Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation in vivo and some of its properties in vitro[1]

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Abstract To gain insight into the role of the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) in cellular cholesterol homeostasis, its regulation in rat liver was investigated both in vivo and in vitro. In vitro assay conditions were optimized and some properties of the microsomal enzyme in vitro were also studied. Arrhenius plots of microsomal ACAT activity showed discontinuities at about 28–29°C and 16–17°C. Detergents above their critical micelle concentrations and organic solvents both inhibited the enzyme. Addition of progesterone or SC 31769, a 7-keto-cholesterol analogue, to microsomes inhibited activity while addition of 25-hydroxycholesterol increased the rate of cholesterol esterification, suggesting that the enzyme is susceptible to both negative and positive modulation by steroids, steroid analogues, or their metabolic products. Increasing the rate of cholesterol biosynthesis had a variable effect on ACAT activity. It was higher at the circadian peak of sterol biosynthesis than at the nadir. Increasing sterol biosynthesis by intragastric administration of mevalonolactone resulted in increased activity. In contrast, increasing the rate of sterol biosynthesis by feeding cholestyramine or administration of Triton WR 1339 had little effect on ACAT. Increasing hepatic cholesterol content by feeding cholesterol, cholate, or an atherogenic diet, fasting or intragastric administration of mevalonolactone all resulted in increased ACAT activity. ACAT activity showed a positive correlation with changes in microsomal free and esterified cholesterol contents. The response of ACAT to changes in hepatic cholesterol concentration in vivo and its response to changes in the rate of cholesterol synthesis support the hypothesis that this enzyme plays an important role in maintenance of hepatic cholesterol homeostasis.—**Erickson, S. K., M. A. Shrewsbury, C. Brooks, and D. J. Meyer.** Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation in vivo and some of its properties in vitro. *J. Lipid Res.* 1980. **21:** 930–941.

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The regulation of cholesterol homeostasis in cells appears to be vital for proper cellular function. In the liver this is achieved by a balance between the rate of entrance of cholesterol via lipoproteins, the intracellular rate of cholesterol biosynthesis, and the rates of exit from the cell via lipoproteins and via bile secretion as free cholesterol or as bile acids. In addition, cholesterol may be stored in the cell after esterification by the intracellular enzyme acyl-coenzyme A:cholesterol acyltransferase, ACAT (EC 2.3.1.26). Thus, one can envision three major hepatic cellular compartments for cholesterol: first, a free cholesterol pool which serves as a membrane structural component, second, a metabolically active pool of free sterol which provides the substrate for bile acid and lipoprotein syntheses, and third, a cholesteryl ester pool which appears to function as a storage reservoir. Whether these three pools are truly distinct, how entry and exit of sterol is regulated, and how these pools relate to aspects of cholesterol metabolism are questions still not resolved.

It is likely that the balance between free and esterified cholesterol may play an important role in regulating cholesterol homeostasis. The factors involved in determining and maintaining this balance are not understood. One important determinant may be the activity of the enzyme ACAT. Increases in cholesteryl ester content are paralleled by increases in ACAT activity in human skin fibroblasts exposed to LDL (3, 4), hepatoma cells exposed to hyperlipemic serum (5), in arteries from rabbits, monkeys, and pigeons fed atherogenic diets (6–8), and in livers of guinea pigs fed cholesterol (9). These observations suggested that the enzyme can be regulated.

In the rat, hepatic ACAT activity was first de-

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonolactone; D-6, the midpoint of the dark phase or peak of the circadian rhythm of cholesterol biosynthesis; D-8, 2 hr after the midpoint; L-6, the midpoint of the light phase or nadir of the circadian rhythm of cholesterol biosynthesis.

[1] Portions of this work were presented at the annual meetings of the American Society of Biological Chemists, Atlanta, June 1978 (1), and of the American Heart Association, Dallas, November, 1978 (2).

scribed by Mukerjee, Kunitake, and Alfin-Slater (10) and characterized by Goodman, Deykin, and Shiratori (11). As in other tissues, the enzyme has a preference for oleoyl coenzyme A, but can utilize other acyl CoA's. Its highest specific activity is in the microsomal fraction (12). However, little is known about the regulation of the hepatic enzyme nor are the mechanisms of ACAT regulation in any tissue well understood.

To gain insight into possible modes of regulation of hepatic cholesterol esterification and how these might be related to the regulation of cholesterol homeostasis, an in vitro assay for rat liver ACAT was first optimized to insure that initial rates of activity were measured. Then the response of the enzyme to a number of in vivo manipulations of the rate of cholesterol biosynthesis and of hepatic cholesterol content was studied. In these experiments, relationships between ACAT activity and changes in whole liver and microsomal total, free and esterified cholesterol were also investigated, as were possible relationships with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol biosynthesis. Finally, some properties of microsomal ACAT were studied in vitro.

METHODS AND MATERIALS

Chemicals

[1,2-^{3}H]Cholesterol (39–52 mCi/mmol), [1-^{14}C]oleoyl coenzyme A (40–60 mCi/mmol), DL-3-hydroxy-3-methyl-[3-^{14}C]glutaric acid (10–25 mCi/mmol), and *R,S*-]5-^{3}H]mevalonic acid (dibenzyldiethylenediamine salt, 1–5 Ci/mmol) were from New England Nuclear; [G-^{3}H]cholesterol (10 Ci/mmol) was from Schwarz-Mann; cholesteryl oleate, oleic anhydride, oleoyl coenzyme A, Triton X-100, propylthiouracil, deoxycholate, progesterone, glucose-6-phosphate (disodium salt), cholic acid, cholesterol, and phenylmethylsulfonyl fluoride were from Sigma; *R,S*-mevalonolactone was from ICN Pharmaceuticals; glucose-6-phosphate dehydrogenase, dithiothreitol, and digitonin were from Calbiochem; Nonidet P40 was from Shell; Tween 20 and Tween 80 were from Atlas Chemical Industries; cetyl triethylammonium bromide was from Sigma; 25-hydroxycholesterol was from Steraloids; Mylar-backed silica gel chromatography sheets were from Eastman; silica gel H was from E. Merck (Darmstadt): Triton WR 1339 was from Winthrop Laboratories; cholestryamine was from Mead Johnson; lard was from Armour; Liquifluor was from New England Nuclear. Filipin was a gift from UpJohn. SC 31769 was a gift from G. D. Searle. All other chemicals were analytical grade.

