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ACYLCOENZYME A:CHOLESTEROL ACYLTRANSFERASE ACTIVITY: SOLUBILIZATION AND RECONSTITUTION IN LIPOSOMES[1]

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SUMMARY: Acylcoenzyme A:cholesterol acyltransferase (ACAT) has been solubilized from Ehrlich ascites cell microsomes by extraction with 2% Triton X-100. Activity was obtained with the solubilized material after removal of the detergent, and it was enhanced when the extract was incorporated into liposomes. The activity of the reconstituted system was dependent upon the amount of cholesterol present in the liposomes. It reached a maximum when the molar ratio of cholesterol to phospholipid was 0.074 and decreased when larger amounts of cholesterol were added. The increase in ACAT activity appears to be a substrate effect of cholesterol, whereas the reduction at higher cholesterol contents probably is due to changes in the physical properties of the liposomes.

Acylcoenzyme A:cholesterol acyltransferase (ACAT), a tightly bound microsomal enzyme (1), catalyzes the synthesis of cholesteryl esters in mammalian tissues. The enzyme is part of the receptor mediated pathway by which cholesterol is taken up from plasma low density lipoproteins and stored within cells as cholesteryl ester (2-4). This process probably is involved in the accumulation of cholesteryl esters within the arterial wall in atherosclerosis (5-7). Recent evidence suggests that ACAT activity may be regulated by changes in the cholesterol content and fatty acid composition of the microsomal membrane (5,8,9). Additional insight into the mechanism of these potentially important regulatory effects might be gained if a solubilized ACAT preparation that was suitable for reconstitution was available. Previous studies indicated, however, that the enzyme contained in liver and aorta was resistant to solubilization (1,5). In the present communication we report a method for solubilizing ACAT from Ehrlich ascites tumor cell microsomes as well as some of the properties of this enzyme preparation when it is combined with liposomes.

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MATERIALS AND METHODS: Liposomes were prepared in most cases from Asolectin, a soybean lipid extract. Before use, the Asolectin was purified by washing with acetone (10) followed by silicic acid column chromatography to remove remaining neutral lipids and plant sterols. The phospholipid fraction then was eluted with methanol. After addition of cholesterol and drying, liposomes were prepared by sonication for 12 min at 40°C under N_2 in a buffer solution containing 100 mM K_2HPO_4 and 1 mM dithiothreitol, pH 7.2. The suspension was centrifuged for 10 min at 15,000 g, annealed at 40°C for 60 min, and sedimented at 105,000 g for 3 hr at 4°C. These liposomes contained 41% phosphatidylcholine, 36% phosphatidylethanolamine, 13% phosphatidylinositol plus phosphatidylserine and 10% minor phospholipids as determined by thin layer chromatography followed by measurement of lipid phosphorus content (11).

Microsomes were prepared from Ehrlich ascites tumor cells (9). The isolated microsomes were extracted for 2 hr at 4°C with 2% Triton X-100 and then sedimented at 105,000 g for 60 min. The supernatant solution was incubated twice with 0.6 g/ml of SM-2 BioBeads and then dialyzed for 16 hr against 1L of 100 mM K_2HPO_4 and 1 mM dithiothreitol, pH 7.2. The resulting protein extract was incubated with the liposomes (12) for 2 hr at 10°C with shaking, and the complex isolated at 105,000 x g for 60 min. After removing the supernatant solution, the pellet was suspended in fresh buffer to a final concentration of 1 mg/ml of phospholipid.

Unless stated otherwise, incubations were done with shaking at 30°C for 10 min using air as the gas phase. The medium contained 5 nmoles of defatted bovine serum albumin (13), 6 nmoles of palmitoyl CoA, 0.1 μCi of [1-^{14}C] palmitoyl CoA, 0.1 M K_2HPO_4 and 1 mM dithiothreitol, pH 7.2. Incubations were terminated by the addition of 3 ml of a chloroform-methanol solution (2:1, v/v), and the amount of cholesteryl palmitate synthesized was measured by thin layer chromatography and liquid scintillation counting (9). Protein (14) and phospholipid (11) contents were measured chemically. Cholesterol content and fatty acid compositions were measured by gas liquid chromatography.

RESULTS AND DISCUSSION: Experiment 1 in Table I shows that ACAT activity was obtained in the solubilized microsomal extract and that the activity was enhanced when the extract was combined with liposomes. Based on microsomal protein content, the specific activity of the system reconstituted with Asolectin liposomes was 70% of that obtained with intact microsomes. No activity was observed with the protein-free liposomes or when Triton X-100 was not removed from the solubilized material. Additional studies with ^{3}H-labeled Triton X-100 revealed that only 0.5% of the detergent added to the microsomes remained after dialysis of the soluble extract and even less, 0.15%, was present after reconstitution with liposomes. When palmitoyl CoA was replaced by palmitate and an acyl CoA generating system, ACAT activity was obtained with the intact microsomes but not with the reconstituted system. It is possible that the acyl CoA ligase was not solubilized from the microsomes by the extraction procedure. Alternatively, the ligase may be present in the liposomes but the CoA, ATP or $MgSO_4$ added to the medium may not have been accessible to the en-

