

# Effect of dietary fat saturation on acylcoenzyme A: cholesterol acyltransferase activity of rat liver microsomes

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**Abstract** The saturation of the fat contained in the diet has been observed to affect the acylcoenzyme A:cholesterol acyltransferase (ACAT) activity of rat liver microsomes. ACAT activity in microsomes ( $M_p$ ) prepared from livers of rats fed a polyunsaturated fat-enriched diet containing 14% sunflower seed oil was 70–90% higher than in microsomes ( $M_s$ ) prepared from livers of rats fed a saturated fat-enriched diet containing 14% coconut oil. This difference was observed within 20 days after the diets were begun, the earliest time tested, and persisted throughout the 70-day experimental period. The difference was noted at all [1- $^{14}$ C]-palmitoyl CoA concentrations tested, 2.5–33  $\mu$ M, and at temperatures between 18 and 40°C. Arrhenius plots revealed a single transition in enzyme activity, occurring at 29°C in both microsomal preparations. Likewise, the activation energy above this transition was the same in  $M_p$  and  $M_s$ , 12.5 KCal/mol. Addition of albumin to the incubation medium increased the ACAT activity of both microsomal preparations, but the difference between  $M_p$  and  $M_s$  persisted.  $M_p$  was enriched in polyenoic fatty acids, primarily 18:2 and 20:4, while  $M_s$  was enriched in monoenoic fatty acids. Although the 20:4 increase in  $M_p$  occurred in all phosphoglycerides, it was especially pronounced in the serine and inositol phosphoglyceride fraction. There were no differences in the phospholipid or cholesterol content, phospholipid head group composition, or protein composition of the two microsomal preparations. The possibility is discussed that the changes in ACAT activity result from the differences in fatty acid composition of the microsomes. Other microsomal enzymes exhibited varying responses to these dietary fatty acid modifications. Palmitoyl CoA hydrolase and NADPH cytochrome c reductase activities were unchanged. UDP glucuronyl transferase activity was 50% higher in  $M_p$ , but glucose-6-phosphatase and NADH cytochrome  $b_5$  reductase activities were 25% higher in  $M_s$ . Therefore, dietary fat modifications do not produce a uniform effect on the activity of microsomal enzymes.—**Spector, A. A., T. L. Kaduce, and R. W. Dane.** Effect of dietary fat saturation on acylcoenzyme A: cholesterol acyltransferase activity of rat liver microsomes. *J. Lipid Res.* 1980. **21:** 169–179.

**Supplementary key words** cholesteryl esters · phospholipids · triglycerides · membranes · acyl CoA hydrolase · NADPH cytochrome c reductase · NADH cytochrome  $b_5$  reductase · UDP glucuronyl transferase · glucose 6-phosphatase

Acylcoenzyme A:cholesterol acyltransferase activity (ACAT) (E.C. 2.3.1.26) catalyzes the synthesis of cholesteryl esters in mammalian cells (1). ACAT is present in many tissues, including the liver, where it is located almost exclusively in the hepatocytes (2). The enzyme is bound tightly to intracellular membranes and is recovered primarily in the rough endoplasmic reticulum fraction (3). ACAT appears to have several important metabolic roles. It protects against unesterified cholesterol accumulation in isolated rat hepatocytes (4). ACAT also esterifies the cholesterol that is released intracellularly during the catabolism of plasma lipoproteins taken up from the extracellular fluid (5, 6). Since ACAT catalyzes cholesteryl ester formation in the arterial intima, it has been implicated in the development of atherosclerosis (7–9). Therefore, it is important to determine the factors which regulate the activity of this enzyme.

Studies with cultured human fibroblasts indicated that oxygenated sterols and progesterone can influence ACAT activity. This suggests that the enzyme contains a regulatory site that interacts with steroids (10). A similar mechanism appears to operate in the liver, for the administration of ethynylestradiol to rats increases hepatic ACAT activity (11). Free fatty acids also are effectors of hepatic ACAT activity (1). They inhibit, probably by competing for the acyl CoA binding site of the enzyme. Another type of control has been observed during the course of dietary studies designed to modify the membrane lipids of Ehrlich ascites cells. ACAT activity, as measured with either radioactive palmitoyl CoA or cholesterol as the tracer, was altered when the fatty acid composition of the microsomal fraction was modified (12). No differences

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase;  $M_s$ , microsomes isolated from the livers of rats fed the saturated fat-rich diet;  $M_p$ , microsomes isolated from the livers of rats fed the polyunsaturated fat-rich diet.

in the phospholipid or cholesterol content of the microsomes was produced by the dietary modifications. Before exploring the mechanism of this effect, we wished to be certain that the phenomenon had some general applicability and was not peculiar to Ehrlich ascites cell. Since the fatty acid composition of rat liver endoplasmic reticulum can be modified extensively by diet (13), we have investigated whether similar effects on ACAT activity would be produced in this more representative experimental system. We found that, as in Ehrlich ascites cells, dietary lipid modifications were associated with appreciable changes in the ACAT activity of rat liver microsomes.

## MATERIALS AND METHODS

### Animals and diets

Weanling male Sprague-Dawley rats weighing  $65 \pm 2$  g were fed a semisynthetic diet consisting of 54% sucrose, 27% casein, 1% vitamin mix, and 4% mineral mix (Teklad Mills, Madison, WI) supplemented with either 14% sunflower oil (Cargill, Inc., Minneapolis, MN) or 14% coconut oil (Ruger Chemical Co., Irvington, NJ). The preparation and fatty acid composition of these diets have been reported (14). The animals were housed in a room maintained at 20–22°C with lights on from 0700 to 1900 hr.

