# Regulation of hepatic cholesterol ester hydrolase and acyl-coenzyme A:cholesterol acyltransferase in the rat

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**Abstract** Cholesterol exists within the hepatocyte as free cholesterol and cholesteryl ester. The proportion of intrahepatic cholesterol in the free or ester forms is governed in part by the rate of cholesteryl ester formation by acyl-coenzyme A:cholesterol acyltransferase (ACAT) and cholesteryl ester hydrolysis by neutral cholesterol ester (CE) hydrolase. In other cell types both ACAT and CE hydrolase activities are regulated in response to changes in the need for cellular free cholesterol. In rats, we performed a variety of experimental manipulations in order to vary the need for hepatic free cholesterol and to examine what effect, if any, this had on the enzymes that govern cholesteryl ester metabolism. Administration of a 20-mg bolus of lipoprotein cholesterol or a diet supplemented with 2% cholesterol resulted in an increase in microsomal cholesteryl ester content with little change in microsomal free cholesterol. This was accomplished by an increase in cholesteryl esterification as measured by ACAT but no change in CE hydrolase activity. An increased need for hepatic free cholesterol was experimentally induced by intravenous bile salt infusion or cholestyramine (3%) added to the diet. ACAT activity was decreased with both experimental manipulations compared to controls, while CE hydrolase activity did not change. Microsomal cholesteryl ester content decreased significantly with little change in microsomal free cholesterol content. Addition of exogenous liposomal cholesterol to liver microsomes from cholestyramine-fed and control rats resulted in a 784 ± 38% increase in ACAT activity. Nevertheless, the decrease in ACAT activity with cholestyramine feeding was maintained. These studies allowed us to conclude that changes in hepatic free cholesterol needs are met in part by regulation of the rate of cholesterol esterification by ACAT without a change in the rate of cholesteryl ester hydrolysis by CE hydrolase. —**Stone, B. G., C. D. Evans, R. J. Fadden, and D. Schreiber.** Regulation of hepatic cholesterol ester hydrolase and acyl-coenzyme A:cholesterol acyltransferase in the rat. *J. Lipid Res.* 1989. **30:** 1681–1690.

**Supplementary key words** hepatic cholesterol esterification • cholesteryl ester hydrolysis • neutral cholesterol hydrolase • acyl-coenzyme A:cholesterol acyltransferase

In the hepatocyte, cholesterol exists as free cholesterol and as cholesteryl esters. The amount of free cholesterol within the cell is tightly regulated by a variety of cellular mechanisms. One important mechanism is the conversion of free cholesterol to cholesteryl esters, and hepatic esterification of free cholesterol plays an important role in protecting the cell from free cholesterol accumulation (1). Hepatic cholesterol esterification is catalyzed by the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) and the majority of ACAT enzyme activity is localized to RNA-rich microsomes (2–4). The newly formed cholesteryl esters are stored in smooth microsomes and the cytoplasm and are thought to represent an inert storage pool of cellular cholesterol (1, 4). However, the cholesterol contained within these esters in ultimately available for secretion in hepatic lipoproteins or bile (5, 6).

Hydrolysis of cholesteryl esters also represents a potential regulatory process that would affect cellular free cholesterol concentrations. In the liver, the rate of lipoprotein cholesteryl ester hydrolysis is determined by the rate of hepatic uptake of the lipoprotein particle (7). After uptake the cholesteryl esters from serum lipoproteins are hydrolyzed to free cholesterol by an acid cholesteryl esterase. This enzyme is localized to lysosomes, has a pH optimum of 4.5, and hydrolyzes exclusively lipoprotein-derived cholesteryl esters (8). In distinction, cytoplasmic cholesteryl esters formed by the action of ACAT are hydrolyzed back to free cholesterol by a separate reaction catalyzed by the enzyme neutral cholesterol ester (CE) hydrolase (EC 3.1.13) (9, 10). Therefore, cellular cholesterol under the influence of ACAT and CE hydrolase potentially can undergo a constant cycling between the free and ester forms.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; CE hydrolase, neutral cholesterol ester hydrolase; GLC, gas–liquid chromatography; HPLC, high pressure liquid chromatography; apoE, apolipoprotein E.

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The rate of cholesterol esterification by ACAT serves to regulate a variety of metabolic processes (11). In the gut it is in part responsible for cholesterol absorption (12). In the adrenal cortex the rate of cholesterol esterification regulates cholesterol availability for steroid production (13). Hepatic cholesterol esterification regulates, in part, biliary cholesterol secretion (14, 15) and VLDL cholesteryl ester secretion (5).

In some tissues the activity of CE hydrolase is responsive to cellular free cholesterol needs. In the adrenal cortex, CE hydrolase activity is stimulated by an increase in adrenocorticotrophic hormone, thus supplying more free cholesterol for steroidogenesis (13). Hormonal regulation of CE hydrolase activity has also been demonstrated in arterial smooth muscle (16), cardiac muscle (17), and in ovarian tissue (18). However, in macrophages hydrolysis of cholesteryl esters by CE hydrolase does not appear to be regulated and the level of free cholesterol in this cell type is determined by the rate of cholesterol esterification by ACAT and the rate of egress of free cholesterol from the cell (19).

