Hepatic acylcoenzyme A:cholesterol acyltransferase activity during diet-induced hypercholesterolemia in cynomolgus monkeys

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Abstract Acylcoenzyme A:cholesterol acyltransferase (ACAT) activity was studied in hepatic microsomes of cynomolgus monkeys fed either commercial chow or an atherogenic diet of high cholesterol and saturated fat content. ACAT activity (pmol/min per mg protein) was 35 in liver microsomes from control monkeys, and 142 and 161 at 10 and 100 days, respectively, after starting the high cholesterol diet. The cholesterol-fed monkeys had about 1.5-fold increase in cholesterol content of hepatic microsomes as compared to control monkeys (94 nmol/mg protein in controls versus 142 nmol/mg protein in the cholesterol-fed group). There was no difference between the two groups in microsomal phospholipid content or distribution of microsomal fatty acids in saturated, monoenoic, or polyenoic acid classes. However, the cholesterol-fed monkeys had relatively lower amounts of linoleic acid and higher amounts of arachidonic acid in the microsomes. To determine whether the increased microsomal cholesterol content might be responsible for the increase in ACAT activity, liver microsomes from control monkeys were incubated for 15–120 min with liposomes composed of cholesterol and dipalmitoyl phosphatidylcholine, 2:1 (mol/mol). The microsomal cholesterol content increased from 90 to 128 nmol/mg protein as the incubation progressed. There was a corresponding increase in ACAT activity from 80 to 240 pmol/min per mg protein. This observation is consistent with the view that the higher hepatic ACAT activity in the cholesterol-fed monkeys is due to the larger amount of cholesterol contained in the microsomes. The increase in hepatic ACAT activity occurs soon after cholesterol feeding is started; this response may be involved in the production of cholesteryl ester-rich lipoproteins by the liver, and thereby may be related to the atherogenic process in these primates.—**Mathur, S. N., M. L. Armstrong, C. A. Alber, and A. A. Spector.** Hepatic acylcoenzyme A:cholesterol acyltransferase activity during diet-induced hypercholesterolemia in cynomolgus monkeys. *J. Lipid Res.* 1981. **22:** 659–667.

Supplementary key words liver · microsomes · cholesteryl esters · dietary fat · atherogenic diet

The intracellular formation of cholesteryl esters is catalyzed by acylcoenzyme A:cholesterol acyltransferase (ACAT) (EC 2.3.1.26) (1). ACAT has been detected in many tissues, including the liver and arteries of monkeys (2, 3). The enzyme is tightly bound to the microsomal fraction of cellular homogenates, and the highest activities are recovered from the rough endoplasmic reticulum (4). A number of factors regulate ACAT activity, including the availability of cholesterol and dietary fat saturation. When either human skin fibroblasts or rat Fu5AH cells are exposed to cholesterol-rich lipoproteins, ACAT activity in the cells increases and intracellular cholesteryl esters accumulate (5–8). Likewise explants of aorta have higher ACAT activity when they are exposed to hypercholesterolemic serum (9, 10). In addition, ACAT activity in isolated hepatocytes increases when cholesterol synthesis is stimulated by exposure to high concentrations of mevalonate (11). Cholesterol probably acts in these cases by providing more substrate for the enzyme, although evidence in the human fibroblast suggests that it may have an additional regulatory effect on ACAT (6, 12). Changes in dietary fat saturation also can influence the ACAT activity of rat liver (13), possibly by modifying the fatty acyl composition of the microsomal phospholipids with which the enzyme interacts.

When cynomolgus monkeys (*Macaca fascicularis*) are fed a high cholesterol diet containing saturated fat, they develop a hypercholesterolemia characterized by an accumulation of low density lipoproteins that are enriched in cholesteryl esters and are increased in size (14, 15). Atherosclerosis results if this diet is continued for 12 to 18 months (16). We wished to determine whether hepatic ACAT activity is affected when nonhuman primates are fed cholesterol and saturated fat. In the present work, we observed that both the cholesterol content and ACAT activity of the microsomes prepared from cynomolgus monkey liver are elevated when the atherogenic diet is fed. Additional studies with the isolated microsomes suggest that the increased ACAT activity can be explained entirely by the elevation in cholesterol content.

Abbreviation: ACAT, acylcoenzyme A:cholesterol acyltransferase.

TABLE 1. Fatty acid composition of the diets

Fatty Acid	Composition of Dietary Fat[a]	
	Control	High Cholesterol
	%	
Individual acids		
14:0	1.8	1.1
16:0	19.6	25.1
16:1	2.7	3.4
18:0	9.8	11.6
18:1	31.0	41.9
18:2	27.9	12.1
Classes		
Saturated	31.2	37.8
Monoenoic	33.7	45.3
Polyenoic	27.9	12.1
Unsaturation index	0.89	0.69

[a] Other fatty acids, i.e., 20:3, 20:4, 22:5, and 22:6 were present only in trace amounts (<0.5%).

MATERIALS AND METHODS

[1-^{14}C]Palmitoyl coenzyme A (57 mCi/mmol) was purchased from New England Nuclear (Boston, MA), palmitoyl coenzyme A was from P-L Biochemical Inc. (Milwaukee, WI) and cholesterol was from the Hormel Institute (Austin, MN).