### Preparation of microsomes

Rats were killed between 0800 and 1000 hr by decapitation. They had free access to food up to the time that they were decapitated. After the livers were perfused with ice-cold isotonic saline, they were removed, blotted dry, weighed, and suspended in a buffered 0.25 M sucrose solution (15). The livers were homogenized by mincing with a scissors, followed by five passes in a Potter-Elvehjem tissue grinder equipped with a motor-driven Teflon pestle. The homogenate was subjected to two preliminary centrifugations, 10,000 *g* for 10 min at 4°C, followed by 20,000 *g* for 10 min, to remove heavier particles. The resulting supernatant solution was filtered through glass wool and then centrifuged at 104,000 *g* for 50 min at 4°C. After the pellet was resuspended in 20 ml of buffer containing 0.1 M  $K_2HPO_4$  and 1 mM dithiothreitol, pH 7.2, the microsomes were sedimented again by centrifugation and then dispersed in this buffer solution. The protein concentration was adjusted to 10–15 mg/ml.

### ACAT assay

Unless noted otherwise, the incubation mixtures consisted of 0.2 mg of microsomal protein, 0.1 M

$K_2HPO_4$  adjusted to pH 7.2, and 1 mM dithiothreitol. Between  $9 \times 10^4$  and  $2.2 \times 10^5$  dpm of [ $1-^{14}C$ ]palmitoyl CoA and 7.5 nmol of palmitoyl CoA was added. The total volume of the incubation was 0.5 ml. Free fatty acid was not incorporated into cholesteryl esters in this assay unless ATP and CoA were added to the incubation medium (12). As will be described, there were fatty acid compositional differences in the two hepatic microsome preparations. We wished to avoid complications in interpretation due to possible dilution of the labeled substrate by the inherent fatty acyl groups. Therefore, none of the assays were performed with radioactive fatty acids as the substrate, and neither ATP nor CoA was added in any of the incubations. Likewise, no cholesterol was added in these assays, and the inherent cholesterol in the microsomes served as the second substrate.

In preliminary tests, cholesterol was incorporated into the microsomes by incubation for up to 2 hr at 37°C with liposomes composed of egg yolk phosphatidylcholine and [ $1,2-^3H$ ]cholesterol. When liver microsomes obtained from rats fed regular rodent chow were tested, incubation with these liposomes produced some increase in ACAT activity. The largest increase occurred after 2 hr of incubation. Therefore, the 2-hr time point was selected for testing the microsomes from the livers of rats fed the coconut oil diet ( $M_s$ ) or the sunflower oil diet ( $M_p$ ). As a control, the ACAT activities of  $M_p$  and  $M_s$  were measured prior to exposure to the liposomes. In these control measurements, the ACAT activities of  $M_p$  and  $M_s$  were  $63 \pm 3$  and  $47 \pm 4$  pmol/mg protein  $\times$  min, respectively. After incubation for 2 hr with the liposomes, the ACAT activities of  $M_p$  and  $M_s$  were  $92 \pm 7$  and  $76 \pm 4$  pmol/mg protein  $\times$  min, respectively. While the activity of both preparations increased by about 50% when they were enriched with cholesterol in this manner, the difference in activity between  $M_p$  and  $M_s$  persisted and was of about the same magnitude. Furthermore, incubation with the liposomes produced alterations in the fatty acid composition of  $M_p$  and  $M_s$ , as measured by gas–liquid chromatography. Since the difference in ACAT activity between  $M_p$  and  $M_s$  was not abolished by incubation with the liposomes containing cholesterol, and the changes produced in the inherent microsomal fatty acid composition might interfere with interpretations of the results, we elected not to add cholesterol to the microsomes in the present experiments.

The incubations usually were carried out for 5 min at 30°C with shaking. The reactions were terminated by addition of 2 ml of chloroform–methanol 2:1 (v/v), immediately followed by vigorous agitation. After the phases separated, an aliquot of the chloroform phase

was taken for measurement of lipid radioactivity. Additional aliquots of the chloroform solution were taken for thin-layer chromatography on silica gel G in order to separate the lipid components (16). A solvent system consisting of hexane–diethyl ether–methanol–acetic acid 170:40:4:4 was used, and standards obtained from Nu-Chek Prep, (Elysian, MN) were added to each chromatogram. Lipids were visualized by exposure of the chromatogram to I<sub>2</sub> vapor. After sublimation of the I<sub>2</sub>, the segments of silica gel containing lipids were scraped directly into liquid scintillation vials containing 10 ml of a Triton X-100 toluene scintillation solution (Budget Solve; Research Products International, Elk Grove Village, IL). Measurements of radioactivity were made with a Packard TriCarb model 2425 refrigerated liquid scintillation spectrometer, and quenching was monitored with a <sup>226</sup>Ra external standard.

### Other microsomal enzyme assays

Acyl CoA hydrolase was measured using the same conditions as those employed for ACAT. In order to measure glucose 6-phosphatase activity, microsomes were prepared in 0.25 M sucrose buffered with 5 mM Tris, pH 7.0, rather than KH<sub>2</sub>PO<sub>4</sub>. The activity of this enzyme and that of UDP-glucuronyl transferase were measured according to Zakim and Vessey (17). NADPH-cytochrome c reductase and NADH-cytochrome b<sub>5</sub> reductase activities were measured spectrophotometrically at 22°C (18, 19).

### Chemical analyses

Protein estimations were done by a slight modification of the Lowry method, in which 1% sodium dodecylsulfate is added in order to solubilize lipid (20). Bovine serum albumin was used as the standard. Sodium dodecylsulfate-polyacrylamide disc gel electrophoresis was carried out using 6.5% gels (21), and Coomassie blue was employed as the stain (22). Palmitoyl CoA concentrations were determined using the adenosine molar extinction coefficient of  $15.4 \times 10^3$  at 260 nM. The palmitoyl CoA and [1-<sup>14</sup>C]palmitoyl CoA samples were saponified, methylated, and assayed for fatty acid composition by gas–liquid chromatography. This analysis revealed that more than 99% of the mass and 97% of the radioactivity migrated as palmitic acid. Similar purities were obtained for the [1-<sup>14</sup>C]oleoyl CoA substrate by this procedure. Further analysis of the radioactive acyl CoA samples indicated that more than 98% of their radioactivity cochromatographed with corresponding acyl CoA standards on thin layers of silica gel G.