TABLE I ACAT Activity Under Various Conditions

Enzyme Preparation	Radioactive Substrate	Activity
		pmol/mg protein x min
Experiment 1		
Microsomes	Palmitoyl CoA	22.20 ± 0.75
Solubilized protein in Triton X-100	Palmitoyl CoA	0.45 ± 0.05
Solubilized protein after dialysis	Palmitoyl CoA	9.40 ± 0.85
Solubilized protein in Asolectin liposomes[a]	Palmitoyl CoA	15.90 ± 0.75
Liposomes[b]	Palmitoyl CoA	0.20 ± 0.15
Microsomes	Palmitate[c]	9.05 ± 0.35
Solubilized protein in Asolectin liposomes[a]	Palmitate[c]	0.30 ± 0.15
Experiment 2		
Solubilized protein after dialysis	Palmitoyl CoA	7.40 ± 0.30
Solubilized protein in Asolectin liposomes	Palmitoyl CoA	17.56 ± 1.50
Solubilized protein in Aso/cer liposomes[d]	Palmitoyl CoA	8.73 ± 0.20
Solubilized protein in DPL/PS liposomes[e]	Palmitoyl CoA	20.13 ± 3.90

[a]These liposomes contained 0.17 mg of microsomal protein and 34 µg of cholesterol per mg of phospholipid derived from purified Asolectin. The medium contained 1.1 mg of Asolectin phospholipid in a total volume of 0.5 ml, and the incubations were done at 30°C.

[b]No microsomal protein was added to the liposomes.

[c]These incubations contained 10 mM ATP, 1 mM CoA and 4 mM $MgSO_4$, 0.01 mM albumin and 0.01 mM [1-^{14}C] palmitate.

[d]These liposomes contained Asolectin and cerebrosides (9:1, mole/mole). The latter was a commercial bovine brain preparation provided by Sigma Chemical Company.

[e]These liposomes contained synthetic dipalmitoyl phosphatidylcholine and beef brain phosphatidylserine (9:1, mole/mole); both were obtained from Sigma Chemical Company.

zyme. As shown in Experiment 2, a similar enhancement of ACAT activity was obtained when the liposomes were prepared from a mixture of dipalmitoyl phosphatidylcholine and phosphatidylserine instead of Asolectin. This indicates that ACAT can tolerate considerable changes in the phospholipid milieu in which it is embedded. By contrast, almost no enhancement of ACAT activity was observed when 10% by weight of cerebrosides were added to the Asolectin liposomes.

TABLE II Fatty Acid Compositions

Fatty Acid	Microsomes	Solubilized Protein	Asolectin Liposomes	Reconstituted Asolectin System
		% of total fatty acids		
Classes				
Saturated	20.0	20.6	14.1	13.8
Monoenoic	19.3	19.1	5.4	6.5
Polyenoic	55.8	49.7	80.3	79.0
Unidentified	4.9	11.6	0.2	0.7
Individual Acids				
16:0	3.8	2.8	11.1	10.0
16:1	1.0	1.6		tr[a]
18:0	16.2	17.8	3.0	3.8
18:1	16.8	15.3	5.4	6.5
18:2	17.3	11.7	70.2	67.9
18:3	tr	tr	10.1	9.7
20:1	1.5	2.2		tr
20:3	1.4	1.2		tr
20:4	14.8	13.4		1.0
22:4	5.6	8.3		tr
22:5	2.1	2.1		tr
22:6	14.6	13.0		0.4

[a]Trace, < 0.4% of total fatty acids.

Table II shows the fatty acid compositions of the various preparations. Some lipid was present in the microsomal extract, and the fatty acid composition of the extract resembled that of the intact microsomes. The fatty acid composition of the Asolectin liposomes, however, was quite different from that of the microsomal extract. The fatty acid composition of the system reconstituted with Asolectin liposomes was similar to that of the liposomes themselves, indicating that only a small amount of the lipid in the reconstituted system was derived from the microsomal extract.

Fig. 1 shows that cholesteryl ester formation in the system reconstituted with Asolectin liposomes was linear for 15 min and was proportional to the amount of microsomal protein present in the liposomes. Washing by ultracentrifugation did not remove ACAT activity from the reconstituted system. Maximum activity occurred at 30°C, and activity decreased when the temperature was raised further.