Animals and diet

The monkeys were adult cynomolgus (*M. fascicularis*) males, 4.7 ± 0.15 kg. They were individually caged in thermo-regulated rooms. Three control monkeys were given commercial laboratory chow (Purina monkey chow, Ralston Purina Company, Richmond, IN). A hypercholesterolemic diet containing high cholesterol (0.8%) and high saturated fat described previously was fed to two groups of monkeys, three in each group, for 10 days or 100 days (17). The fatty acid compositions of these diets are shown in **Table 1.** The plasma total cholesterol was measured as previously described (17), and the values at the time of study were 145 ± 11, 368 ± 43, and 822 ± 54 mg/dl for control monkeys, monkeys fed the hypercholesterolemic diet for 10 days, and monkeys fed the hypercholesterolemic diet for 100 days, respectively.

At the end of the study periods, the animals were tranquilized with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) and then exsanguinated from the abdominal aorta. The liver was removed and placed in an ice-cold solution containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl buffer, pH 7.4 (buffered sucrose solution). Liver samples (10 g) used for preparation of microsomes were injected with buffer with a syringe and 22 gauge needle and flushed until clear of residual blood.

Microsome preparation

All microsome preparations were carried out immediately after autopsy. The liver samples were minced with scissors and homogenized in 4 volumes of buffered sucrose solution with six strokes in a Potter-Elvehjem homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 *g* for 30 min at 4°C. After the fat layer was removed, the supernatant solution was collected and centrifuged at 4°C for 60 min at 105,000 *g*. The sediment was suspended in buffered sucrose solution and recentrifuged. After discarding the supernatant wash, the microsomal pellet was dispersed in buffered sucrose solution to give 10 mg protein/ml. The preparations were stored in liquid N_2 and assayed within 21 days. No loss of ACAT activity was found during storage, similar to findings in rat liver microsomes in which ACAT activity was unaltered from the value obtained in fresh tissue after intervals of storage at −70°C for up to 6 weeks (18). Protein estimations were done by a slight modification of the Lowry method, in which 1% sodium dodecylsulfate was added in order to solubilize lipids (19). Bovine serum albumin was used as the standard for this assay.

Enzyme assays

ACAT activity was measured in 0.5 ml of incubation mixture containing 0.1 mg of microsomal protein, 0.1 M K_2HPO_4 adjusted to pH 7.4, 1 μmol of dithiothreitol, 15 nmol of fatty acid-free bovine serum albumin, and 15 nmol of palmitoyl coenzyme A (1.7 × 10^5 dpm [1-^{14}C]palmitoyl coenzyme A). The mixture was incubated for 10 min at 37°C before the reaction was initiated by adding the palmitoyl CoA. Unless noted otherwise, the reaction was carried out for 10 min at 37°C. It was stopped by addition of 3 ml of chloroform–methanol 2:1 (v/v), and the lipids were extracted into chloroform (20). Neutral lipid standards obtained from Nu-Chek-Prep (Elysian, MN) were added to each sample. The cholesteryl esters were isolated by thin-layer chromatography on silica gel G (Analabs, Inc., North Haven, CT) using a solvent system consisting of hexane–diethyl ether–methanol–acetic acid 170:40:2:2. Lipids were visualized by exposure of the chromatogram to I_2 vapor. After sublimation of the I_2, the outlined segments of the silica gel were scraped directly into scintillation vials containing 10 ml of scintillation fluid (Budget-Solve; Research Products International Corp., Elk Grove Village, IL). Measurements of radioactivity were made with a Beckman LS7000 liquid scintillation spectrometer, and quenching was monitored with a ^{137}Cs external standard.

Acyl CoA hydrolase activity was measured using the same conditions as those employed for ACAT except that the radioactivity contained in the free fatty acid isolated by thin-layer chromatography was measured.

To assay cholesteryl ester hydrolase, [4-^{14}C]cholesteryl oleate was incorporated into egg lecithin liposomes (21). The liposomes were prepared by sonication of this mixture at 50°C for 15 min under an N_2 atmosphere. This mixture was centrifuged at 12,000 *g* for 30 min to remove any metal particles released from the tip of a Branson sonifier. Cholesteryl ester hydrolase activity was assayed in a 0.5 ml volume containing 0.1 M K_2HPO_4, pH 7.4; 1 μmol of dithiothreitol, 15 μmol of fatty acid-free bovine serum albumin, and 0.2 mg of microsomal protein. To this reaction mixture, 5.9 nmol of cholesteryl oleate containing 0.02 μCi [4-^{14}C]cholesteryl oleate was added, and the incubation was continued for 60 min at 37°C. The reaction was terminated as described above, and the radioactivity in unesterified cholesterol isolated by thin-layer chromatography was determined by liquid scintillation counting.

Preparation of liposomes

A chloroform solution containing dipalmitoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) or cholesterol–dipalmitoyl phosphatidylcholine 2:1 (mol/mol) was evaporated under N_2 to dryness. Buffered sucrose solution was added to give 6.7 μmol/ml of phospholipid. This mixture was dispersed for 2 min with a Vortex mixer and sonicated under N_2 at 10 watts output for 15 min at 50°C using a Branson sonifier. The liposome solution was then centrifuged at 12,000 *g* for 30 min to remove any metal particles released by the sonifier.