Lipids were isolated by extraction with chloroform–methanol 2:1 (v/v) (23), and aliquots of the isolated,

washed chloroform phase were taken for analysis. Phospholipid classes were separated by thin-layer chromatography on silica gel H with a solvent system containing chloroform–methanol–acetic acid–water 100:60:16:8 (24). The phospholipids were eluted by the method of Raheja et al. (25) with dipalmitoyl lecithin as the standard. Triglycerides were measured with the Technicon Auto Analyzer II method (26). Free and total cholesterol were measured enzymatically with the commercially available cholesterol oxidase method (Cholesterol Reagent Set, Boehringer-Mannheim Corp., Indianapolis, IN). In this assay, 10 mg of Triton X-100 was added to the chloroform solution. After removing the organic solvent by evaporation under N<sub>2</sub>, 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. The lipid samples were saponified, methylated with 14% BF<sub>3</sub> in methanol, and separated using a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector (27). SP-2340 on Chromasorb WAW was used in a 1.9 M × 2 mm ID glass column, and N<sub>2</sub> (20 ml/min) served as the carrier gas. Peak areas were measured with a Hewlett-Packard 3380A integrator. Fatty acid methyl ester standards were obtained from Supelco, Inc. (Bellefonte, PA) and Nu-Chek Prep (Elysian, MN).

## RESULTS

### Microsomal lipid modifications

Differences were observed in the fatty acid composition of microsomes isolated from the livers of rats fed the saturated and polyunsaturated fat-enriched diets. **Table 1** shows the phospholipid fatty acyl composition of microsomes obtained from rats fed these diets for 20, 40 or 70 days. Although some time-dependent variations were observed, the results were generally similar in each case. The microsomes isolated from rats fed the diet enriched in saturated fat (M<sub>s</sub>) contained more monoenoic and less polyenoic fatty acids than those isolated from the rats fed the polyunsaturated fat-enriched diet (M<sub>p</sub>). These differences are accounted for primarily by increases in the 16:1<sup>1</sup> and 18:1 content of M<sub>s</sub>, as opposed to increases in the 18:2 and 20:4 content of M<sub>p</sub>. The unexpected increases in the 20:4 and 22:6 content of

<sup>1</sup> Fatty acids are abbreviated as, number of carbon atoms: number of double bonds.

TABLE 1. Fatty acid composition of microsomes

Fatty Acid	Percentage Composition <sup>a</sup>					
	Day 20 <sup>b</sup>		Day 40		Day 70	
	M <sub>s</sub> <sup>c</sup>	M <sub>p</sub>	M <sub>s</sub>	M <sub>p</sub>	M <sub>s</sub>	M <sub>p</sub>
	%		%		%	
Classes <sup>d</sup>						
Saturated	41	39	41	41	39	41
Monoenoic	27	17	25	12	21	11
Polyenoic	26	44	32	46	38	46
Major individual acids						
16:0 <sup>e</sup>	22.9 ± 1.4	18.8 ± 1.8	19.0 ± 0.6	16.0 ± 0.4 <sup>g</sup>	14.4 ± 1.0	15.4 ± 0.4
18:0	17.8 ± 1.1	19.9 ± 1.0	20.7 ± 0.7	24.2 ± 0.6 <sup>g</sup>	24.4 ± 0.7	24.5 ± 1.1
16:1	5.8 ± 0.4	1.4 ± 0.4 <sup>g</sup>	4.6 ± 0.5	0.9 ± 0.1 <sup>g</sup>	1.9 ± 0.2	0.6 ± 0.1 <sup>g</sup>
18:1	20.5 ± 0.7	8.3 ± 0.4 <sup>g</sup>	18.4 ± 0.5	6.9 ± 0.2 <sup>g</sup>	12.9 ± 0.2	7.4 ± 0.4 <sup>g</sup>
18:2	6.4 ± 0.4	14.3 ± 0.2 <sup>g</sup>	8.9 ± 0.2	12.5 ± 0.3 <sup>g</sup>	6.7 ± 0.2	12.1 ± 0.3 <sup>g</sup>
20:2	2.8 ± 0.2	0.6 ± 0.1 <sup>g</sup>	7.3 ± 0.8	1.1 ± 0.1 <sup>g</sup>	5.0 ± 0.6	1.1 ± 0.1 <sup>g</sup>
20:3	0.8 ± 0.1	0.6 ± 0.1	2.0 ± 0.1	0.6 ± 0.2 <sup>g</sup>	2.1 ± 0.2	0.3 ± 0.1 <sup>g</sup>
20:4	13.5 ± 1.5	25.7 ± 1.5 <sup>g</sup>	12.3 ± 0.6	29.2 ± 0.8 <sup>g</sup>	23.3 ± 0.6	29.2 ± 1.6
22:4	tr <sup>f</sup>	1.2 ± 0.1	0.4 ± 0.3	1.4 ± 0.2	1.2 ± 0.1	2.5 ± 0.7
22:6	2.9 ± 0.4	1.6 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	6.7 ± 0.2	0.7 ± 0.1 <sup>g</sup>

<sup>a</sup> Mean ± SE of four separate microsomal preparations.

<sup>b</sup> Length of time that the animals were fed the diets.

<sup>c</sup> M<sub>s</sub> refers to the microsomal preparations from the livers of the rats fed the saturated fat diet (coconut oil enriched); M<sub>p</sub> refers to the microsomal preparations from the livers of the rats fed the polyunsaturated fat diet (sunflower seed oil enriched).

<sup>d</sup> The totals for the fatty acid classes do not add up to 100% in most cases because some of the fatty acid methyl esters were not identified.