Animals

Male, Sprague-Dawley rats, 50–100 g, were housed individually under normal or reversed lighting (lights on 4:00 PM, lights off 4:00 AM; the peak of cholesterol biosynthesis was at 6 hr after the beginning of the dark phase denoted D-6; the midpoint of the light phase is denoted L-6). Commercial rat chow and water were given ad libitum. Animals were allowed at least 10 days to stabilize their cholesterol biosynthesis circadian rhythm before beginning an experiment. They weighed 200–250 g at the time of killing.

Preparation of [^{3}H]cholesteryl oleate

[^{3}H]Cholesterol was diluted with unlabeled cholesterol sequentially recrystallized from glacial acetic acid, ethanol, and acetone. The sterol was then incubated in pyridine with a twofold excess of unlabeled oleic anhydride overnight under nitrogen at room temperature followed by 1 hr at 60°C. The labeled cholesteryl oleate was separated by thin-layer chromatography on silica gel H using petroleum ether–diethyl ether–glacial acetic acid 75:25:1. The chromatogram was visualized in iodine vapor and the band corresponding to cholesteryl oleate was scraped from the plate. The ester was eluted with chloroform, concentrated under N_2, and then rechromatographed on silica gel H using hexane–ethyl acetate 9:1. The band corresponding to cholesteryl oleate was scraped from the plate and eluted. The radiochemical purity was determined in the hexane system and was found to be greater than 95%.

Preparation of diets

Cholesterol, dissolved in diethyl ether, was added to ground rat chow to give the desired percent in the diet; the ether was allowed to evaporate at room temperature. Cholestyramine powder was mixed with ground rat chow. The atherogenic diet consisted of 1% cholesterol, 0.3% cholate, 5% lard, and 0.1% propylthiouracil added to ground rat chow (13). Cholate was added as a solution in ethanol–water to give 1% of the diet by weight and the solvent was evaporated at room temperature.

Feeding experiments

For each experiment, two or three pairs of rats were matched by weight and one of each pair was given the rat chow containing the addition while the other was maintained on rat chow alone. Both were allowed free access to water. For each animal, the amount of

food consumed was estimated and, for experiments longer than 18 hr, the change in whole body weight of each animal determined.

Intragastric administration of mevalonolactone

Mevalonolactone was dissolved in water and given by stomach tube to lightly etherized rats. Control rats were given the same volume of water.

Intraperitoneal administration of Triton WR 1339

The detergent was dissolved in normal saline and administered intraperitoneally at a dose of 100 mg/100 g body weight. Controls were given the same volume of saline alone.

Preparation of microsomes

Animals were killed by decapitation; the livers were rapidly excised and placed in 0.9% NaCl at 4°C. All further steps were at 4°C unless otherwise indicated. Each liver was weighed, minced, and homogenized in a Potter-Elvejhem glass-Teflon homogenizer by three strokes at moderate speed in five volumes (w/v) of 0.25 M sucrose, 1 mM EDTA, pH 7.4. Aliquots of this homogenate were removed for determination of total, free, and esterified cholesterol and the remainder was centrifuged 15 min at 12,000 *g*. The floating fatty layer was removed and the supernatant was centrifuged 60 min at 105,000 *g*. The resultant microsomal pellet was washed once by resuspension in one-half the original volume of buffer, followed by centrifugation for 60 min at 105,000 *g*; it was then resuspended in buffer at approximately 10 mg protein/ml.

Preparation and assay of subcellular fractions

Plasma membranes were prepared by the method of Neville (14). Mitochondria were prepared essentially by the method of Colbeau, Nachbaur, and Vignais (15). Microsomes were prepared as described by Wirtz and Zilversmit (16). The following markers were used: for plasma membrane, 5′-nucleotidase (17), for mitochondria, rotenone-sensitive NADH cyt *c* reductase (18), for endoplasmic reticulum, glucose-6-phosphatase (19).

Assay of HMG-CoA reductase

DL-[3-^{14}C]HMG-CoA was prepared and reductase assayed as previously described (20).

Assay of ACAT activity

Each assay contained 200–500 μg of microsomal protein in 0.1 M Tris-HCL, 0.25 M sucrose, 1 mM EDTA, pH 7.5, in a final volume of 1.0 ml. This mixture was incubated for 6 min at 37°C with shaking and the reaction was initiated by addition of 20–25 nmol of [^{14}C]oleoyl coenzyme A. Routinely, the assay was stopped after 4 min by addition of 10 ml of chloroform–methanol 2:1. Tracer [^{3}H]cholesteryl oleate (approximately 10,000 cpm) was added followed by 15 ml chloroform–methanol 2:1 and 5 ml of acidic H_2O saturated with chloroform–methanol 2:1. The tubes were left overnight at 4°C for phase separation after which the aqueous phase was aspirated and the remainder was taken to dryness under N_2. The residue was dissolved in chloroform and plated on silica gel H (250 μm thickness). Authentic cholesteryl oleate was plated as marker. The chromatograms were developed in hexane–ethyl acetate 9:1 which gave complete separation of cholesteryl esters from triglycerides. Experimental R_f values were 0.64 for cholesteryl esters, 0.32 for triglycerides, and 0.13 for free fatty acids; phospholipids remained at the origin. The plates were visualized in iodine vapor and the cholesteryl ester band scraped into scintillation vials containing toluene-Liquifluor and counted in a Beckman liquid scintillation counter.

