to fully realize the uses and limitations of this procedure for studying the effect of various lipid molecules on ACAT activity in liposomes and for reconstitution studies of other membrane-bound proteins.

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