<sup>e</sup> The fatty acids are abbreviated as number of carbon atoms: number of double bonds.

<sup>f</sup> <0.5% of the total fatty acids.

<sup>g</sup> P < 0.01.

M<sub>s</sub> after 70 days of feeding cannot be explained. No difference was noted in the saturated fat content of two microsomal preparations. More than 90% of the fatty acyl groups in both microsomal preparations were contained in phospholipids, and additional studies revealed that the phospholipid fatty acid compositions presented in Table 1 are representative of those of the unfractionated microsomal lipid extract. It should be noted that eicosatrienoic acid did not accumulate in the microsomes of the rats fed the saturated fat diet or, as demonstrated by additional analyses, in the lipids of the intact liver homogenate. The coconut oil used in the saturated fat diet contained 1.3% linoleic acid (28), an amount probably sufficient to prevent essential fatty acid deficiency. Medium-chain-length fatty acids also did not accumulate in the microsomes, or in the liver homogenate, when the rats were fed the saturated fat diet. Yet, 83% of the fatty acids of the coconut oil contained 8 to 14 carbon atoms. The elongation mechanism in the liver apparently is effective enough to prevent medium-chain fatty acid build-up in spite of this rather large dietary load.

Table 2 shows that there were no appreciable differences in the phospholipid or cholesterol content of

M<sub>s</sub> and M<sub>p</sub>. There was an increase in the lipid content on day 20 as compared with the later times, but this was found in both sets of microsomes. At each time, the molar ratio of phospholipid to cholesterol in M<sub>s</sub> and M<sub>p</sub> was similar. Moreover, most of the cholesterol recovered from the microsomes was in unesterified form. These results indicate that the fatty acid differences noted in Table 1 represent compositional changes in a relatively fixed quantity of phospholipid and are not due to an added amount of phospholipid in one of the two microsomal preparations. In addition, the differences in ACAT activity, which will be described below, cannot be explained on the basis of differences in the cholesterol content of the two microsomal preparations.

As seen in Table 3, there were no major differences in the phospholipid composition in the two microsomal preparations. In both cases, choline phosphoglycerides accounted for more than 50% of the total phospholipids. Table 4 shows the fatty acyl composition of the three main phosphoglyceride fractions contained in these microsomes. The choline and ethanolamine phosphoglycerides from M<sub>s</sub> contained more 18:1 and less 18:2 and 20:4 than the corresponding fractions from M<sub>p</sub>. By contrast, there was little dif-



TABLE 2. Lipid composition of microsomes

Dietary Fat	Time on Diet	Microsomal Lipid Content			
		Phospholipids <sup>a</sup>	Unesterified Cholesterol		Phospholipid/ Cholesterol
			Amount <sup>a</sup>	Percent of Total	
	days	μg/mg protein	μg/mg protein	%	mol/mol
Saturated	20	508 ± 12	44 ± 2	92	5.7
Polyunsaturated	20	514 ± 22	49 ± 2	93	5.2
Saturated	40	426 ± 12	38 ± 2	92	5.5
Polyunsaturated	40	397 ± 22	36 ± 2	91	5.4
Saturated	70	428 ± 8	37 ± 1	93	5.7
Polyunsaturated	70	405 ± 10	33 ± 1	94	6.0

<sup>a</sup> Mean ± SE of four separate microsomal preparations. None of the differences between the two dietary fat groups are statistically significant,  $P > 0.05$ .

ference in the 18:1 and 18:2 contents of the serine and inositol phosphoglycerides of  $M_s$  and  $M_p$ . This fraction, however, contained more than twice as much 20:4 in  $M_p$  as compared with  $M_s$ .

These differences in fatty acid composition were not accompanied by any major changes in the protein composition of the microsomes as determined by one-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis. The protein electrophoretic patterns were qualitatively similar, and no differences were noted when the gels were scanned densitometrically. Taken together, these findings indicate that the changes in hepatic microsomal fatty acid composition that were produced by the fat-enriched diets are localized to the fatty acyl groups and that neither the lipid content nor protein composition of the microsomes were affected.

### ACAT activity

In every case that we tested and under all conditions of assay, the ACAT activity of  $M_p$  was greater than that of  $M_s$ . **Fig. 1** (left side) illustrates that linear rates were obtained during the first 12 min of incubation with [ $1-^{14}C$ ]palmitoyl CoA as the substrate. The ACAT activity of  $M_p$  was 1.5- to 2.6-times higher than in  $M_s$ . In most subsequent experiments, a 5-min incubation time was employed. **Fig. 1** (right side) shows that the ACAT activity was linearly dependent on microsomal protein content between 0.05 and 0.25 mg under these conditions. Again, the activity with  $M_p$  was 1.3- to 1.8-times higher than with  $M_s$ . In order to more thoroughly assess the difference between  $M_p$  and  $M_s$ , ACAT activity was compared throughout the course of the 70-day feeding period. The data, listed in **Table 5**, demonstrate that the activity of  $M_p$  was 1.7 to 1.9-times greater than that of  $M_s$  at each time.

The above experiments were done with 15 μM pal-

mitoyl CoA as the substrate. As seen in **Fig. 2**, however, similar results were obtained when the palmitoyl CoA concentration was varied between 5 and 33 μM. In each case, the activity with  $M_p$  was considerably higher than with  $M_s$ . In a similar experiment in which oleoyl CoA was used as the substrate, the ACAT activity also was 2.1- to 3.3-times higher with  $M_p$  than with  $M_s$ .