The hepatic form of CE hydrolase has not been well studied. First described by Deykin and Goodman (9), it has a pH optimum of 7.0 and the majority of the enzyme activity is found in the cytosolic fraction. Since the first description, no data defining the role of CE hydrolase, if any, in regulating hepatic free cholesterol levels have been published. Furthermore, it is not known whether fluctuations in the activity of CE hydrolase occur in response to changes in cellular needs for free cholesterol.

A supply of free cholesterol is necessary for bile salt synthesis and biliary cholesterol secretion, and both these processes are regulated in part by the availability of intracellular free cholesterol (15, 20). When a need for intracellular free cholesterol develops, this need can be met by an increase in new cholesterol synthesis and an enhanced uptake of lipoprotein cholesterol (21). Another potential mechanism for supplying the needed free cholesterol would be a decrease in cellular cholesteryl ester formation. To date, a decrease in cholesteryl ester formation in response to cellular free cholesterol depletion has not been reported.

Alternately, when the supply of free cholesterol exceeds that amount necessary to satisfy cellular needs, the excess free cholesterol is quickly converted to intracellular cholesteryl esters (15, 22–24). Since the formation of cholesteryl esters by the action of ACAT and cholesteryl ester hydrolysis by CE hydrolase are opposing reactions, a change in the activity of both enzymes might regulate free cholesterol levels more precisely than a change in the activity of either enzyme alone. To date, whether the activities of CE hydrolase and ACAT both respond to changes in hepatic free cholesterol requirements has not been investigated. Therefore, the goal of these experiments was first to regulate the free cholesterol needs of the hepatocyte, and second to investigate how these manipulations influenced the opposing rates of hepatic cholesterol esterification (ACAT activity) or hydrolysis (CE hydrolase activity).

## MATERIALS AND METHODS

### Materials

Cholesteryl [1-$^{14}$C]oleate (53.1 mCi/mmol) and [1-$^{14}$C]oleoyl coenzyme A (55–60 mCi/nmol) were obtained from Amersham (Arlington Hills, IL). [9,10-$^{3}$H(N)]Oleic acid (13.3 Ci/mmol) and [1,2-$^{3}$H(N)]cholesterol (40–60 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Cholesteryl oleate, oleic acid, and taurocholic acid (99% pure) were from Sigma (St. Louis, MO).

### Animals

Male Sprague-Dawley rats (Zivic Miller, Pittsburgh, PA) weighed between 300 and 400 g at the time of killing. Groups of age- and weight-matched animals were housed under normal lighting conditions and were killed between 8 and 10 AM. All animals were fed standard laboratory chow with the exception of the groups fed: *1*) a 3% cholestyramine diet for 2 or 3 weeks; *2*) a 2% cholesterol diet for 2 weeks; or *3*) an atherogenic diet consisting of 5% lard, 1% cholesterol, 0.1% propylthiouracil, and 3% taurocholic acid for 3 weeks (22). Control groups for these experiments received the identical diet without the added cholestyramine, cholesterol, or atherogenic mixture. In one set of experiments rats received progesterone (40 mg/kg subcutaneously) or the vehicle for 4 days as described by Nervi et al. (25). The cholesterol rich lipoproteins were prepared as described previously (22).

### Bile infusion studies

For the bile infusion experiments, rats were anesthetized with pentobarbital and the bile duct and a femoral vein were cannulated with polyethylene tubing (PE-10, Clay Adams, Parsippany, NJ). In the initial experiments rats were infused with either 8 $\mu$mol/min · kg body weight for 2 h or 6 $\mu$mol/min · kg body weight for 3 h. In these experiments the control group was treated identically except normal saline was substituted for the taurocholate infusion. To extend the length of bile salt administration, rats in a separate group were intravenously infused with 4 $\mu$mol taurocholic acid/min · kg body weight overnight (18 h) and bile was collected for the entire period. To avoid bile depletion in the animals not receiving the overnight bile infusion, the control group for this experiment underwent a sham operation in which the peritoneal cavity was entered but the bile duct was not cannulated. After closure of the peritoneal cavity, a femoral vein catheter was placed for normal saline infusion. After 18 h the peri-

toneal cavity was reopened and the bile duct was cannulated for bile collection.

### Liver preparation

Five g of liver was minced and homogenzied in 12.5 ml of buffer (0.25 M sucrose, 1 mM EDTA, pH 7.2). The homogenate was centrifuged at 10,000 *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 105,000 *g* for 60 min. After centrifugation a discrete fat layer could be identified and this layer was carefully removed and discarded. The final supernatant (cytosol), containing a protein concentration of approximately 20 mg protein/ml, was assayed for CE hydrolase activity. The pellet from the 105,000 *g* centrifugation was washed by resuspension and recentrifugation. The final pellet (microsomal fraction) was resuspended in buffer to a protein concentration of approximately 10 mg/ml and was used for determination of ACAT and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activities.