Spillover and counting efficiencies were estimated using ^{3}H-labeled and ^{14}C-labeled toluene standards (New England Nuclear). ^{3}H Efficiency averaged 50% and ^{14}C efficiency, 65%. Spillover averaged 1% for ^{3}H and 15% for ^{14}C.

The identity of cholesteryl [^{14}C]oleate synthesized via ACAT was confirmed by scraping the region on the thin-layer plate corresponding to authentic cholesteryl oleate, eluting the ester from the silica gel with $CHCl_3$, determining the ^{14}C/^{3}H ratio, and rechromatographing the material in two systems: heptane–diethyl ether–glacial acetic acid 85:15:2 (4) and petroleum ether–diethyl ether–glacial acetic acid 75:25:1, scraping the areas corresponding to cholesteryl oleate, and determining the ^{14}C/^{3}H ratios. The ratio remained constant within 5% suggesting that the ^{14}C-labeled product behaved the same as the [^{3}H]cholesteryl oleate internal standard.

Chemical methods

Total, free, and esterified cholesterol were determined as previously described (20). Phosphorus was measured according to Bartlett (21) after total lipid extraction (20). Protein was estimated by the biuret method (22) or according to Lowry et al. (23) using bovine serum albumin as reference standard.

Statistical analysis

The paired *t*-test was used on the data after log transformation; for data on the circadian rhythm, the unpaired *t*-test was used on the data directly.

RESULTS

Assay conditions

ACAT activity was estimated by measuring the incorporation of [^{14}C]oleate from [^{14}C]oleoyl coenzyme A into cholesteryl esters utilizing endogenous cholesterol. The cholesteryl esters were separated from the other lipids derived from oleoyl coenzyme A by thin-layer chromatography. Incorporation of [^{14}C]-oleate into cholesteryl esters from [^{14}C]oleoyl CoA was chosen to follow the reaction for the following reasons: *1*) oleoyl CoA is relatively water-soluble and hence no carriers or organic solvents are introduced into the assay; *2*) the endogenous acyl CoA pool is very small (24), therefore dilution of radiolabeled oleoyl CoA by endogenous substrate is minimal; and *3*) addition of oleoyl CoA directly instead of indirectly via an oleoyl CoA-generating system insures direct quantitive measurement of ACAT activity. Utilizing the endogenous cholesterol pool, rather than following the reaction by adding labeled exogenous cholesterol, precludes use of organic solvents and no assumptions need be made regarding the size or constancy of the cholesterol substrate pool.

Microsomal ACAT assay conditions were optimized with respect to incubation time, protein concentration, and oleoyl CoA concentration. The reaction rate was linear over 4–5 min and diminished to zero by 10–15 min (**Fig. 1A**). In contrast, incorporation of [^{14}C]oleate into triglyceride, which is low in rat liver microsomes relative to incorporation into cholesteryl esters (11) was linear over 10–15 min (not shown). The amount of recovered cholesteryl [^{14}C]-oleate remained constant after 10–15 min incubation. The fraction of total microsomal free cholesterol which could be esterified within 30 min did not exceed 4%, nor did it increase when the assay was extended to 60 min, or after addition of more oleoyl CoA. Cholesterol esterification was linear over the protein concentration range of 0.1–0.7 mg/ml (Fig. 1B). Maximal rates of esterification were observed at oleoyl CoA concentrations of 20–30 μM (Fig. 1C) which is well above the critical micelle concentration for oleoyl CoA (25). Concentrations above 30 μM were inhibitory. A broad pH optimum was observed between 7.5 and 8.5 as has been reported previously (11); at pH 6.5, half maximum activity was recovered.

Incubating microsomes at 37°C for 30 min or longer before assay resulted in loss of ACAT activity. Dithiothreitol (DTT), a thiol group-protecting agent, had no effect on this loss, and indeed accelerated it at concentrations greater than 1 mM. Inclusion in the incubations of phenylmethylsulfonyl fluoride, a serine specific protease inhibitor, also had little effect on the loss of activity.

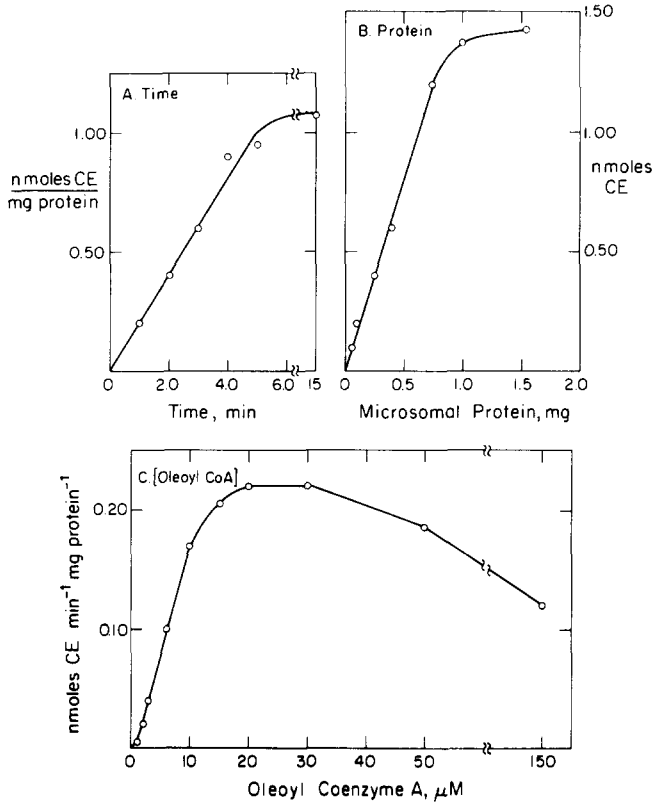

Fig. 1. Some parameters of the assay for rat liver microsomal acyl coenzyme A:cholesterol acyltransferase (ACAT). Microsomes were prepared from control animals and assayed as described in Methods. A. Protein dependence-time = 4 min; oleoyl CoA conc = 25 μM. B. Time dependence-protein = 500 μg; oleoyl CoA conc = 25 μM. C. Oleoyl CoA concentration dependence-time = 4 min; protein concentration = 500 μg protein.