Addition of fatty acid-free bovine serum albumin produced a considerable increase in ACAT activity, but the difference between  $M_p$  and  $M_s$  was maintained. As shown in **Fig. 3** (left side), increasing the albumin concentration from 5 to 20 μM led to a marked increase in ACAT activity. At higher concentrations, either no further stimulation or some inhibition of activity was observed. The ACAT activity with  $M_p$  remained 1.5- to 2.3-times higher than that of  $M_s$  over this range of albumin concentrations. Furthermore, albumin did not have to remain in the incubation medium during the assay in order to produce a stimulatory effect. In another series of experiments, aliquots of the microsomal suspension were incubated

TABLE 3. Phospholipid composition of microsomes

Phospholipid Fraction	Composition <sup>a</sup>	
	$M_s$	$M_p$
	%	
Choline phosphoglycerides	53.9 ± 1.3	60.8 ± 2.8
Ethanolamine phosphoglycerides	24.9 ± 1.1	20.6 ± 1.7
Serine plus inositol phosphoglycerides	8.0 ± 0.2	5.3 ± 0.8 <sup>b</sup>
Choline lysophosphoglycerides	0.5 ± 0.1	0.4 ± 0.1
Sphingomyelin	2.8 ± 0.3	3.1 ± 1.0
Unidentified	10.0 ± 2.4	10.2 ± 2.8

<sup>a</sup> Mean ± SE of four separate microsomal preparations obtained from rats that have been fed the special diets for 50 days.

<sup>b</sup> 0.01 <  $P$  < 0.02.

TABLE 4. Fatty acid composition of the individual phosphoglyceride fractions isolated from the microsomes<sup>a</sup>

Fatty Acid <sup>b</sup>	Percentage Composition <sup>c</sup>					
	Choline Phosphoglycerides		Ethanolamine Phosphoglycerides		Serine plus Inositol Phosphoglycerides	
	M <sub>s</sub>	M <sub>p</sub>	M <sub>s</sub>	M <sub>p</sub>	M <sub>s</sub>	M <sub>p</sub>
	%					
16:0	17.0 ± 2.2	16.9 ± 2.4	13.0 ± 3.0	8.8 ± 2.0	6.7 ± 1.5	4.3 ± 2.1
18:0	24.4 ± 1.5	25.6 ± 0.5	24.8 ± 1.0	26.6 ± 1.0	43.5 ± 1.5	41.9 ± 0.5
16:1	1.7 ± 0.7	0.5 ± 0.5	0.7 ± 0.4	nd	nd	0.2 ± 0.2
18:1	16.6 ± 0.7	12.4 ± 4.4	16.5 ± 0.6	13.5 ± 0.8 <sup>d</sup>	12.5 ± 1.1	9.4 ± 0.6
18:2	11.6 ± 1.1	17.8 ± 1.8 <sup>d</sup>	9.8 ± 0.4	16.8 ± 2.0 <sup>d</sup>	7.3 ± 1.3	6.7 ± 1.4
20:2	4.1 ± 0.5	0.2 ± 0.2 <sup>e</sup>	2.7 ± 0.5	nd	8.3 ± 1.7	nd
20:4	16.7 ± 0.5	23.1 ± 5.0	23.7 ± 2.0	26.4 ± 2.0	16.9 ± 1.6	35.4 ± 2.0 <sup>e</sup>
22:4	1.5 ± 0.2	1.0 ± 0.3	3.7 ± 0.6	2.5 ± 0.5	2.0 ± 0.8	0.7 ± 0.3
22:6	1.3 ± 0.2	0.2 ± 0.2 <sup>d</sup>	3.9 ± 0.6	2.0 ± 0.9	2.1 ± 1.3	0.2 ± 0.2

<sup>a</sup> The abbreviations used are the same as those listed in Table 1.

<sup>b</sup> Only the major individual fatty acids are listed.

<sup>c</sup> Mean ± SE of four separate microsomal preparations.

<sup>d</sup> 0.01 < P < 0.05.

<sup>e</sup> P < 0.001.

for 10 min at 30°C with 0.17 mM albumin. They were then sedimented, washed, and assayed for ACAT activity in a medium that did not contain any added protein. As a control, microsomes were similarly incubated in the absence of albumin, washed and assayed for ACAT activity. The activity in M<sub>p</sub> increased from 22.4 ± 0.5 pmol/mg protein × min in the control preparations to 88.6 ± 1.6 in those exposed to albumin (n = 4). Likewise, the activity in M<sub>s</sub> increased from 11.6 ± 0.5 pmol/mg protein × min to 50.8 ± 8.0 after incubation with albumin (n = 4). Although the activities were higher, the difference between M<sub>p</sub> and M<sub>s</sub> was maintained. Since albumin was

effective even when added prior to the assay, the stimulatory effect probably is due to removal of an inhibitor, perhaps free fatty acid (1) or progesterone (10). Whatever the mechanism, it apparently is not related to the basic difference in the ACAT activity of M<sub>p</sub> and M<sub>s</sub>.

In addition to utilizing palmitoyl CoA for cholesterol esterification, these hepatic microsomes also hydrolyzed large quantities of this substrate. As shown in Fig. 3 (right side), the hydrolytic activity was increasingly inhibited as more albumin was added to the incubation medium. Because free fatty acids are known to inhibit hepatic ACAT (1), it is possible that the stimulation of ACAT activity observed when the medium contained albumin was secondary to inhibition of acyl CoA hydrolysis. Fig. 3 (right side) also shows that there was little difference in the hydrolase activity of M<sub>p</sub> and M<sub>s</sub> over this range of albumin concentrations.