### HMG-CoA reductase, ACAT, and CE hydrolase assays

ACAT activity (as a measure of the rate of intracellular cholesteryl ester formation) and HMG-CoA reductase (as a measure of the rate of intracellular free cholesterol synthesis) were assayed in the microsomal fraction as described previously (15). In selected experiments exogenous cholesterol was added to microsomes in the form of cholesterol–dipalmitoyl lecithin 2:1 liposomes prepared by the method of Rothblat, Arbogast, and Ray (26). In these experiments microsomes (150 $\mu$g protein) were preincubated for 30 min at 37°C with 70 $\mu$l of liposomes (1.97 mg cholesterol/ml) or the identical volume of buffer (27). The reaction was initiated with the addition of 10 nmol of [$^{14}$C]oleoyl coenzyme A (10 mCi/mmol) and terminated after an additional 5 min by the addition of chloroform–methanol 2:1. [$^{3}$H]Cholesteryl oleate was added to assess recovery and the product was isolated by thin-layer chromatography on silica gel G plates developed in hexane–diethyl ether–acetic acid 85:15:1.

Cytosolic CE hydrolase activity was assayed by an adaption of the method of Hoeg, Demosky, and Brewer (28). An aliquot (0.2 ml) of cytosol was incubated with 40 nmol of cholesteryl [1-$^{14}$C]oleate (3.3-13.0 dpm/pmol) delivered in 5 $\mu$l absolute ethanol. The mixture was shaken at 37°C for 1 h. The reaction was terminated by the addition of 3 ml of a 0.1 mM oleate solution in benzene–chloroform–methanol 1:0.5:1.2. An internal standard of [$^{3}$H]oleate (20,000 dpm) was added to each tube to assess recovery. After addition of 0.7 ml of 0.3 M NaOH, the mixture was immediately centrifuged for 10 min at 1,000 *g* to separate and recover the newly released [$^{14}$C]oleate in the aqueous phase. An aliquot (1 ml) of the aqueous upper layer was added to Aquasol-2 (New England Nuclear) and counted in a Packard liquid scintillation counter. The amount of [$^{14}$C]oleate released by hydrolysis of the radiolabeled cholesteryl ester was calculated by correcting for recovery of the internal standard and subtracting the [$^{14}$C]oleate recovered from a reaction mixture containing buffer (0.2 ml) in place of the cytosolic protein. The efficiency of extraction ranged from 80 to 90% and the specific activity of CE hydrolase was expressed as pmol oleate/min · mg cytosolic protein.

### Liver cholesterol determinations

The cholesterol (free and esterified) content of the cytosolic fraction was determined by gas–liquid chromatography (GLC) as previously described (15). This method calculates the cholesteryl ester content as the difference between the total and free cholesterol contents in the same sample. In microsomes, the cholesteryl ester content was routinely only 10% of the total cholesterol content. Therefore, it was difficult to accurately quantitate changes in microsomal cholesteryl ester content by this method. In order to overcome this problem, a method was developed to directly measure the microsomal cholesterol (free and esterified) contents by isocratic normal phase high pressure liquid chromatography (HPLC).

Chromatography was performed using a Shimadzu (Kyoto, Japan) model LC-6A solvent delivery system on a 25.0 cm × 4.6 mm Zorbax (Du Pont, Wilmington, DE) silicon oxide column. Desmosterol (150 $\mu$g) was added to each sample to assess recovery. Cholesterol was extracted by the addition of 7 ml of hexane-isopropanol-basic water 1.0:4.0:0.1 (v/v) followed by 2.25 ml basic water (pH 10) and 4.5 ml of hexane. The mixture was separated into two distinct layers by low speed centrifugation. The upper hexane layer was loaded onto prewashed silica Sep-Pak columns (Waters, Milford, MA) and the cholesteryl esters were eluted with 12 ml of 1% (v/v) ethyl acetate in hexane. The free cholesterol was then eluted off the column with 15% (v/v) ethyl acetate in hexane (29). This technique of extraction and sequential elution by increasing solvent polarity resulted in greater than 88% recovery of the internal standard and complete separation of free cholesterol and cholesteryl ester when analyzed by HPLC (data not shown).

The solvent containing the free cholesterol was evaporated under a $N_2$ stream and the free cholesterol was redissolved in 240 $\mu$l of mobile phase composed of hexane–butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01 (30). The mobile phase was delivered at a rate of 2.5 ml/min. A 20-$\mu$l sample was injected using a fixed volume flow loop. The free cholesterol was detected at 206 nm using a Shimadzu ultraviolet spectrophotometric detector model SPD-6A and quantitated with a Shimadzu Chromatopac Model C-R3A by integrating the area under the signal. The mass was calculated by comparison to a free cholesterol standard after correcting for recovery.

The fraction containing the eluted cholesteryl esters was evaporated to dryness under $N_2$ and the cholesteryl

esters were saponified to free cholesterol as previously described (15). The liberated cholesterol was extracted with two volumes of hexane (5 ml) and the cholesterol was quantitated as described above.

### Other determinations

Biliary lipids were determined as previously described (15). The protein contents were determined by the biuret method with bovine serum albumin as a standard (31).

## RESULTS

### Determinations of assay conditions

In the CE hydrolase assay described by Deykin and Goodman (9) the cholesteryl ester substrate was added to the reaction mixture dissolved in acetone. However, as has been previously noted for arterial CE hydrolase (16), a significant increase in CE hydrolase activity was measured when the radiolabeled substrate was delivered in ethanol (10.4 pmol oleate/mg · min) compared to the same mass of substrate delivered in acetone (4.3 pmol oleate/mg · min). Therefore, the substrate was delivered in ethanol in all experiments.