Lipid analysis

Lipids were extracted from the microsomes using chloroform–methanol 2:1 (v/v) (20). The phospholipid content was estimated according to the procedure of Raheja et al. (22). The total and unesterified cholesterol contents were determined enzymatically with the commercially available cholesterol oxidase method (Cholesterol Reagent Set, Boehringer-Mannheim Corp., Indianapolis, IN). This assay was modified by adding 10 mg of Triton X-100 to the chloroform solution and, after removing the organic solvent by evaporation under N_2, 1 ml of the commercial reagent was added. The samples were mixed thoroughly, incubated for 1 hr at 37°C with shaking, and the absorbance was measured at 410 nm.

Fatty acid composition was determined by gas–liquid chromatography after the samples were saponified and then methylated with 14% BF_3 in methanol (23). The fatty acids were separated using a Hewlett-Packard 5840A gas chromatograph equipped with a flame ionizer detector. SP-2330 on Chromosorb WAW was contained in a 1.9 m × 2 mm glass column, and N_2 (20 ml/min) served as the carrier gas for the separation. Temperature was programmed to 170°C for 4 min, followed by 2°C/min increments to a final temperature of 210°C for 8 min. Fatty acid methyl ester standards were obtained from Supelco, Inc. (Bellefonte, PA) or Nu-Chek-Prep (Elysian, MN).

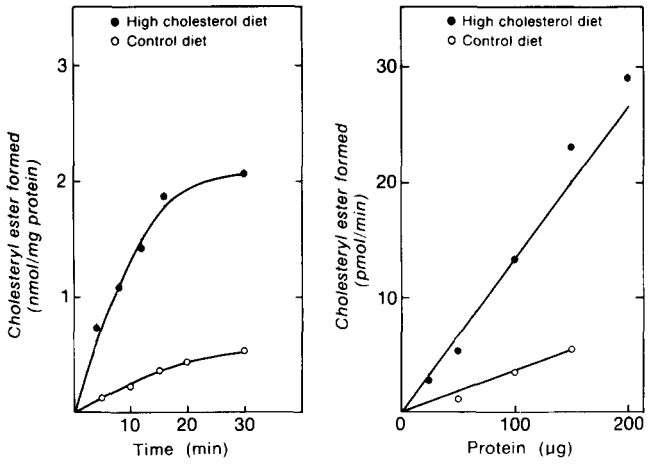

Fig. 1. Effect of incubation time and microsomal protein content on ACAT activity. In the time-course study, each incubation contained 100 μg of microsomal protein. In the protein-concentration study, the time of incubation was 10 min. The values are averages of two microsomal preparations.

RESULTS

Microsomal enzyme activities

ACAT activity was higher under all conditions of assay in hepatic microsomes from the cholesterol-fed monkeys as compared with those fed ordinary monkey chow. **Fig. 1** (left side) shows that the ACAT activity increased proportionately during the first 15 min of incubation and that, at each time point, higher activity was obtained with the microsomes from the cholesterol-fed animals. As seen on the right side of Fig. 1, ACAT activity was linear when up to 200 μg of microsomal protein was added. Again, the ACAT activity of the microsomes isolated from the monkeys fed the high cholesterol diet was greater at each microsomal protein content tested.

In the above experiments, ACAT activity was determined with 30 μM palmitoyl CoA as substrate. As illustrated in **Fig. 2,** the microsomes from the cholesterol-fed group had higher activities at all palmitoyl CoA concentrations tested between 1 and 40

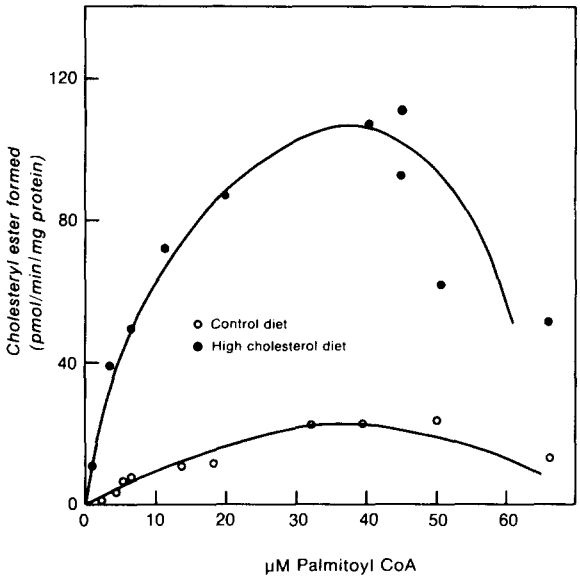

Fig. 2. Effect of palmitoyl CoA concentration on ACAT activity. Each point represents the average of duplicate assays of two microsomal preparations.

μM. However, in both microsome preparations, 14–50% inhibition of ACAT activity was observed when the palmitoyl CoA concentration was raised from 40 to 66 μM. In these experiments 30 μM bovine serum albumin was present in the incubation medium, and no cholesterol was added.