Characteristics of microsomal ACAT

Microsomal ACAT activity was stable with up to three freeze-thaw cycles. It could be stored frozen for up to 2 weeks without significant loss of activity. The activity was completely destroyed by heating at 80°C for 10 min.

Hepatic ACAT appears to be an intrinsic membrane protein since it could not be solubilized from microsomes by a controlled freeze-thawing technique (26) or by extraction with 0.5 M KCl.

The activities of intrinsic membrane enzymes are often dependent on the state of the membrane. Temperature changes are known to alter the physical state of the membrane. Arrhenius plots of microsomal ACAT activity from normal livers showed two discontinuities, one at about 28–29°C and one at about 16–18°C (**Fig. 2**).

To investigate further the role of the membrane in determining ACAT activity, the effects of organic

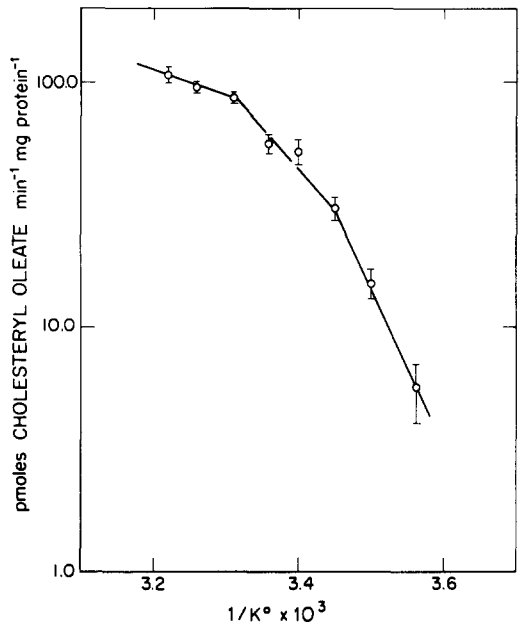

Fig. 2. Variation of microsomal ACAT activity with temperature. Microsomes were prepared and ACAT activity assayed as described over the temperature range given. Arrhenius plots were then constructed. Each point is the average of three separate determinations. The error bars represent the S.E.. The lines were drawn by linear regression analyses. In all cases, the correlation coefficient was 0.98 or greater.

solvents and detergents which perturb membrane structures were studied. At the 1% level (v/v) ethanol, butanol, and acetone inhibited the activity 30–50%. The inhibitory actions of ethanol and butanol on ACAT were prevented by addition of cholesterol to the solvent.

The enzyme was also very sensitive to a wide range of anionic, cationic, and nonionic detergents above the critical micelle concentration including cholate, deoxycholate, cetyltriethylammonium bromide, Triton X-100, Tween 20, Tween 80, and Nonidet P40. The inhibition could be partially reversed by washing unsolubilized microsomes.

Digitonin and filipin specifically complex 3β-hydroxysterols and in doing so can disrupt cholesterol-containing membranes (27, 28). Both agents inhibited ACAT activity by 84–90%.

TABLE 1. Effect of steroid and sterol analogues on ACAT activity in vitro

Inhibition	% Control
Cholesterol	116 ± 18
25-Hydroxycholesterol	171 ± 25
Progesterone	40 ± 20
SC-31769	36 ± 4

Microsomes were prepared and assayed as described. The sterols were added in 10 μl ethanol to give 100 μM final concentration before addition of microsomes to the assay mixture. The control value was taken as that with ethanol. The values are the average of four determinations. Control values without ethanol averaged 190 ± 20 pmol cholesteryl oleate/min/mg protein; while with ethanol, the values averaged 144 ± 60 pmol cholesteryl oleate/min/mg protein.

Effect of exogenous steroids or sterol analogues in vitro

Unlike the human skin fibroblast enzyme (29), rat liver microsomal cholesterol esterification via ACAT was enhanced in vitro by addition of 25-hydroxycholesterol (**Table 1**). As was observed in the fibroblast system (29), both progesterone and SC 31769, a 7-ketocholesterol analogue, were inhibitory in vitro. Cholesterol itself, when added in vitro, had little effect on the initial rate of ACAT activity when compared to the control with no additions. However, when compared with the control plus organic solvent, the activity could be increased up to 50% over the control value.

Subcellular distribution

Because the presence of ACAT activity has been reported in cellular organelles other than endoplasmic reticulum (11, 30), the subcellular localization of the enzyme was reinvestigated using the direct assay with oleoyl CoA at pH 7.5.