A maximal rate of hydrolysis of the added cholesteryl [1-$^{14}$C]oleate was achieved with the addition of 40 nmol of the radiolabeled substrate to the reaction mixture. This was true with addition of the substrate either to 0.8 mg cytosolic protein (**Fig. 1A**, main figure) or 4 mg of cytosolic protein (Fig. 1A, insert). Therefore, for all determinations of CE hydrolase activity, 40 nmol of cholesteryl [1-$^{14}$C]oleate was added to the reaction mixture. The rate of CE hydrolysis was linear for 60 min (Fig. 1B) over a wide range of cytosolic protein concentrations (Fig. 1C). Routinely, between 0.8 and 4 mg of cytosolic protein was utilized for measuring CE hydrolase activity.

### Effect of increasing hepatic cholesterol availability on ACAT and CE hydrolase activities

The initial series of experiments was designed to create a relative excess of cholesterol in the cell to determine what effect, if any, this had on the enzymes governing cellular cholesteryl ester metabolism. An acute excess of

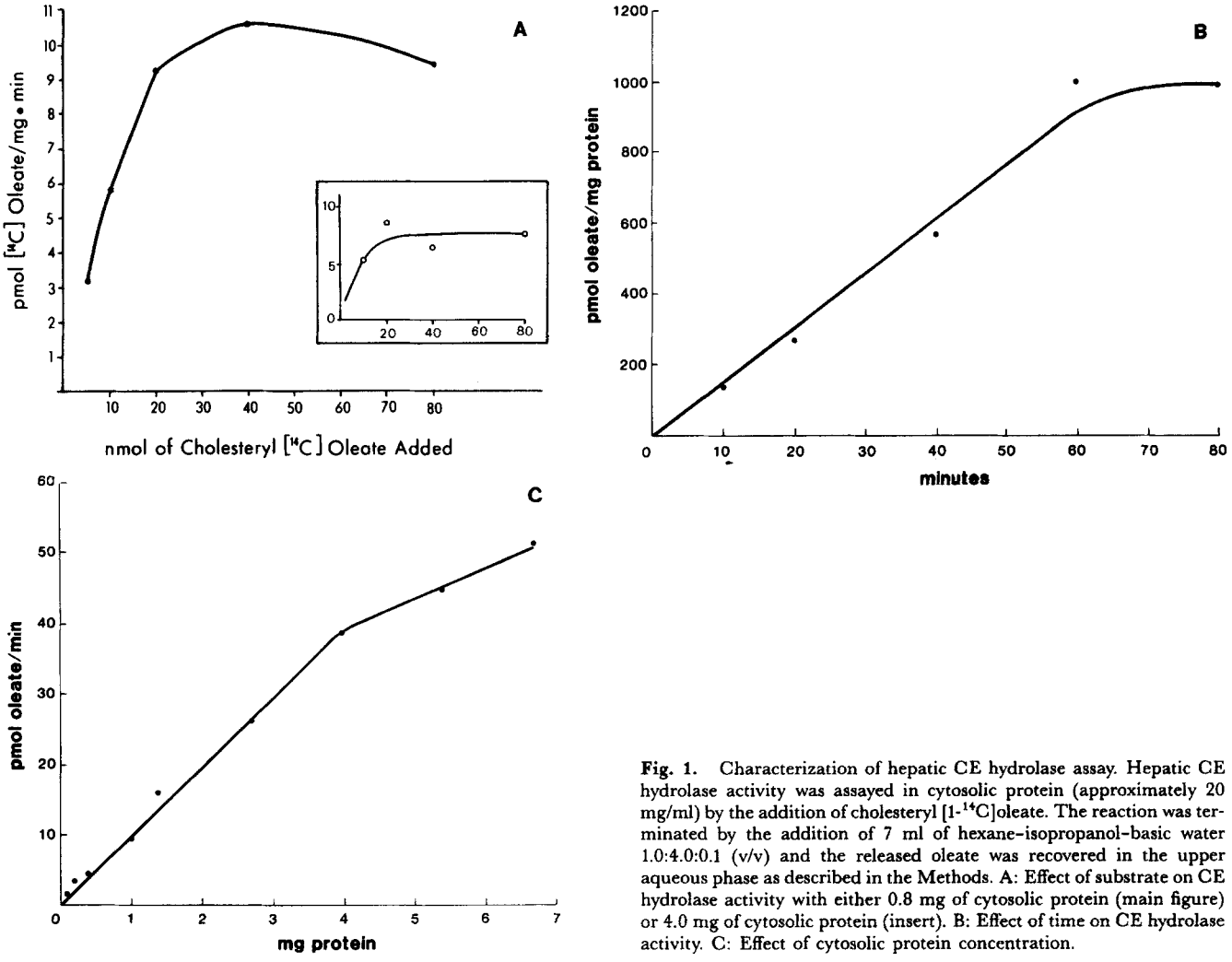


**Fig. 1.** Characterization of hepatic CE hydrolase assay. Hepatic CE hydrolase activity was assayed in cytosolic protein (approximately 20 mg/ml) by the addition of cholesteryl [1-$^{14}$C]oleate. The reaction was terminated by the addition of 7 ml of hexane-isopropanol-basic water 1.0:4.0:0.1 (v/v) and the released oleate was recovered in the upper aqueous phase as described in the Methods. A: Effect of substrate on CE hydrolase activity with either 0.8 mg of cytosolic protein (main figure) or 4.0 mg of cytosolic protein (insert). B: Effect of time on CE hydrolase activity. C: Effect of cytosolic protein concentration.