Under optimal conditions of assay, the ACAT activity in hepatic microsomes from monkeys fed the high cholesterol diet for 10 or 100 days was four-fold greater than in corresponding microsomes from monkeys fed the control diet (**Table 2**). Moreover, as shown in Table 2, the rate of hydrolysis of palmitoyl CoA was similar in both microsomal preparations so that the acyl CoA substrate was depleted at approximately the same rate in both cases.

We could not detect cholesteryl ester hydrolase activity in the hepatic microsomes prepared from either the normal or cholesterol-fed monkeys under the conditions of the ACAT assay. Therefore, the differences in ACAT activity cannot be explained on the basis of different rates of hydrolysis of the newly formed cholesteryl ester in the two microsomal preparations. This was further confirmed by measuring the rate of hydrolysis of 4-methyl umbelliferyl oleate (24) in microsomes from two controls and three monkeys fed the high cholesterol diet for 100 days. Microsomes from both groups had similar hydrolytic activity at pH 7.4. At pH 4.2, however, control values (nmol/min per mg protein) were 5.7 and 6.3, whereas values in cholesterol-fed monkeys were 8.5, 14.4, and 15.5.[1] Therefore, it is possible that a difference in hydrolytic activity occurs in the lower pH range, but this difference was not manifested at the pH of ACAT assay.

Lipid composition of microsomes

To determine whether the enhanced cholesterol esterification in the cholesterol-fed monkeys might be related to the lipid composition of the microsomes, we measured the cholesterol, cholesteryl ester, and phospholipid content of the hepatic microsomes from the cholesterol-fed and the normal monkeys. As seen in **Table 3,** the hepatic microsomes from the cholesterol-fed monkeys had 2.6-fold and 1.5-fold higher cholesteryl ester and cholesterol contents, respectively. There was no significant difference in the phospholipid contents, so that the cholesterol to phospholipid ratio (mol/mol) was higher in the hepatic microsomes from the cholesterol-fed monkeys.

As shown in **Table 4,** the fatty acid composition also was different in the two microsomal preparations. The microsomes from the cholesterol-fed monkeys contained less linoleic acid but more arachidonic and docosapentaenoic acids. In spite of these changes in individual fatty acids, the distribution of the fatty acids in saturation classes was similar in both microsomal preparations. Due to the relative decrease in linoleic acid and increase in arachidonic acid, the microsomes from the cholesterol-fed animals had a higher unsaturation index.

The lipid compositional changes produced by the high cholesterol, saturated fat-rich diet were essentially the same after both 10 and 100 days of feeding.

[1] Sando, G. N., and S. N. Mathur. Unpublished observations.

TABLE 2. Enzyme activity in liver microsomes

Diet	ACAT Activity[a]	Acyl CoA Hydrolase[a]
	pmol/min/mg protein	
Control	35 ± 13	4016 ± 946[c]
High cholesterol		
10 days	142 ± 10[b]	4319 ± 213
100 days	161 ± 24[b]	2866 ± 219

[a] Mean ± S.E.M. of values obtained from three separate microsomal preparations.
[b] ACAT values obtained during feeding of atherogenic diet differ significantly from the values obtained on the control diet, $P < 0.05$.
[c] The differences in hydrolase activity are not statistically significant, $P > 0.1$.
In all tables, Duncan's multiple range test was used to show statistically significant differences between means, $P < 0.05$.

TABLE 3. Lipid composition of liver microsomes

Diet	Cholesterol[a]		Phospholipids[a]	Free Cholesterol/ Phospholipid
	Esterified	Free		
	nmol/mg protein			*mol/mol*
Control	5.3 ± 2.2	94 ± 5	727 ± 57[c]	0.13 ± 0.004
High cholesterol				
10 days	14.0 ± 3.2[b]	142 ± 12[b]	814 ± 29	0.18 ± 0.022
100 days	13.0 ± 0.0[b]	137 ± 3[b]	772 ± 42	0.18 ± 0.006

[a] Mean ± SE of values obtained from three separate microsomal preparations.
[b] The differences in free and esterified cholesterol content between the control monkeys and those fed the atherogenic diet are statistically significant, $P < 0.05$.
[c] The differences in phospholipid content are not statistically significant.

Enrichment of microsomes with cholesterol

One possible explanation for the higher ACAT activity in the cholesterol-fed monkeys is the larger unesterified cholesterol content of the microsomes, since cholesterol is one of the substrates for the reaction. In other systems, ACAT activity has been shown to be greater when the cholesterol content is increased (6–11, 25, 26). To test this possibility, we examined the effect of adding cholesterol in vitro to microsomal preparations isolated from the livers of monkeys fed the control diet. Both the cholesterol content and ACAT activity were measured in the preparations before and after cholesterol loading. Two methods were employed to increase the microsomal cholesterol content: addition of cholesterol dissolved in a small volume of acetone, and incubation of the microsomes with liposomes containing a relatively large quantity of cholesterol. In the latter case, the liposomes were separated from the microsomes by sedimentation through a sucrose solution prior to assay for ACAT activity and cholesterol content.