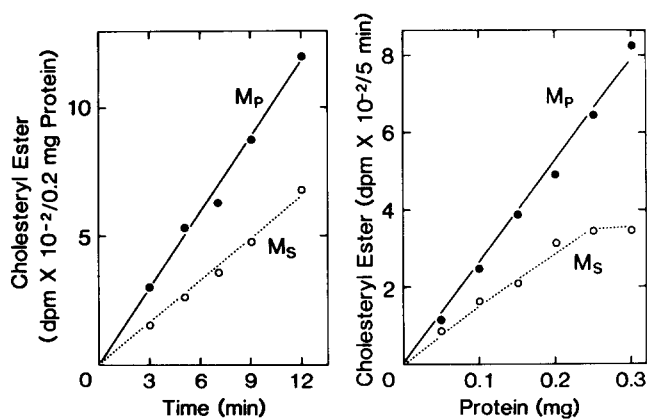
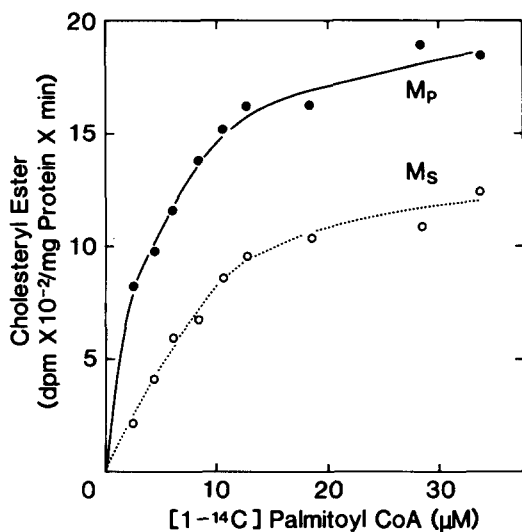


Fig. 1. Effects of incubation time and microsomal protein content on ACAT activity. The media contained 7.65 nmol of palmitoyl CoA and 146,000 dpm of [1-<sup>14</sup>C]palmitoyl CoA in 0.5 ml of incubation buffer. The incubations were carried out at 30°C with shaking. In the time course study, the media contained 0.2 mg of microsomal protein. In the protein concentration study, the time of incubation was 5 min. Each point represents the average of two separate incubations.

TABLE 5. Comparison of ACAT activity in liver microsomes<sup>a</sup>

Length of Feeding Period	Cholesteryl Ester Formation	
	M <sub>s</sub>	M <sub>p</sub>
days	<i>pmol/mg protein × min</i>	
20	26.8 ± 2.6	50.1 ± 5.9
38	29.0 ± 2.0	51.0 ± 5.0
40	29.5 ± 1.8	50.7 ± 5.3
54	29.0 ± 2.0	51.0 ± 5.0
70	28.4 ± 1.5	54.5 ± 7.6

<sup>a</sup> Incubations were for 5 min at 30°C. The medium contained 15 μM [1-<sup>14</sup>C]palmitoyl CoA and 0.2 mg of microsomal protein. Each value is the mean ± SE of four separate microsomal preparations. The differences between M<sub>p</sub> and M<sub>s</sub> are statistically significant (P < 0.02).

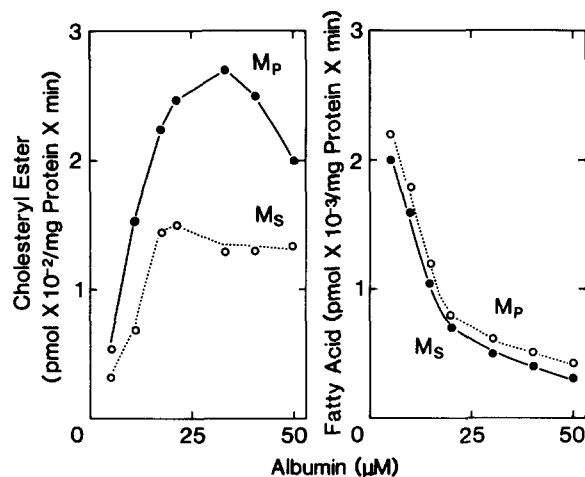


**Fig. 2.** Effect of palmitoyl CoA concentration on ACAT activity. The incubations were carried out for 5 min at 30°C with 0.2 mg of microsomal protein. Each point represents the average of two separate incubations.

The temperature dependence of ACAT activity was compared in the two microsomal preparations, and Arrhenius plots of these results are shown in **Fig. 4**. Each plot was biphasic, and contained a break point at about 29°C. Above this temperature, the calculated activation energy was similar in both cases, about 12.5 Kcal/mol. The activation energies below the break point, however, were different in the two preparations. A value of 27 Kcal/mol was calculated for  $M_p$  and 33 Kcal/mol for  $M_s$ . This difference is consistent with the fact that the fatty acid composition of  $M_p$  is more unsaturated than that of  $M_s$  (Table 1). Over the entire range of temperatures tested, 14 to 40°C, the ACAT activity of  $M_p$  was greater than that of  $M_s$ .

#### Other microsomal enzymes

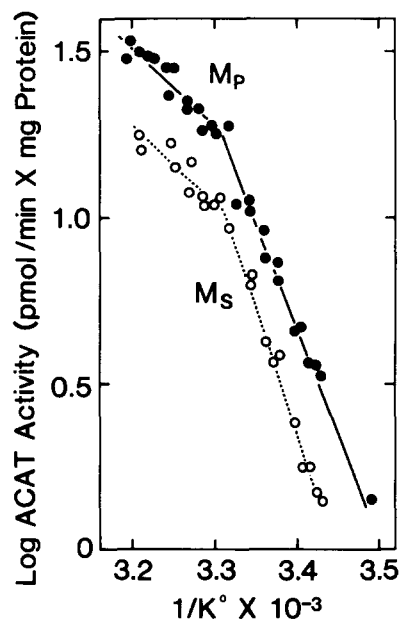
In addition to ACAT, five other enzymatic activities were compared in the two microsomal preparations. The results, listed in **Table 6**, were obtained in animals fed the special diets for 38 days. No consistent pattern was observed, although some statistically significant differences were noted. Acyl CoA hydrolase activity, assayed with [1-<sup>14</sup>C]palmitoyl CoA as the substrate in a medium that did not contain any albumin, was not significantly different in the two microsomal preparations. These results are consistent with those presented in Fig. 3. Likewise, there was no significant difference in NADPH cytochrome c reductase activity. UDP glucuronyl transferase activity was about 50% higher in  $M_p$ . By contrast, glucose 6-phosphatase and NADH cytochrome b<sub>5</sub> reductase were about 25% higher in  $M_s$ . These differences, however, were significant only at the  $P < 0.05$  level.