cholesterol was created in the liver by delivering an intravenous bolus of apoE and cholesterol-rich lipoproteins (20 mg of cholesterol) obtained from donor rats fed an atherogenic diet. As we have previously reported (15), the administration of the cholesterol-rich lipoproteins increased ACAT activity 46% over controls, but did not change CE hydrolase activity. This was accompanied by a small increase in microsomal free cholesterol content (24.07 ± 1.12 μg/mg protein vs 20.19 ± 1.17 μg/mg protein control, $P < 0.05$), and a relatively greater increase in microsomal cholesteryl ester content (2.12 ± 0.25 μg/mg protein vs 1.07 ± 0.13 μg/mg protein control, $P < 0.01$).

We next examined the effect of chronic cholesterol feeding on intracellular cholesteryl ester metabolism. A 2% cholesterol-enriched diet resulted in a significant increase in the rate of cholesteryl ester formation as measured by ACAT (**Table 1**). Using 4 mg of cytosolic protein per assay, there was an accompanying decrease in CE hydrolase activity compared to controls. The measured change in CE hydrolase activity in response to cholesterol feeding might represent a real inhibition of cholesteryl ester hydrolysis within the cell. Alternatively, the measured decrease in CE hydrolase activity may be due to an increase in cytosolic cholesteryl ester content. The more abundant unlabeled cholesteryl esters would effectively dilute the added cholesteryl [$^{14}$C]oleate. The net result would be a decrease in the rate of hydrolysis of the radiolabeled substrate and, therefore, the apparent CE hydrolase activity.

We therefore measured the cholesterol contents (free and esterified) of the cytosolic preparations from the cholesterol-fed and control rats. Cholesterol feeding resulted in an elevated cytosolic free cholesterol content (2.4 ± 0.5 μg chol/mg protein, n = 6) compared to the free cholesterol content of controls (0.9 ± 0.2 μg chol/mg protein, n = 6, $P < 0.02$). The cytosolic cholesteryl ester content was increased to a greater degree with cholesterol feeding (7.4 ± 0.9 μg chol/mg protein, cholesterol fed vs 0.8 ± 0.1 μg chol/mg protein, control, n = 6, $P < 0.001$). This tenfold increase in cytosolic cholesteryl esters would result in the dilution of the added radiolabeled cholesteryl [$^{14}$C]oleate substrate and a decrease in the calculated specific activity (dpm cholesteryl [$^{14}$C]oleate/pmol cholesteryl ester) of the cholesterol fed cytosols to 41% of that calculated for the radiolabeled substrate added to the control cytosols. This calculated decrease agrees well with the measured decrease in CE hydrolase enzyme activity with cholesterol feeding (47% of control).

We also measured the CE hydrolase activity in cholesterol-fed and control rats using 0.8 mg of cytosolic protein to decrease the dilution of the radiolabeled substrate by endogenous cholesteryl esters. No difference was apparent between the cholesterol-fed rats (5.16 ± 0.35 pmol/mg · min, n = 6) and the controls (6.05 ± 0.57 pmol/mg · min, n = 6) at this lower protein concentration. These results suggest that the decrease in CE hydrolase activity with cholesterol feeding was due to radiolabeled substrate dilution rather than a diminished enzyme activity. Cholesterol feeding also resulted in a modest increase in free cholesterol content and a greater increase in microsomal cholesteryl ester content (Table 1).

Finally, we measured cytosolic CE hydrolase in rats fed an atherogenic diet for a 3-week period. This diet has previously been shown to markedly enhance ACAT activity (3). Again, no significant change in CE hydrolase activity was appreciated between the animals fed the atherogenic diet (6.17 ± 1.17 pmol/mg · min, n = 3) and age- and weight-matched controls (6.62 ± 0.62 pmol/mg · min, n = 2).

### Effect of decreasing hepatic cholesterol availability on ACAT and CE hydrolase activities

The goal of this series of experiments was to create a need for free cholesterol within the cell. To create this need we utilized an intravenous bile salt infusion to stimulate bile salt-coupled biliary cholesterol secretion. In initial studies we infused a group of rats equipped with bile fistulae with either 8 μmol taurocholate/min · kg body weight (n = 6) or a control group (n = 4) receiving an equal amount of normal saline for 2 h. No change in ACAT activity (210 ± 33 pmol/mg · min vs 171 ± 28 pmol/ mg · min, control) or CE hydrolase activity

TABLE 1. Effect of a 2% cholesterol diet on CE hydrolase, ACAT, and microsomal cholesterol content

| | Cholesterol-fed (12) | Control (12) |
|---|---|---|
| ACAT (pmol/mg · min) | 251 ± 25[a] | 129 ± 9 |
| CE hydrolase (pmol/mg · min) | 3.2 ± 0.5[a] | 6.8 ± 0.7 |
| Microsomal free cholesterol (μg/mg) | 23.84 ± 0.94[b] | 19.95 ± 0.54 |
| Microsomal cholesteryl ester (μg/mg) | 5.42 ± 0.38[a] | 0.95 ± 0.03 |

Groups of rats were fed either a diet supplemented with 2% cholesterol or the control diet for a 2-week period. The number of determinations is in parentheses and the values are expressed as the mean ± SE.
[a]Different from control at $P < 0.001$.
[b]Different from control at $P < 0.01$.