As shown in **Fig. 3,** only a slight stimulation of ACAT activity was observed when the cholesterol content of the incubation system was raised from 84 nmol of cholesterol, the inherent cholesterol content of the microsomes per mg microsomal protein, to 284 nmol by addition of cholesterol dissolved in acetone. By contrast, large increases in ACAT activity were produced when the microsomes were exposed to cholesterol-rich liposomes. In these experiments, the normal monkey hepatic microsomal cholesterol content was raised by incubation with choles-

TABLE 4. Fatty acid composition of liver microsomes[a]

Fatty Acid	Control Diet	High Cholesterol Diet	
		10 Days	100 Days
	%	%	
Individual acids			
16:0	19.3 ± 0.3	17.0 ± 0.7	17.9 ± 0.3
18:0	21.3 ± 0.2	21.4 ± 1.0	23.0 ± 0.5
18:1	11.8 ± 0.2	13.6 ± 0.9	12.9 ± 0.6
18:2	22.2 ± 0.7	16.8 ± 0.2[b]	15.6 ± 1.1[b]
20:3	2.3 ± 0.3	2.2 ± 0.2	1.6 ± 0.1
20:4	11.6 ± 0.5	16.3 ± 1.2[b]	17.2 ± 0.8[b]
22:5	0.6 ± 0.1	2.2 ± 0.2[b]	2.5 ± 0.1[b]
22:6	5.8 ± 0.4	6.3 ± 1.1	5.5 ± 0.7
Classes			
Saturated	40.6 ± 0.6	38.5 ± 0.7	40.9 ± 0.6
Monoenoic	12.9 ± 0.4	14.6 ± 1.2	13.8 ± 0.7
Polyenoic	45.7 ± 0.7	45.6 ± 0.8	44.2 ± 0.1
Unsaturation index	1.57	1.76	1.71

[a] Mean ± SE of values obtained from three separate microsomal preparations.
[b] $P < 0.05$, high cholesterol diet as compared to control diet.

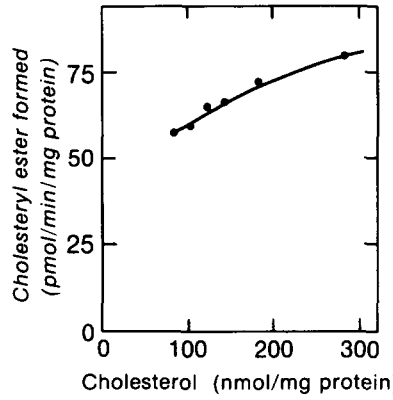

Fig. 3. Effect of cholesterol added in acetone on the ACAT activity of normal monkey liver microsomes. The reaction mixture contained 100 μg microsomal protein (8.4 nmol of cholesterol), 15 nmol of bovine serum albumin, 1 nmol dithiothreitol, and 0.1 M K_2HPO_4 buffer, pH 7.4 in a total volume of 0.4 ml. To this mixture 0, 2, 4, 6, 10, or 20 nmol of cholesterol in 10 μl acetone was added with vigorous agitation using a Vortex mixer. The contents were incubated with shaking for 10 min at 37°C, and the reaction was initiated by adding 15 nmol of [^{14}C]palmitoyl CoA in 0.1 ml of 0.1 M K_2HPO_4, pH 7.4. The incubation was for 10 min at 37°C. The abscissa of the graph gives the total cholesterol content of the incubation system following addition of the material in acetone.

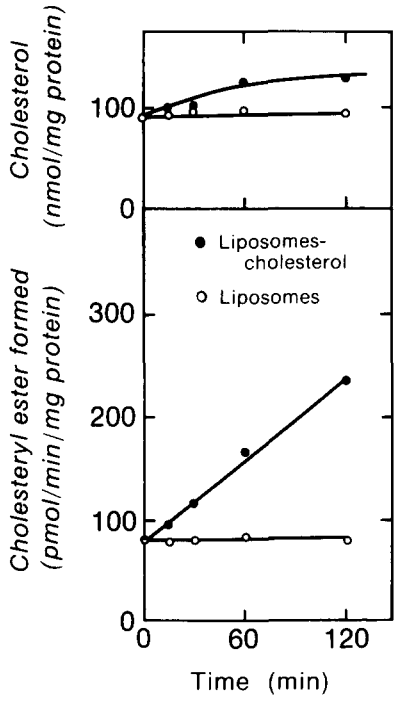

Fig. 4. Effect of cholesterol added in the form of a liposome complex on the ACAT activity of normal monkey liver microsomes. The microsomes were incubated with cholesterol–dipalmitoyl phosphatidylcholine liposomes (2:1, mol/mol) for the times indicated on the abscissa, to increase the microsomal unesterified cholesterol content. As a control, additional aliquots of microsomes were incubated with dipalmitoyl phosphatidylcholine liposomes containing no cholesterol. The incubation mixtures contained 0.9 μmol of phospholipid in liposomes, 3.6 μmol of microsomal phospholipids, and 5 mg of fatty acid-free bovine serum albumin in 1 ml of buffered sucrose solution. After incubation, the mixture was cooled in ice, layered over 5 ml of 20% sucrose solution, and sedimented at 4°C for 1 hr at 105,000 *g*. The microsomal pellet was dispersed in 2 ml of buffered sucrose solution, layered over 5 ml of 20% sucrose solution, and again sedimented at 105,000 *g* for 1 hr. The pellet was dispersed in buffered sucrose solution to give 2.2 mg protein/ml. In the top panel, the microsomal cholesterol content relative to time of incubation with liposomes is shown, and the ACAT activity of these microsomes is shown in lower panel. The values at 0 time are those for microsomes that were not incubated with liposomes.