As had been reported previously (11, 12), the highest specific activity was recovered in the microsomal fraction (**Table 2**). Little activity was recovered

TABLE 2. Subcellular distribution of ACAT activity

Fraction	ACAT	5′ Nucleotidase	Glucose-6-phosphatase	NADH Cyt C Reductase
	pmol cholesteryl oleate min⁻¹ mg protein⁻¹			*(Rotenone sensitive)*
Plasma membrane (3)	3 ± 2	25.0 ± 1.0	0.05 ± 0.01	0.0
Microsomes (3)	180 ± 3	3.0 ± 0.5	3.5 ± 0.2	1.0
Mitochondria	25 ± 10	1.0	0.1 ± 0.1	10.0
Nuclei (3)	3 ± 5	ND	0.7 ± 0.5	ND
Cytosol (3)	0 ± 0	ND	ND	ND

Fractions were prepared and assayed as described. All ACAT assays were at pH 7.5 with a 6-min incubation and 4-min assay. The number of determinations is in parentheses. Marker enzyme activities are given relative to the whole homogenate as 1.0; ND, not determined.

in cytosol. Upon subfractionation of microsomes into smooth and rough endoplasmic reticulum, the highest specific activity was recovered in the rough fraction (data not shown) confirming the observations of Balasubramaniam et al. (32). Little ACAT activity was detected in plasma membrane or nuclei enriched fractions (Table 2). However, low levels of ACAT activity were recovered in a fraction highly enriched for mitochondria (Table 2). This low level of ACAT activity could not be entirely explained by contamination with endoplasmic reticulum as judged by the ratio of activity of rotenone-sensitive NADH cytochrome *c* reductase to that of glucose-6-phosphatase, suggesting that this organelle may have intrinsic ACAT activity. Mitochondrial activity has also been reported to be present in arteries (31).

In vivo regulation

In order to gain more information on modes of regulation of hepatic cholesterol esterification in vivo, microsomal ACAT activity was investigated under two sets of physiological conditions: those known to increase the rate of cholesterol biosynthesis with no apparent change in hepatic cholesterol content and those known to increase hepatic cholesterol concentration.

In all cases, initial rates of incorporation of [^{14}C]-oleate from [^{14}C]oleoyl CoA into cholesteryl ester were determined to minimize changes in ACAT activity due simply to cholesterol substrate limitation.

Circadian rhythm

Hepatic cholesterol biosynthesis exhibits a circadian rhythm with up to 10-fold higher activity at midnight than at noon despite little detectable change in hepatic cholesterol content (33, 34). Thus it was important to ascertain whether ACAT activity responded to this dramatic change in biosynthetic rate. Hepatic ACAT activity in animals killed at midnight (D-6, the peak of cholesterol biosynthetic rate) was higher, 176 ± 17 pmol cholesteryl oleate/min/mg protein (average of 25 animals), than in those killed at noon, (L-6, the nadir of cholesterol biosynthetic rate) 122 ± 9 pmol cholesteryl oleate/min/mg protein (average of 13 animals ± S.E.; $P < 0.05$) suggesting that ACAT may have a rhythm of low amplitude which parallels that of cholesterol biosynthesis. Further evidence for a circadian rhythm is presented in **Table 3** where ACAT activity in control animals is clearly dependent upon the point in the circadian rhythm of sterol synthesis at which the animals were killed.

TABLE 3. Effect of treatments known to decrease HMG-CoA reductase activity and to increase the hepatic cholesterol content

Treatment	ACAT	% of Control	HMG-CoA Reductase
	pmol cholesteryl oleate min^{-1} mg protein^{-1}		*nmol MVA min^{-1} mg protein^{-1}*
A. Fasting[a]			
Control (8)	105 ± 15		0.38 ± 0.03
Experimental (8)	169 ± 8	161, $P < 0.005$	0.01 ± 0.01
B. Cholesterol fed			
Control (14)	203 ± 42		0.66 ± 0.25
Experimental, 18 h (12)	407 ± 84	201, $P < 0.001$	0.08 ± 0.04
Experimental, 1 wk (6)	568 ± 92	280, $P < 0.001$	0.03 ± 0.01
C. Cholate fed[a]			
Control (6)	122 ± 10		1.12 ± 0.20
Experimental (6)	177 ± 24	145, $P < 0.04$	0.30 ± 0.06
D. Atherogenic diet[b]			
Control (3)	115 ± 15		0.13 ± 0.04
Experimental, 10 days (4)	461 ± 54	401, $P < 0.005$	0.02 ± 0.01
Experimental, 3 wks (3)	402 ± 72	610, $P < 0.001$	0.01 ± 0.01
E. Mevalonolactone			
Control (8)	162 ± 25		0.93 ± 0.10
Experimental (9)	324 ± 75	200, $P < 0.005$	0.28 ± 0.04

[a] Rats killed at D8 (2 h after the peak of cholesterol biosynthetic rate).
[b] Rats killed at L6 (midpoint of the light phase or nadir of cholesterol biosynthetic rate).
Animals were paired by weight and the experimental groups were fasted for 48 h, fed cholesterol for 18 h or 1 week, or fed cholate for 1 week. Animals were fed the atherogenic diet (14) for 10 days or 6 weeks. Another experimental group was given a 200-mg bolus of mevalonolactone (MVA) intragastrically. The animals were killed at D6, or the peak of cholesterol biosynthetic rate, unless otherwise stated; microsomes prepared and assayed as described. All values are ±S.E. The number in parentheses is the number of animals. At least three experiments were done for each group with the exception of D.

TABLE 4. Effect of treatments known to increase the rate of cholesterol biosynthesis with no apparent increase in hepatic cholesterol content

Treatment	ACAT	% Control	HMG-CoA Reductase
	pmol cholesteryl oleate min^{-1} mg protein^{-1}		*nmol MVA min^{-1} mg protein^{-1}*
A. Cholestyramine			
Control (10)	126 ± 20		0.68 ± 0.25
Experimental (11)	132 ± 28	105[a]	3.32 ± 0.27
B. Triton WR 1339			
Control (12)	211 ± 48		0.66 ± 0.14
Experimental (12)	174 ± 44	82[a]	1.36 ± 0.40

[a] Not significant.