**Fig. 3.** Effect of addition of albumin on ACAT and palmitoyl CoA hydrolase activities. Each incubation flask contained 7.8 nmol of [1-<sup>14</sup>C]palmitoyl CoA and 0.2 mg of microsomal protein in a total volume of 0.5 ml of buffer solution. The incubations were carried out for 5 min at 30°C with shaking. Each point represents the average of two separate incubations.

#### Liver and plasma lipid concentrations and growth

Liver and plasma lipid measurements were made on a separate group of rats that completed the 70-day feeding period. The results are listed in **Table 7**. The only difference noted in the liver was an increase



**Fig. 4.** Temperature dependence of microsomal ACAT activity. 0.2 mg of microsomal protein in a volume of 400  $\mu$ l of buffer was incubated at the desired temperature for 5 min. To initiate the reaction, 7.49 nmol of [1-<sup>14</sup>C]palmitoyl CoA in 100  $\mu$ l was added, and the incubation continued for 5 min. Each point represents the mean of two separate incubations, each with a different microsomal preparation. The lines were fitted by a linear regression least squares analysis, and the correlation coefficients were at least 0.96 or greater.

TABLE 6. Comparison of enzyme activities in liver microsomes

Enzyme	Activity <sup>a</sup>		Significance of Difference
	M <sub>s</sub>	M <sub>p</sub>	
	<i>nmol/mg protein × min</i>		
Acyl CoA hydrolase	1.56 ± 0.07	1.38 ± 0.04	>0.1
NADPH cytochrome c reductase	146 ± 9	157 ± 12	>0.1
UDP glucuronyl transferase	2.34 ± 0.27	3.49 ± 0.25	<0.02
Glucose 6-phosphatase	765 ± 55	598 ± 36	<0.05
NADH cytochrome b <sub>5</sub> reductase	3690 ± 24	2890 ± 190	<0.05

<sup>a</sup> Mean ± SE of four separate microsome preparations obtained from rats fed the special diets for 38 days.

in triglyceride content in the rats fed the saturated fat diet. This group also had higher plasma triglyceride and cholesterol concentrations. The plasma cholesteryl ester concentration also was higher in the rats fed saturated fat.

There was no difference in the growth of the rats fed the two diets, and both groups of animals appeared to be healthy. The rats weighed 65 ± 2 g when they were placed on these diets. After 20 days, the group fed the polyunsaturated fat diet weighed 108 ± 4 g, whereas those fed the saturated fat diet weighed 111 ± 5 g. The livers were the same size in both groups of animals, 5.4 ± 0.5 g. After 70 days, the rats fed polyunsaturated fat weighed 352 ± 13 g, while those fed saturated fat weighed 361 ± 14 g. The livers from the saturated fat group were slightly larger, 14.7 ± 0.6 g as compared with 12.6 ± 1.0 g.

TABLE 7. Liver and blood plasma lipid contents

Lipid	Content <sup>a</sup>	
	Saturated Fat <sup>b</sup>	Polyunsaturated Fat
	<i>μg/mg protein</i>	
Liver homogenate		
Phospholipid	29.0 ± 2.4	23.5 ± 1.8
Cholesterol	3.2 ± 0.2	3.3 ± 0.06
Cholesteryl esters	0.76 ± 0.2	0.90 ± 0.2
Triglycerides	139 ± 19	88 ± 12 <sup>c</sup>
	<i>mg/dl</i>	
Blood plasma		
Cholesterol	14.6 ± 0.5	11.2 ± 0.2 <sup>d</sup>
Cholesteryl esters	22.2 ± 0.1	16.2 ± 1.0 <sup>d</sup>
Triglycerides	225 ± 66	63 ± 9 <sup>e</sup>

<sup>a</sup> The values for the homogenate are the mean ± SE of four separate livers in each group. Those for the plasma are the mean ± SE of blood samples from 3 separate animals in each group.

<sup>b</sup> Type of fat present in the diet. These rats were kept on the diet for 70 days before they were studied, and separate rats were used for the liver and blood plasma experiments.

<sup>c</sup> 0.01 < *P* < 0.05.

<sup>d</sup> *P* < 0.01.

<sup>e</sup> 0.05 < *P* < 0.1.

This increase may be due in part to the increase in hepatic triglyceride content.

## DISCUSSION

These findings indicate that ACAT activity in rat liver microsomes can be influenced by changes in dietary fat composition. A similar effect was observed previously in mouse Ehrlich ascites tumor cells, but the direction of the change was different (12). Polyenoic fatty acid enrichment reduced microsomal ACAT activity in Ehrlich cells, an opposite effect from that observed in rat liver microsomes. This difference could be due to species or tissue variations. The lipid modifications produced in the two microsomal preparations, however, are somewhat different. In both cases, polyunsaturated fat feeding raised the polyenoic fatty acid content. Even though the 18:2 content increased 3.6-fold in the Ehrlich cell microsomes, there was no appreciable change in 20:4 content (12). By contrast, a large increase in both 18:2 and 20:4 occurred in the hepatic microsomes when the rats were fed the polyunsaturated fat enriched diet. Furthermore, M<sub>s</sub> from the tumor cells contained less saturated than monoenoic fatty acid (12), whereas the M<sub>s</sub> from rat liver contained 39–41% saturated and only 21–27% monoenoic fatty acids (Table 1). These differences possibly account for the different ACAT responses that result from polyunsaturated fatty acid feeding in the two systems. While the difference between rat liver and Ehrlich cells certainly is of interest, the striking finding in our opinion is that both systems respond to fatty acid modifications. This suggests that dependence on dietary lipid composition may be a general property of ACAT and, therefore, that the process warrants further study.