terol–dipalmitoyl phosphatidylcholine liposomes (2:1, mol/mol) for various time intervals at 37°C. As illustrated in **Fig. 4,** no change in cholesterol content or ACAT activity was observed when the microsomes were incubated with either buffer alone (value indicated on the ordinate) or dipalmitoyl phosphatidylcholine liposomes containing no cholesterol. However, the cholesterol content of the microsomes was raised from 90 nmol to 128 nmol/mg protein after 2 hr of incubation when the microsomes were incubated with the cholesterol–dipalmitoyl phosphatidylcholine liposomes. This was associated with a time-dependent increase in ACAT activity, which was 2.6-fold higher after 2 hr of incubation with these liposomes.

DISCUSSION

This work demonstrates that hepatic cholesterol esterification in monkeys can be influenced by the lipid composition of the diet. The response is evident within 10 days after a high cholesterol and saturated diet is begun, the earliest time tested. There was a 1.5-fold increase in the cholesterol content of the hepatic microsomes, accompanied by a 4-fold enhancement in ACAT activity. A similar enhancement of ACAT activity in response to an atherogenic diet containing an elevated cholesterol content has been reported in aortic microsomes from pigeons (27), rabbits (28–30), and monkeys (3). Higher activity of ACAT also has been observed in other experimental systems when the cellular cholesterol content is raised. For example, human skin fibroblasts in culture esterify cholesterol at higher rates when the cells take up cholesterol contained in low density lipoproteins (5, 6). Likewise, the Fu5AH hepatoma cells in culture exhibit higher cholesterol esterification when when the cellular cholesterol content is increased, either by incubation with cholesterol-rich liposomes (7) or with hyperlipemic serum (8). A stimulation of cholesterol esterification has also been demonstrated in normal segments of pigeon aorta (9) and in explants of the aortic media from rhesus monkeys in culture (10) when these tissues are exposed to hypercholesterolemic serum. Finally, stimulation of de novo synthesis of cholesterol with mevalonate, either by administration to rats by stomach tube (31) or by incubation with rat hepatocytes (11), results in a higher cellular cholesterol content as well as increased cholesteryl ester formation. These findings, together with the present results of both feeding cholesterol and enriching microsomes with cholesterol in vitro, indicate that ACAT in many different tissues is not saturated with respect to this substrate under ordinary physiologic conditions. The enzyme apparently has a large capacity to respond to elevations in intracellular cholesterol content, accomodating for this imbalance by increasing the synthesis of cholesteryl esters within the cell.

The molecular mechanism by which augmentation of cellular cholesterol content brings about an increase in ACAT activity is not well understood. One possibility is that cholesterol provides additional substrate for the reaction, the cholesterol entering a metabolic pool which is accessible to the enzyme (11, 12, 28, 30). Alternatively, like other oxygenated sterols and progesterone, cholesterol or one of its metabolites may modulate ACAT activity by interactions with one or more regulatory sites of the enzyme (6, 12, 32). In addition, it is possible that the increase

in cholesterol content may alter the fluidity of the microsomal membrane and thereby influence ACAT activity, for the molar ratio of microsomal cholesterol to phospholipid increases. Which of these possible mechanisms is primarily responsible for the diet-induced increase in the monkeys fed the high cholesterol diet remains to be elucidated.

These results demonstrate that the increase in ACAT activity is not directly proportional to the increase in microsomal cholesterol content. For example, the ACAT activity was about four to five times higher in the hepatic microsomes from the cholesterol-fed monkeys, whereas the unesterified cholesterol content of these microsomes increased only about 50%. Likewise, the percentage increase in ACAT activity of the microsomes exposed to liposomes containing cholesterol was considerably higher than the actual cholesterol enrichment. While such observations suggest that cholesterol may act by a mechanism other than simply providing more substrate, it must be remembered that there are at least two pools of microsomal cholesterol, only one of which is accessible to ACAT (4). It is likely that a considerable fraction of the membrane cholesterol is in the inaccessible pool. If most of the added cholesterol should enter the accessible pool, then a relatively small total increase actually may represent a large increment in substrate availability. Therefore, the failure to observe a direct correlation between increases in microsomal cholesterol content and ACAT activity does not exclude the possibility that cholesterol enhances the activity primarily or even entirely by providing more substrate. These studies also point out the importance of the manner in which cholesterol is presented in vitro in assessing effects of added cholesterol. Addition of cholesterol in acetone produced only a small enhancement in ACAT activity. It is possible that when added in this way, much of the cholesterol was adsorbed in microcrystalline form to the membrane so that it was not accessible to the enzyme. Furthermore, only a small percentage of the exogenously added cholesterol may have been in contact with the microsomes, much less have entered a pool available to ACAT. The actual distribution of the added cholesterol may depend on exactly how the procedure is carried out, leading to additional variability. In contrast, prior exposure of the microsomes to liposomes containing cholesterol produced a large increase in activity relative to the actual enrichment with cholesterol. This difference should be kept in mind in considering the effects of added cholesterol on other experimental systems.