TABLE 2. Effect of a 3-h infusion of taurocholate on CE hydrolase, ACAT, and HMG-CoA reductase activities

| | Bile Infused | Control |
|---|---|---|
| CE hydrolase (pmol/mg · min) | 11.6 ± 2.8 (6) | 12.7 ± 2.2 (6) |
| ACAT (pmol/mg · min) | 144 ± 21 (6) | 167 ± 12 (6) |
| HMG-CoA reductase (pmol/mg · min) | 169 ± 18 (5) | 171 ± 15 (4) |

CE hydrolase, HMG-CoA reductase, and ACAT activities were determined in rats with bile fistulae after a 3-h infusion of taurocholate (80 mM) at a rate of 6 μmol/min · kg body weight. The control group was fitted with bile duct catheters and received an identical volume of normal saline for 3 h. No significant differences in enzyme activities were observed. The number of animals is in parentheses and the values are expressed as the mean ± SE.

(8.9 ± 0.7 pmol/mg · min vs 8.1 ± 0.6 pmol/mg · min, control) could be observed between the two groups. In addition, a 3-h infusion of 6 μmol taurocholate/mg · kg body weight did not alter ACAT, CE hydrolase, or HMG-CoA reductase activities (**Table 2**). These results suggested that intracellular cholesterol depletion had not yet occurred by 3 h. We therefore intravenously administered a solution of taurocholate (60 mM) at a rate of 4 μmol/min · kg body weight for 18 h to a group of rats. A marked and sustained increase in bile flow, and bile salt, cholesterol and phospholipid secretion occurred over that observed in a control group receiving a normal saline infusion (**Table 3**). At the end of the 18-h infusion period there was a significant decrease in ACAT activity, but no significant change in HMG-CoA reductase or CE hydrolase activities (**Table 4**). The decrease in ACAT activity was associated with a significant reduction in the microsomal cholesteryl ester content without a significant change in free cholesterol content compared to controls.

Cholestyramine feeding, by depleting the bile salt pool, should create a need for free cholesterol in the cell. Therefore, a diet of 3% cholestyramine was administered for either 2 or 3 weeks to groups of rats. After 2 weeks of a cholestyramine-supplemented diet there was no change in CE hydrolase activity (12.8 ± 3.2 pmol/mg · min, n = 3) compared to controls (11.3 ± 2.2 pmol/mg · min, n = 4); however, there was a decrease in ACAT activity (116 ± 13 pmol/mg · min, n = 3) compared to controls (213 ± 24 pmol/mg · min, n = 3, $P<0.05$). Cholestyramine feeding for 3 weeks resulted in a significant decrease in the activity of ACAT, a marked increase in HMG-CoA reductase activity, but no change in the activity of CE hydrolase compared to rats receiving an identical diet but without the added cholestyramine (**Table 5**). This was accompanied by a small decrease in microsomal free cholesterol concentration and a greater decrease in microsomal cholesteryl ester concentration compared to controls.

The observed decrease in ACAT activity with cholestyramine feeding might be due to a change in the enzyme activity per se or to a decreased availability of microsomal free cholesterol substrate to the enzyme. We therefore supplied additional free cholesterol to microsomes by preincubating with cholesterol-rich liposomes (**Table 6**). The preincubation of microsomes from both the cholestyramine-fed and control rats with liposomal cholesterol effected a marked increase in ACAT activity over both types of microsomes preincubated for 30 min at 37°C without added liposomes (A), and microsomes not subjected to preincubation (unincubated microsomes). Nevertheless, a similar difference was maintained between the cholestyramine-fed and control ACAT activities in the cholesterol-supplemented microsomes (B) as had been

TABLE 3. Biliary lipid secretion in response to an overnight intravenous bile salt infusion

| | Bile Flow | Bile Salt | Cholesterol | Phospholipid |
|---|---|---|---|---|
| | *μl/kg · hr* | | *μmol/kg · hr* | |
| Bile infused (n = 7) | | | | |
| First hour | 5.3 ± 0.3[a] | 365 ± 16[a] | 4.2 ± 1.3[b] | 21.1 ± 3.9[a] |
| Last hour | 5.3 ± 0.3[a] | 260 ± 21[a] | 3.0 ± 0.4[b] | 25.0 ± 3.4[a] |
| Control (n = 11) | 3.1 ± 0.3 | 94 ± 9 | 1.8 ± 0.3 | 10.2 ± 0.8 |

Rats were fitted with bile duct catheters and taurocholate (60 mM) was infused through a surgically placed femoral catheter at a rate of 4 μmol/kg · min for 18 h. A 1-h bile sample was collected after the first and last hour of bile infusion. Control animals received a sham operation and a similar volume of normal saline over the 18-h period, after which the bile duct was cannulated and a 1-h bile collection was obtained. The number of animals is given in parentheses and the values are expressed as the mean ± SE.

[a]Different from control at $P < 0.001$.

[b]Different from control at $P < 0.05$.