The differences detected in these *in vitro* assays cannot be due to the composition of the fatty acids available as substrates for ACAT. The assay was done with substrate amounts of radioactive acyl CoA, under con-



ditions where free fatty acid was not incorporated into cholesteryl esters (12). Several other explanations involving microsomal lipids also appear to be excluded by the present results. There were no appreciable changes in phospholipid head group composition, phospholipid to protein ratio, molar ratio of phospholipid to cholesterol, or overall protein composition. Since the cholesterol content of the two microsomal preparations is very similar, the effect also probably is not due to differences in the availability of cholesterol as a substrate. Conclusions regarding cholesterol, however, are uncertain because ACAT utilizes a small cholesterol subfraction rather than the entire microsomal cholesterol pool as substrate (29). Therefore, it is possible that lipid redistribution occurs as a result of the fatty acid modifications, making different amounts of membrane cholesterol available to ACAT in the two microsomal preparations.

It is possible that the accessibility of the added acyl CoA to the enzyme is different in  $M_p$  than in  $M_s$ . While this cannot be excluded, it is unlikely in view of the acyl CoA hydrolase results. Both microsomal preparations hydrolyzed palmitoyl CoA to about the same extent, indicating roughly equal availability of the substrate in each case. Access to individual enzymes still could be different, but this is unlikely because palmitoyl CoA has limited penetration into microsomal vesicles (30). Therefore, it is probably utilized near the cytoplasmic surface of the microsomal vesicle in both the ACAT and hydrolase reactions. Furthermore, the lesser ACAT activity in  $M_s$  is not overcome by raising the palmitoyl CoA concentration, again suggesting that acyl CoA availability probably is not the explanation of the difference.


Another possible explanation is that the two microsomal preparations contain different amounts of ACAT per unit weight of protein and lipid. Although ACAT has been solubilized (31), it has not been purified and antibodies are not available to test this point. The results with the other microsomal enzymes (Table 6), however, argue against a non-specific inactivation or loss of enzymes in  $M_s$ . Furthermore, NADPH cytochrome c reductase and ACAT are contained in microsomal vesicles of about the same density (3). If  $M_p$  and  $M_s$  were enriched to different extents with membrane fragments in this density range, the NADPH cytochrome c reductase activity should have been reduced in  $M_s$  to the same extent as ACAT. There was, however, no difference in the NADPH cytochrome c reductase activity of  $M_s$  and  $M_p$ . Although these results as well as the chemical analyses (Tables 2 and 3) tend to rule out an artifact, the possibility of specific loss or inactivation of ACAT during the isolation of  $M_s$  cannot be excluded.

Fatty acid compositional changes have been shown to influence the activity of many enzymes that are tightly bound to membranes (32–40). Moreover, dietary lipid modification can affect the fluidity of rat liver microsomes and the transition temperatures of microsomal enzymes (41). Therefore, it is possible that the differences in ACAT activity in  $M_p$  and  $M_s$  are due at least in part to the differences in their fatty acid compositions. Two observations make us suspect, however, that the mechanism probably is more complex than an overall change in membrane fluidity. First, the Arrhenius plots show that the activity transition occurs at the same temperature in both microsomal preparations (Fig. 4). Second, the activation energy above this 29°C transition was the same in both cases, but the differences in ACAT activity in  $M_p$  and  $M_s$  persisted at temperatures up to 40°C. Assuming that the difference in ACAT activity is related in some way to the change in microsomal fatty acid composition, these results are more consistent with a specific micro-environmental or fatty acid effect on ACAT than with a bulk membrane fluidity effect.

Fatty acid compositional effects on ACAT activity have been reported following exposure to phospholipid dispersions in vitro (42). ACAT was stimulated by incubating rat liver microsomes with certain phosphatidylcholines, and the degree of stimulation depended on the fatty acid composition of the added phospholipid. ACAT activity was increased by saturated and dioleoyl phosphatidylcholine but not by dilinoleoyl phosphatidylcholine. Since the polyenoic phosphatidylcholine did not stimulate ACAT activity, it is unlikely that this in vitro response is related to the changes that we have noted as a result of dietary fat modification.

The increase in hepatic ACAT activity in the polyunsaturated fat-fed rats was not associated with any cholesteryl ester increase in either the liver or plasma (Table 7). Therefore, the physiologic importance of this effect is uncertain. Since the diets used in the present work did not contain any added cholesterol, substrate availability may have limited any response in the intact animal. Previous work indicates that if rats are fed 1% cholesterol, a much larger accumulation of cholesteryl esters occurs in the liver when the diet contains polyunsaturated instead of saturated fat (43). Assuming that the availability of dietary cholesterol does not change the hepatic ACAT response, the increased hepatic cholesteryl ester accumulation could result at least in part from higher microsomal ACAT activity.

Two other hepatic microsomal enzymes involved in cholesterol metabolism are influenced by changes in dietary fat composition. Cholesterol 7 $\alpha$ -hydroxylase

activity was reduced in the livers of rats fed triolein or trilinolein as compared with tripalmitin (44). This is opposite from the dietary lipid effect on ACAT, where polyunsaturated fat increased the activity. Like ACAT, however, the HMGCoA reductase activity of hepatic microsomes was higher when rats were fed a polyunsaturated fat enriched diet as compared with coconut oil (45). On the other hand, the feeding of long-chain saturated fats such as tristearin produced higher HMGCoA reductase activities than either corn oil or safflower oil. Furthermore, the activity was 3- to 5-times greater when the diet contained long-chain saturated fats than either trilaurin or trioctanoin. These additional results suggest that the difference between polyunsaturated fats and coconut oil in terms of hepatic HMGCoA reductase is related to fatty acid chain length rather than to the degree of unsaturation (45). This also must be considered as a possible explanation for the differences in ACAT activity produced by feeding these dietary fats. 

These studies were supported by research grants HL 14,781 and HL 14,230 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

Manuscript received 12 June 1979; accepted 10 September 1979.

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