Like many other membrane-bound enzymes, the activity of ACAT can be influenced by the fatty acid composition of the microsomal membranes (25, 33, 34). This dependence on membrane fatty acid composition has been demonstrated in microsomes enriched in saturated or unsaturated fatty acids, but having similar cholesterol and phospholipid content (13, 33). Besides increasing the cholesterol content, the atherogenic diet produced some alteration in the fatty acid composition of the microsomes. Therefore, it is possible that this change also contributed to the enhancement of ACAT activity in these microsomes. While differences in some individual fatty acids were appreciable, for example, a 45% difference in arachidonic acid, the changes produced in fatty acid class distribution or unsaturation index are extremely small. This, coupled with the increase in cholesterol content and the effects produced by enriching the normal monkey hepatic microsomes with cholesterol in vitro, suggest that the fatty acid compositional differences probably have only a minor influence in the present system.

The high cholesterol-saturated fat diet used in this study was investigated to determine whether liver ACAT activity changed in monkeys under conditions of intense atherogenesis, in comparison to a control diet known to have no atherogenic effect. Alternative diet selections that would have permitted evaluation of the independent effects of cholesterol and saturated fat would not have provided this unequivocal contrast with respect to atherogenesis and plasma lipid levels. We have emphasized the role of cholesterol in regulating ACAT activity, but the net effects of the atherogenic diet may also include effects caused by the large intake of saturated fat.

High cholesterol-saturated fat diets profoundly alter lipoprotein patterns in monkeys (14, 15, 35–37). The lower density lipoproteins are increased in particle size and number, and these high molecular weight particles have increased amounts of cholesteryl esters. The latter have more saturated and monoenoic, and fewer polyenoic fatty acids, than the cholesteryl esters of control lipoproteins (15). Since hepatic cholesteryl esters may contribute to the cholesteryl ester content of lipoproteins (37), it is plausible that one source of the increased core lipid of the altered lower density lipoproteins may be cholesteryl esters formed in the liver in response to pathogenic high cholesterol diets. Therefore, we suggest that the increase in hepatic ACAT activity may play a role in the production of abnormal lipoproteins found in nonhuman primates fed diets high in cholesterol and saturated fat.