TABLE 4. Effect of an overnight bile salt infusion on enzyme activities and microsomal cholesterol content

| | Bile-Infused | Control |
|---|---|---|
| ACAT (pmol/mg · min) | 195 ± 19[a] (6) | 260 ± 22 (7) |
| HMG-CoA reductase (pmol/mg · min) | 146 ± 24 (8) | 125 ± 20 (9) |
| CE hydrolase (pmol/mg · min) | 17.2 ± 2.1 (8) | 14.8 ± 1.3 (9) |
| Microsomal free cholesterol (μg/mg) | 19.65 ± 0.05 (8) | 19.94 ± 0.59 (8) |
| Microsomal cholesteryl ester (μg/mg) | 0.64 ± 0.04[a] (8) | 0.98 ± 0.08 (8) |

CE hydrolase, HMG-CoA reductase, and ACAT activities were measured in bile-infused and control rats as described in the legend to Table 3 and the Methods. The number of determinations is in parentheses and the values are expressed as the mean ± SE.

[a]Different from control at $P < 0.01$.

demonstrated in the unsupplemented microsomes (A) and the unincubated microsomes.

### Effect of progesterone treatment on ACAT and CE hydrolase activities

Chronic progesterone therapy at a dose of 40 mg/kg body weight administered subcutaneously to rats for 3–14 days has been shown to inhibit ACAT activity in harvested microsomes (25). We therefore tested whether this might influence CE hydrolase activity. In agreement with previously published work, progesterone treatment (40 mg/kg body weight for 4 days) decreased microsomal ACAT activity from 126 ± 9 pmol/mg · min in controls receiving the corn oil vehicle to 60 ± 17 pmol/mg · min ($P<0.05$, n = 2). Again, no change in CE hydrolase activity was apparent between the progesterone-treated (13.7 ± 0.6 pmol/mg · min, n = 5) and the vehicle-treated controls (13.9 ± 0.4 pmol/mg · min, n = 5).

## DISCUSSION

Neelon and Lack (32) demonstrated that the in vitro addition of bile salts to liver homogenates stimulated CE hydrolase activity and inhibited ACAT activity. These authors suggested that in vivo bile salts may alter the proportion of hepatic cholesterol in the free or esterified form by regulating the rates of cholesterol esterification and cholesteryl ester hydrolysis. Addition of taurocholate (0.5 mM) in vitro to cytosolic protein resulted in a significant increase in CE hydrolase activity (12.03 pmol/mg · min) over that measured in the same sample not incubated with taurocholate (8.86 pmol/mg · min), confirming the findings of Neelon and Lack. Therefore, we tested whether bile salts influence ACAT and CE hydrolase activities in vivo. Infusion of bile salts at a rate 2–3 times the normal secretion rate for 2 or 3 h did not affect ACAT or CE hydrolase activities. These results do not support the concept that CE hydrolase is directly regulated in vivo by bile salts.

The administration of taurocholate for 18 h at a rate that was twice the bile salt secretion rate of the controls resulted in a sustained increase in bile flow and bile salt, phospholipid and biliary cholesterol secretion (Table 3). The increased secretion of free cholesterol in the bile should create a relative deficiency of free cholesterol in the cell. Cholestyramine administration by promoting fecal losses of bile salts creates a cellular need for free cholesterol to replace cholesterol utilized for bile salt synthesis. This need for free cholesterol is met, in part, by an enhanced hepatic uptake of lipoprotein cholesterol and an increase in hepatic cholesterol synthesis (21). In agreement, our data demonstrated an increase in microsomal HMG-CoA reductase activity with cholestyramine feed-

TABLE 5. Effect of a 3% cholestyramine diet administered for 3 weeks on enzyme activities and microsomal cholesterol content

| | Cholestyramine-Fed | Control |
|---|---|---|
| ACAT (pmol/mg · min) | 128 ± 3[a] | 207 ± 8 |
| CE hydrolase (pmol/mg · min) | 9.1 ± 1.1 | 12.0 ± 1.6 |
| HMG-CoA reductase (pmol/mg · min) | 487 ± 29[b] | 84 ± 14 |
| Microsomal free cholesterol (μg/mg) | 17.20 ± 0.42[b] | 20.80 ± 0.61 |
| Microsomal cholesteryl ester (μg/mg) | 0.66 ± 0.04[c] | 1.04 ± 0.17 |

Groups of rats were fed either a diet containing 3% cholestyramine or the control diet for a 3-week period. Each value represents the mean ± SE of five animals.

[a]Different from control at $P < 0.02$.

[b]Different from control at $P < 0.01$.

[c]Different from control at $P < 0.05$.

TABLE 6. Effect of the addition of liposomal cholesterol to microsomes from cholestyramine-fed and control rats

| | ACAT Activity | |
|---|---|---|
| | Cholestyramine-Fed (5) | Control (4) |
| | *pmol/mg · min* | |
| Unincubated microsomes | 123 ± 15[a] | 205 ± 28 |
| Preincubated microsomes | | |
| A) Without added liposomes | 113 ± 13[a] | 180 ± 27 |
| B) With added liposomes | 1041 ± 80[a] | 1404 ± 143 |

Aliquots of microsomes (150 μg protein) from rats treated with 3% cholestyramine for 3 weeks were preincubated for 30 min at 37°C with and without cholesterol dipalmitoyl lecithin 2:1 liposomes containing 300 nmol of cholesterol. Aliquots of microsomes from control animals were preincubated under the same circumstances. ACAT activity was determined in both sets of preincubated microsomes and in microsomal aliquots not subject to preincubation. In all cases ACAT activity in the microsomes from cholestyramine-fed rats was significantly decreased compared to controls. Although preincubation with liposomal cholesterol markedly enhanced ACAT activity in microsomes from both the cholestyramine-fed and control rats, the increase was comparable. The number of rats in each group is in parentheses and the values are expressed as the mean ± SEM.