Animals were paired by weight and the experimental groups were fed 4% cholestyramine for 2 weeks or given Triton WR 1339 by intraperitoneal injection. The animals were killed at D6, the peak of cholesterol biosynthetic rate, and microsomes were prepared and assayed as described. All values are ±S.E. The number in parentheses is the number of animals. At least three separate experiments were done for each group.

Cholestyramine feeding

Feeding cholestyramine, a bile acid sequestering agent, decreases cholesterol and bile acid absorption resulting in increased bile acid synthesis, cholesterol biosynthesis, and HMG-CoA reductase activity (35). This regimen had no effect on ACAT activity (**Table 4**). The activity was the same as that in control animals despite a 2- to 3-fold increase in HMG-CoA reductase activity. Moreover, no statistically significant differences were noted in whole liver, or in microsomal total, free, or esterified cholesterol content (**Table 5**).

Triton WR 1339 treatment

Triton WR 1339 inhibits lipoprotein metabolism resulting in an increase in cholesterol biosynthesis and HMG-CoA reductase activity (36). Intraperitoneal administration of Triton WR 1339 18 hr before killing increased HMG-CoA reductase activity and resulted in an average 18% decrease in ACAT activity relative to controls (Table 4). Although this change was not statistically significant, in three of five experiments ACAT activity was decreased up to 50% while in the other two experiments, there was no change from control values. There were no statistically significant differences observed in whole liver or microsomal total, free or esterified cholesterol measured at this time point after administration (Table 5).

TABLE 5. Effect of various treatments on hepatic microsomal free and esterified cholesterol content

Treatment	Free Cholesterol	Esterified Cholesterol
	μg sterol/mg protein	
Control	20.1 ± 0.9	1.2 ± 0.1
Cholestyramine	20.2 ± 1.0	1.0 ± 0.2
Triton WR 1339	18.8 ± 0.7	1.4 ± 0.2
Fasting	25.4 ± 3.7	3.0 ± 1.2
Cholesterol	24.6 ± 2.6	3.5 ± 0.4
Cholate	22.5 ± 4.7	2.1 ± 0.4
Atherogenic diet	29.7 ± 2.7	6.9 ± 0.3
Mevalonolactone	18.3 ± 2.5	1.8 ± 0.2

Microsomes were prepared from the animals treated as described in Tables 1 and 4. They were analyzed for free and esterified cholesterol as described in Methods. Values are ±S.E.

Fasting

Fasting increases hepatic cholesterol content and causes an inhibition of cholesterol synthesis (37). A 48-hr fast increased ACAT activity to 161% of control values (Table 3). Both whole liver and microsomal total cholesterol contents increased while HMG-CoA reductase fell to 5–10% of the control value. Microsomal cholesteryl esters increased 130%. (Table 5).

Cholesterol feeding

Cholesterol feeding is well-known to increase hepatic cholesterol content, and to inhibit HMG-CoA reductase and cholesterol biosynthesis (37–39). Inclusion in the diet of 0.1–5% cholesterol for 18 hr before killing resulted in a two-fold increase in ACAT activity (Table 3). Whole liver total and esterified cholesterol were elevated (Table 5), while HMG-CoA reductase was inhibited over 90%. Microsomal cholesteryl esters increased 200%. Feeding 1% cholesterol in the diet for 7 days increased ACAT activity 2.5-fold (Table 3), which was not markedly different from that observed at 18 hr. Microsomal cholesteryl ester content had not changed compared to that from livers of animals fed 18 hr although whole liver total and esterified cholesterol contents were higher.

Atherogenic diet

Feeding an atherogenic diet containing cholesterol, cholate, lard, and propylthiouracil (13) increased

ACAT to 4 to 6 times that of control values (Table 3). Reductase activity was inhibited by over 90% and whole liver total and esterified cholesterol had increased dramatically (Table 5). Microsomal free cholesterol increased 175% and esterified cholesterol, 480%. There was, however, little change in the microsomal free cholesterol:phospholipid mole ratio, 0.13 ± 0.01 versus 0.10 ± 0.02 for controls.

Cholate feeding

Feeding cholate increases hepatic cholesterol content and decreases cholesterol biosynthesis and the activity of HMG-CoA reductase (40). Feeding 1% cholate in the diet for 1 week resulted in an increase in ACAT activity to 145% of the control levels (Table 3). Reductase activity was inhibited 67% in these experiments and microsomal cholesterol esters had increased to 170% of control. (Table 5)

Intragastric administration of mevalonolactone

Mevalonolactone bypasses a major rate-limiting step for cholesterol biosynthesis; therefore, it provided a method of acutely increasing both the rate of endogenous cholesterol synthesis and the endogenous sterol concentration. Intragastric administration of MVA rapidly inhibits HMG-CoA reductase activity and results in accumulation of whole liver and microsomal cholesterol esters (41, 42). Mitropolous et al. (43) confirmed these observations and also reported that ACAT activity increased when measured 2 hr after a 400-mg bolus was administered intragastrically. However, they did not study any of the details of this MVA-induced increase in ACAT.

ACAT responded rapidly to a 200-mg bolus of MVA. By 15 min the activity was 150% of control, increasing to 200% by 30 min and remaining at close to that level for up to 2 hr (**Fig. 3**). By 18 hr after a single bolus, ACAT activity had returned to control levels. At the 30-min time point after MVA administration, the twofold increase in ACAT activity (Table 3) was accompanied by 80% inhibition of HMG-CoA reductase (not shown). There was little detectable change in whole liver or microsomal total or free cholesterol (Table 5) nor was there a change in the free cholesterol:phospholipid mole ratio, 0.07 ± 0.01 versus 0.07 ± 0.01 for controls. However, the microsomal esterified cholesterol had increased 50% of the control value.