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REFERENCES

1. Goodman, D. S., D. Deykin, and T. Shiratori. 1964. The formation of cholesterol esters with rat liver enzymes. *J. Biol. Chem.* **239:** 1335–1345.
2. Stokke, K. T. 1974. Cholesteryl ester metabolism in liver and blood plasma of various animal species. *Atherosclerosis.* **19:** 393–406.
3. Brecher, P. I., and A. V. Chobanian. 1974. Cholesteryl ester synthesis in normal and atherosclerotic aortas of rabbits and rhesus monkeys. *Circ. Res.* **35:** 692–701.
4. Balasubramaniam, S., S. Venkatesan, K. A. Mitropoulos, and T. Peters. 1978. The submicrosomal localization of acyl coenzyme A-cholesterol acyltransferase and its substrate, and of cholesteryl esters in rat liver. *Biochem. J.* **174:** 863–872.
5. Brown, M. S., J. R. Faust, and J. L. Goldstein. 1975. Role of the low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.* **55:** 783–793.
6. Goldstein, J. L., J. R. Faust, J. H. Dygos, R. J. Chorvat, and M. S. Brown. 1978. Inhibition of cholesteryl ester formation in human fibroblasts by an analogue of 7-ketocholesterol and by progesterone. *Proc. Natl. Acad. Sci. USA.* **75:** 1877–1881.
7. Arbogast, L. Y., G. H. Rothblat, M. H. Leslie, and R. A. Cooper. 1976. Cellular cholesterol ester accumulation induced by free cholesterol-rich lipid dispersions. *Proc. Natl. Acad. Sci. USA.* **73:** 3680–3684.
8. Rothblat, G. H., L. Arbogast, D. Kritchevsky, and M. Naftulin. 1976. Cholesteryl ester metabolism in tissue culture cells. II. Source of accumulated esterified cholesterol in Fu5AH rat hepatoma cells. *Lipids.* **11:** 98–108.
9. St. Clair, R. W., and G. J. Harpold. 1975. Stimulation of cholesterol esterification in vitro in organ cultures of normal pigeon aorta. *Exp. Mol. Pathol.* **22:** 207–219.
10. St. Clair, R. W. 1977. The arterial smooth muscle cell: stimulation of cholesterol esterification by plasma lipoproteins. *Adv. Exp. Med. Biol.* **82:** 921–927.
11. Nilsson, A. 1975. Increased cholesterol-ester formation during forced cholesterol synthesis in rat hepatocytes. *Eur. J. Biochem.* **51:** 337–342.
12. Goldstein, J. L., and M. S. Brown. 1976. The LDL pathway in human fibroblasts: a receptor-mediated mechanism for the regulation of cholesterol metabolism. *Curr. Top. Cell Regul.* **11:** 147–181.
13. Spector, A. A., T. L. Kaduce, and R. W. Dane. 1980. Effect of dietary fat saturation on acylcoenzyme A: cholesterol acyltransferase activity of rat liver microsomes. *J. Lipid Res.* **21:** 169–179.
14. Rudel, L. L., L. L. Pitts, and C. A. Nelson. 1977. Characterization of plasma low density lipoproteins of nonhuman primates fed dietary cholesterol. *J. Lipid Res.* **18:** 211–222.
15. Tall, A. R., D. M. Small, D. Atkinson, and L. L. Rudel. 1978. Studies on the structure of low density lipoproteins isolated from *Macaca fascicularis* fed an atherogenic diet. *J. Clin. Invest.* **62:** 1354–1363.
16. Kramsch, D. M., and W. Hollander. 1978. Occlusive atherosclerotic disease of the coronary arteries in monkey (*Macaca irus*) induced by diet. *Exp. Mol. Pathol.* **9:** 1–22.
17. Armstrong, M. L., M. B. Megan, and E. D. Warner. 1978. The relation of hypercholesterolemic fatty streaks to intimal permeability changes shown by Evans blue. *Atherosclerosis.* **31:** 443–452.
18. Lichtenstein, A. H., and P. Brecher. 1980. Properties of acyl CoA:cholesterol acryltransferase in rat liver microsomes. Topological localization and effect of detergents, albumin, and polar steroids. *J. Biol. Chem.* **255:** 9098–9104.
19. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.* **47:** 184–192.
20. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
21. Brecher, P., J. Chobanian, D. M. Small, and A. V. Chobanian. 1976. The use of phospholipid vesicles for in vitro studies on cholesteryl ester hydrolysis. *J. Lipid Res.* **17:** 239–247.
22. Raheja, R. K., C. Kaur, A. Singh, and I. S. Bhatia. 1973. New colorimetric method for the quantitative estimation of phospholipids without acid digestion. *J. Lipid Res.* **14:** 695–697.
23. Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* **38:** 514–515.
24. Cortner, J. A., P. M. Coates, E. Swoboda, and J. D. Schnatz. 1976. Genetic variation of lysosomal acid lipase. *Pediatr. Res.* **10:** 927–932.
25. Hashimoto, S., and S. Dayton. 1977. Studies of the mechanism of augmented synthesis of cholesteryl ester in atherosclerotic rabbit aortic microsomes. *Atherosclerosis.* **28:** 447–452.
26. Hashimoto, S., and S. Dayton. 1979. Stimulation of cholesterol esterification in hepatic microsomes by lipoproteins from normal and hypercholesterolemic rabbit serum. *Biochim. Biophys. Acta* **573:** 354–360.
27. St. Clair, R. W., H. B. Lofland, and T. B. Clarkson. 1970. Influence of duration of cholesterol feeding on esterification of fatty acids by cell-free preparations of pigeon aorta. Studies on the mechanism of cholesterol esterification. *Circ. Res.* **27:** 213–225.
28. Brecher, P., and C. T. Chan. 1980. Properties of acyl CoA:cholesterol *O*-acyltransferase in aortic microsomes from atherosclerotic rabbits. *Biochim. Biophys. Acta* **617:** 458–471.
29. Proudlock, J. W., and A. J. Day. 1972. Cholesterol esterifying enzymes of atherosclerotic rabbit intima. *Biochim. Biophys. Acta* **260:** 716–723.
30. Hashimoto, S., S. Dayton, R. B. Alfin-Slater, P. T. Bui, N. Baker, and L. Wilson. 1974. Characteristics of the cholesterol-esterifying activity in normal and atherosclerotic rabbit aortas. *Circ. Res.* **34:** 176–183.
31. Mitropoulos, K. A., S. Balsubramaniam, S. Venkatesan, and B. E. A. Reeves. 1978. On the mechanism for the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A

reductase, of cholesterol 7α-hydroxylase and of acyl-coenzyme A:cholesterol acyltransferase by free cholesterol. *Biochim. Biophys. Acta.* **530:** 99–111.
32. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Cholesterol ester formation in cultured human fibroblasts. *J. Biol. Chem.* **250:** 4025–4027.
33. Brenneman, D. E., T. Kaduce, and A. A. Spector. 1977. Effect of dietary fat saturation on acylcoenzyme A: cholesterol acyltransferase activity of Ehrlich cell microsomes. *J. Lipid Res.* **18:** 582–591.
34. Hashimoto, S., and S. Dayton. 1978. Stimulation of acyl CoA:cholesterol acyltransferase activity in rat liver microsomes by phosphatidylcholine. *Biochem. Biophys. Res. Commun.* **82:** 1111–1120.
35. Mahley, R. W., K. H. Weisgraber, and T. Innerarity. 1976. Atherogenic hyperlipoproteinemia induced by cholesterol feeding in the Patas monkey. *Biochemistry.* **15:** 2979–2985.
36. Paula, R. K., L. L. Rudel, and T. B. Clarkson. 1977. A comparative study of the effect of dietary cholesterol on plasma lipoproteins in nonhuman primates. *Adv. Exp. Med. Biol.* **82:** 256–258.
37. Mahley, R. W. 1978. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietschy, A. M. Gotto, Jr., and J. A. Ontko, editors. American Physiological Society, Bethesda, MD. 181–197.