[a]Different from control at $P < 0.05$.

ing (Table 5). With the overnight taurocholate infusion, no change in HMG-CoA reductase occurred (Table 4). The difference between the effect of cholestyramine feeding for 3 weeks and the overnight bile salt infusion on HMG-CoA reductase activity may relate to *1*) a greater depletion of microsomal free cholesterol, *2*) a longer time of exposure of the liver to cholesterol depletion, and *3*) the additional stimulus of bile salt depletion that occurs with cholestyramine feeding.

The hepatic need for free cholesterol could also be met by a decrease in cholesteryl ester formation or an increase in cholesteryl ester hydrolysis. Previous work by Erickson et al. (3) did not demonstrate an effect of a cholestyramine-supplemented diet on ACAT or microsomal cholesterol content. However, we observed a significant decrease in ACAT activity which, in turn, maintained the microsomal free cholesterol content near the control levels (Table 5). This occurred at the expense of the cholesteryl ester content, which was only 63% of the control amount. The difference between our results and those reported by Erickson et al. may be related to the differing cholestyramine content in the diet or the length of cholestyramine feeding. In our study a decrease in ACAT activity and microsomal cholesteryl ester content occurred in both the overnight taurocholate infusion and cholestyramine feeding. We suggest that these changes serve to replete the cell with free cholesterol by decreasing the amount of free cholesterol converted to the esterified form. With cholestyramine feeding, an increased export of cholesteryl esters in very low density lipoproteins may also contribute to the observed decrease in microsomal cholesteryl ester content (21). In distinction, hepatic CE hydrolase activity did not appear to be regulated by an increased need for free cholesterol in the cell.

It has been suggested that an increase in the intrahepatic free cholesterol concentration is rapidly balanced by an increase in the rate of cholesterol esterification by ACAT in order to prevent excess cellular free cholesterol accumulation (1). With cholesterol feeding and lipoprotein cholesterol administration, we observed a marked increase in ACAT activity and microsomal cholesteryl ester content with only a modest increasse in microsomal free cholesterol content. Although the effect of an increase in hepatic free cholesterol availability on hepatic ACAT activity has been previously published (3, 22, 23), the effect on CE hydrolase has not been described. With lipoprotein cholesterol administration and cholesterol feeding, CE hydrolase activity did not change compared to controls. Therefore, in both the acute and chronic situations, a free cholesterol excess in the cell stimulated ACAT activity without changing CE hydrolase activity. In turn, the increase in ACAT activity resulted in a net increase in microsomal cholesteryl esters while maintaining constant the free cholesterol content.

Together these results reaffirm the concept that ACAT serves to protect the cell by providing a defense against excess free cholesterol accumulation (1). The data reported here also extend this concept by suggesting that a need for cellular free cholesterol can be met, in part, by a decrease in cholesteryl ester formation. A decrease in hepatic ACAT activity in response to microsomal free cholesterol depletion has, to our knowledge, not been previously demonstrated. Therefore, the rate of cholesterol esterification within the hepatocyte appears to be a highly regulated process, responding to changes in the levels of intrahepatic free cholesterol. The regulation of ACAT activity by free cholesterol is thought to occur both by changes in enzyme substrate availability and by nonsubstrate modulation of enzyme activity (33). Our data demonstrating an increase in ACAT activity when exogenous cholesterol was supplied to microsomes are consistent with both mechanisms regulating ACAT activity

(Table 6). The marked increase in ACAT activity with liposomal cholesterol addition in microsomes from both the control and cholestyramine-fed rats suggest that cholesterol substrate availability is a major regulator of ACAT activity. However, a difference in ACAT activity between the cholestyramine-fed rats and the control rats was maintained, suggesting that nonsubstrate modulation of ACAT enzyme activity also had occurred in response to cholestyramine feeding.

Recently, several studies have equated a change in ACAT activity in response to experimental manipulation (including progesterone administration) with a comparable change in hepatic cholesteryl ester formation (3, 13, 14). This interpretation is only valid if the opposing rate of hydrolysis of cholesteryl esters is not affected. The lack of demonstrated change in CE hydrolase activity supports the validity of equating in vitro ACAT activity with cholesteryl ester formation in the liver. Furthermore, these data demonstrate that hepatic cholesteryl ester hydrolysis by CE hydrolase does not respond to changes in hepatic free cholesterol levels. This is in contrast to the rate of cholesteryl ester hydrolysis found in other tissues, where CE hydrolase activity is regulated in response to the needs of the cell for free cholesterol (10, 13). It should be noted that the activity of CE hydrolase represents only a relative measure of the in vivo rate of cholesteryl ester hydrolysis. Nevertheless, comparing the experimental and appropriate controls under the conditions studied, these data suggest that regulation of cytosolic CE hydrolase enzyme activity is not an important mechanism in maintaining free cholesterol levels in the hepatocyte.

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