Because the increase in ACAT activity was expressed so rapidly after MVA administration, the effect on ACAT of adding MVA directly to microsomes was tested. There was no effect with up to 4 mM MVA.

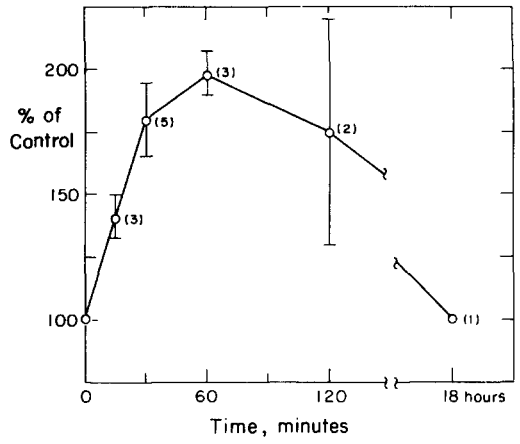

Fig. 3. Time dependence of the increase in ACAT activity after intragastric administration of a 200-mg bolus of mevalonolactone. A 200 mg-bolus of MVA was administered intragastrically at time intervals before killing. All animals were killed at D6–6.5. Microsomes were prepared and assayed as described. At least two control and two experimental animals were used in each experiment. The number in parentheses is the number of experiments at that time point. The error bars are the standard error.

Variation of ACAT activity with microsomal cholesterol content

ACAT activity in other tissues varied with the cellular cholesterol content (4–9), and in hepatoma cells in culture (5) it could be correlated with microsomal cholesterol. Therefore, the relationship of hepatic microsomal ACAT with microsomal free and esterified cholesterol in rat liver was studied. A positive correlation was obtained when initial rates of ACAT activity and microsomal esterified cholesterol content (both measured after a variety of physiological manipulations) were plotted against each other (**Fig. 4**). Initial rates of ACAT activity also showed a positive correlation with microsomal free cholesterol content (**Fig. 5**). Plots of ACAT activity against whole liver total, free or esterified cholesterol content also showed positive correlations (not shown).

Relationship with HMG-CoA reductase

It has been suggested (3) that HMG-CoA reductase and ACAT may be regulated coordinately. Both activities show correlations with cellular cholesterol content, further suggesting a possible common regulatory mechanism. However, when ACAT activity was plotted against HMG-CoA reductase activity, both enzymes having been measured after a variety of in vivo manipulations, a complex relationship was observed (**Fig. 6**).

DISCUSSION

To study mechanisms of the regulation of ACAT either in vitro or in vivo, it was necessary first to

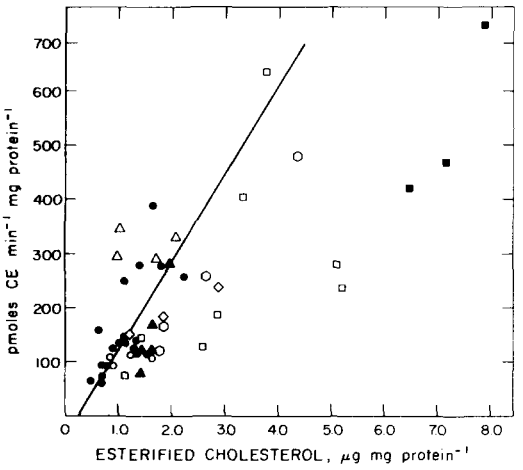

Fig. 4. Relationship between microsomal cholesteryl ester content and ACAT activity. Liver microsomes were prepared from animals after a variety of treatments in vivo and esterified cholesterol content and ACAT activity were measured. Each point represents the average of at least two animals in either control or experimental groups. Correlation coefficient, $r = 0.729$; $P < 0.001$. Control (●); cholestyramine (○); atherogenic diet (■); cholesterol (□); Triton WR 1339 (▲); mevalonolactone (△); cholate (×); fasting (⬡).

define and optimize assay conditions for the enzyme. The assay was linear with respect to protein concentration and length of assay. The concentration of oleoyl CoA required for maximal activity was determined. Substrate-enzyme interactions appear to be complex because the optimal oleoyl CoA concentration is well above its CMC (25). The optimal oleoyl

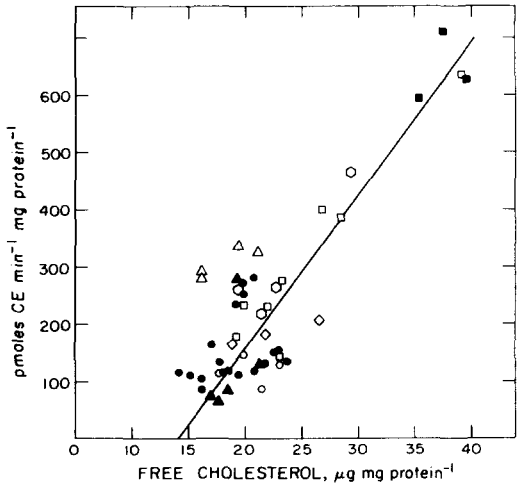

Fig. 5. Relationship between microsomal free cholesterol content and ACAT activity. Liver microsomes were prepared from animals after a variety of treatments in vivo and free cholesterol content and ACAT activity were measured. Each point represents the average value for at least two animals. Correlation coefficient, $r = 0.820$, $P < 0.001$. Control (●); cholestyramine (○); atherogenic diet (■); cholesterol (□); Triton WR 1339 (▲); mevalonolactone (△); cholate (×); fasting (⬡).