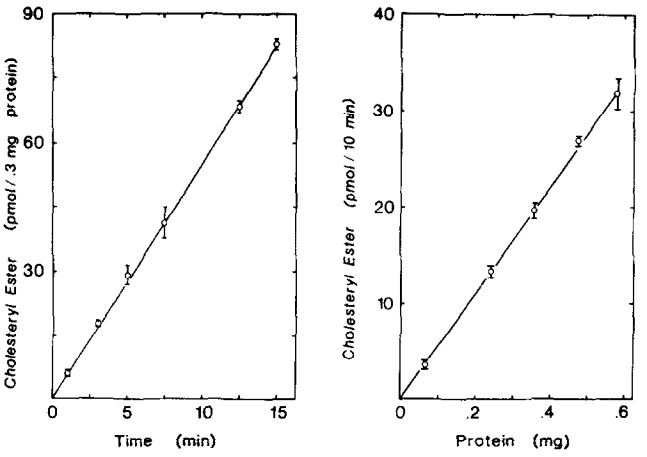

Fig. 1. Properties of ACAT activity in Asolectin liposomes. The time course of the reaction is shown on the left. The liposomes added to each flask contained 0.19 mg of microsomal protein, 1.1 mg of phospholipids from Asolectin and 38 µg of cholesterol. Dependence on the content of microsomal protein is shown on the right. These liposomes contained 3.2 mg of phospholipids, 105 µg cholesterol and the amount of microsomal protein shown on the graph. The time of incubation was 10 min. In both cases, the media contained 6.2 nmoles of [1-^{14}C]-palmitoyl CoA, 5 nmoles of albumin, 50 µmoles of K_2HPO_4 and 0.5 µmoles of dithiothreitol in a total volume of 0.5 ml, and the incubation was carried out at 30°C.

Fig. 2 shows the dependence of ACAT activity in the reconstituted system on the amount of palmitoyl CoA added to the medium and the cholesterol content of the liposomes. The apparent K_m and V_{max} values for palmitoyl CoA in the presence of 10 µM albumin were 38 µM and 53 pmol/mg protein x min, respectively. Inhibition was observed at higher palmitoyl CoA concentrations, possibly due to a detergent effect (9). The molar ratios of cholesterol to phospholipid in the liposomes used for the second part of this experiment varied from 0.033 to 0.56. Increasing ACAT activity was noted until the molar ratio reached 0.074, but it then decreased when the cholesterol content of the liposomes was raised further. Chemical analysis revealed that approximately the same amount of microsomal protein was incorporated in each case, suggesting that the cholesterol dependent changes in ACAT activity probably are not due to differences in the enzyme content of the various liposome preparations.

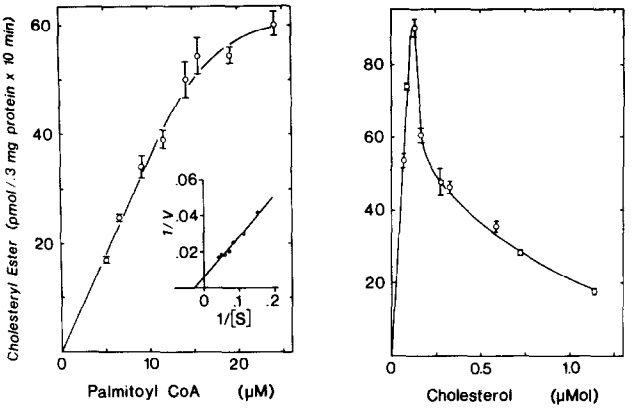

Fig. 2. Dependence of ACAT activity on [1-^{14}C] palmitoyl CoA concentration and cholesterol content. In the experiments shown on the left testing palmitoyl CoA concentration, the liposomes contained 350 μg of cholesterol and 0.34 mg of microsomal protein per mg of phospholipid from Asolectin. In the experiments shown on the right testing cholesterol content, the liposomes contained 0.18 mg of microsomal protein and the amount of cholesterol shown on the graph per mg of phospholipid. The palmitoyl CoA concentration was 13.8 μM. In both cases, the medium contained 5 nmoles of albumin, 50 μmoles of K_2HPO_4 and 0.5 μmoles of dithiothreitol per 0.5 ml, and the incubations were done at 30°C.

Increases in ACAT activity with increasing cholesterol content, as noted in this system, have been reported in microsomes (5,8), intact tissues (7,15) and isolated cells (2,16-18). Since cholesterol is a substrate for the ACAT reaction, we believe that these increases can best be explained as an effect of increasing substrate concentration. On the other hand, the reduction in ACAT activity that occurred when larger amounts of cholesterol were present probably is a physical effect resulting from cholesterol induced alterations in liposome structure or fluidity (19). This interpretation is supported by results with another Ehrlich cell microsomal enzyme, acylcoenzyme A hydrolase, which also can be solubilized and incorporated into Asolectin liposomes. Acylcoenzyme A hydrolase activity decreased from 94 ± 4 to 56 ± 6 pmol/mg protein x min (n = 4) when the molar ratio of cholesterol to phospholipid in the liposomes was raised from 0.074 to 0.56. Since cholesterol is neither a substrate nor known effector of this enzyme, the reduction in activity is due most likely to physical changes in the liposomes, produced by the increasing cholesterol content.

In summary, the availability of a soluble ACAT preparation that can be incorporated into liposomes provides a new approach for studying the regulation of intracellular cholesteryl ester formation, especially the role of membrane lipid composition in this process.

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