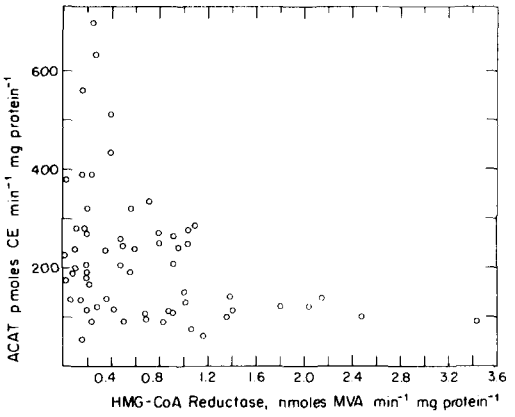

Fig. 6. Relationship between HMG-CoA reductase and ACAT activities. Liver microsomes were prepared from animals after a variety of treatments and reductase and ACAT activities were measured. Each point represents the average of two animals in a control or experimental group.

CoA concentration is also dependent on microsomal protein concentration and further, double reciprocal plots of microsomal ACAT activity relative to oleoyl CoA concentration suggested positive cooperativity effects.[2]

In order to compare ACAT activity after different in vivo or in vitro manipulations, initial rates of cholesterol esterification were measured so that changes would reflect alterations in enzyme activity rather than changes in substrate pool size.

Hepatic ACAT activity appeared to have a circadian rhythm of low amplitude paralleling that of HMG-CoA reductase. Increasing both the rate of sterol biosynthesis and the activity of HMG-CoA reductase in vivo by cholestyramine feeding or intraperitoneal injection of Triton WR 1339 had little effect on ACAT. However, increasing the rate of sterol synthesis in vivo by intragastric MVA administration resulted in a rapid increase in ACAT activity.

Further, ACAT activity could be altered in vivo by altering the level of cholesterol in the liver. When hepatic cholesterol concentration was increased by cholesterol or cholate feeding, an atherogenic diet, fasting, or MVA administration, ACAT activity increased. Increases in rat hepatic ACAT due to cholesterol feeding and MVA administration have also recently been reported by others (43, 44). The positive correlation of initial rates of ACAT activity with changes in microsomal free cholesterol induced by these treatments suggested that the sterol itself plays an important role in modulating the activity of the enzyme. Similar conclusions have been reached to explain the response of the arterial

[2] Erickson, S. K. Unpublished observations.

enzyme to atherogenic diets (7, 8) and the response of ACAT in cells in tissue culture to LDL or hyperlipemic serum (3–5).

Cholesterol is both a constituent of the membranes containing ACAT as well as a substrate for the enzyme. Thus, alterations in microsomal free cholesterol could affect ACAT activity by simply changing the substrate pool size, by acting at an enzyme protein modulation site, or by altering the membrane environment. At present it is not possible to discriminate amongst these possibilities. The studies with steroids and sterol analogues in vitro support the hypothesis that ACAT activity may be modulated by such mechanisms.

The in vitro investigations of the actions of detergents and organic solvents and the temperature dependence of the activity suggested that variations in ACAT activity in vivo may at least partially reflect alterations in the membrane environment of the enzyme. Although interpretation of Arrhenius plots is complex, the discontinuities observed might be explained by the hypothesis that alterations in membrane fluidity could play a role in regulating ACAT activity. Such changes might be expressed as alterations in substrate availabilities or as changes in lipid-protein interactions. Such alterations would also explain the actions of detergents and organic solvents on ACAT activity.

The inhibitory effect on ACAT of progesterone, which cannot be esterified, may be due to competition of the steroid for either substrate or modulator sites as has been suggested for the fibroblast enzyme (29). The stimulating actions of 25-hydroxycholesterol on cholesterol esterification may be due to interactions at modulator site(s). Cholesterol, when added as hyperlipemic lipoproteins to microsomes from hematoma cells (5) or liver (45), also resulted in increased ACAT activity. However, addition of cholesterol itself had little effect on initial rates of cholesterol esterification. Elucidation of the details of action of any of these steroids, including cholesterol itself, will have to await detailed kinetic analyses of ACAT activity and purification of the enzyme. Such studies are presently in progress.

The rapid increase in ACAT activity induced by MVA administration in vivo was probably due to a metabolite because MVA in vitro had no effect. However, whether this was a response to an increase in sterols from endogenous synthesis or some other factor is unclear. Previous studies with ^{3}H-labeled MVA administration in vivo indicated that the sterol newly synthesized from MVA was esterified preferentially (42).

The responses of ACAT and HMG-CoA reductase to cholesterol or cholate feeding, fasting, and atherogenic diet and MVA administration, all treatments known to increase hepatic cholesterol content, show a reciprocal relationship in that ACAT increases and reductase decreases. This suggests that they may share a common modulator of activity, perhaps activated as a result of increased hepatic sterol concentration. However, increases in reductase activity were not necessarily paralleled by decreases in ACAT activity (e.g., cholestyramine feeding, Triton WR 1339 treatment, the circadian rhythm) indicating control of the activity of these two enzymes is not directly linked in a reciprocal fashion.

On the basis of the results reported here, it appears that hepatic ACAT can respond rapidly to perturbations in cholesterol metabolism. Thus it may play a critical role in the maintenance of cholesterol homeostasis in the liver. ACAT, by esterifying free cholesterol not immediately necessary for cellular needs, may play a role in the maintenance of membrane integrity and function. Altering the free cholesterol:phospholipid ratio of membranes has been shown to alter permeability properties of membranes and to influence enzyme activities in the plasma membrane and in mitochondria (46–51). Further, by regulating the proportions of free and esterified cholesterol in the liver, ACAT may be a determinant of the rate of secretion of cholesterol into the bile and may also play a role in lipoprotein metabolism. Thus, this enzyme may be an important focal point for further investigations into the causes of both atherogenesis and lithogenesis.

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