

## Regulation of Biliary Cholesterol Secretion in the Rat

### Role of Hepatic Cholesterol Esterification

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**A**bstract. Although the significance of the enterohepatic circulation of bile salts in the solubilization and biliary excretion of cholesterol is well established, little is known about the intrahepatic determinants of biliary cholesterol output. Studies were undertaken to elucidate some of these determinants in the rat. Feeding 1% diosgenin for 1 wk increased biliary cholesterol output and saturation by 400%. Bile flow, biliary bile salt, phospholipid and protein outputs remained in the normal range. When ethynyl estradiol (EE) was injected into these animals, biliary cholesterol output decreased to almost normal levels under circumstances of minor changes in the rates of biliary bile salt and phospholipid outputs. Similarly, when chylomicron cholesterol was intravenously injected into diosgenin-fed animals, biliary cholesterol output significantly decreased as a function of the dose of chylomicron cholesterol administered.

Relative rates of hepatic cholesterol synthesis and esterification were measured in isolated hepatocytes. Although hepatic cholesterogenesis increased 300% in diosgenin-fed animals, the contribution of newly synthesized cholesterol to total biliary cholesterol output was only  $19 \pm 9\%$ , compared with  $12 \pm 6\%$  in control and  $15 \pm 5\%$  in diosgenin-fed and EE-injected rats. The rate of oleate incorporation into hepatocytic cholesterol esters was 30% inhibited in diosgenin-fed rats. When EE was injected into these animals, the rate of cholesterol esterification increased to almost 300%. To investigate further

the interrelationship between hepatic cholesterol esterification and biliary cholesterol output, we studied 21 diosgenin-fed rats. Six of them received in addition EE and 10 received chylomicron cholesterol. The relationships between biliary cholesterol output as a function of both microsomal acyl-CoA:cholesterol acyltransferase (ACAT) activity and hepatic cholesterol ester concentration were significantly correlated in a reciprocal manner. From these results it is concluded that the size of the biliary cholesterol precursor pool can be rapidly modified through changes in the activity of the hepatic ACAT.

### Introduction

Biliary cholesterol represents one of the two major excretory pathways of sterol elimination from the organism. In the last few years, considerable attention has been paid to the regulatory mechanisms of biliary cholesterol output, because a significant number of patients with gallstone disease present abnormally high rates of biliary cholesterol secretion as the primary pathogenic event (1-3).

Biliary cholesterol originates from a preformed pool of hepatic free cholesterol (4-8). Approximately 15-20% of this precursor pool derives from newly synthesized hepatic cholesterol in man and rat (6, 9). However, experimental conditions associated with high rates of hepatic cholesterogenesis do not induce absolute changes in biliary cholesterol secretion (7). Similarly, the hepatic overload with dietary cholesterol does not drive the excretion of free cholesterol through the canalicular membrane (7, 10). It is unknown whether some specific serum lipoprotein can deliver preferentially cholesterol into the bile.

Regulation of the rate of biliary cholesterol output is complex and is known to be primarily influenced by the rate of biliary bile salt output in man, rhesus monkey, dog, sheep, and rat (11-16). The composition of the bile acid pool is also important in determining biliary cholesterol output. For example, the chronic administration of chenodeoxycholic acid or ursodeoxycholic acid to patients with cholesterol gallstones induces a significant reduction in the rate of biliary cholesterol output (12, 17). This effect, however, is not apparent in the rat (18).

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Recent data from this laboratory have suggested the presence of an important intrahepatic determinant of the rate of biliary cholesterol secretion. This regulatory mechanism, dependent on the size of the hepatic free cholesterol pool destined for biliary secretion, is closely connected with the activity of the microsomal acyl-CoA:cholesterol acyltransferase (ACAT)<sup>1</sup> (EO 2.3.1.26) (19, 20).

The present investigation was undertaken to define further the interrelationships between the hepatic precursor free cholesterol pool and the rate of biliary cholesterol output. Taking advantage of the observation that diosgenin (5,20 $\alpha$ ,22 $\alpha$ ,25 D-Spirosten-3 $\beta$ -ol) increases hepatic cholesterologenesis, fecal neutral sterol output, and biliary cholesterol concentration in the rat (21), we studied: the kinetic characteristics of biliary cholesterol output in diosgenin-fed rats; the effect of ethynyl estradiol (EE) and chylomicron cholesterol on the high biliary cholesterol output induced by diosgenin; the interrelationships between biliary cholesterol output, and hepatic cholesterol synthesis and esterification as two possible determinants of the size of the biliary cholesterol precursor pool. These studies demonstrate that biliary cholesterol can be easily dissociated from the rate of biliary bile salt output and that hepatic cholesterol esterification may be the major intrahepatic determinant of the size of the biliary cholesterol precursor pool.

## Methods

**Materials and animals.** Oleic acid oleoyl-Coenzyme A (CoA), cholesteryl oleate, cholesterol, diosgenin, ethynyl estradiol, triolein, taurocholate, albumin, collagenase, and hydroxysteroid dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-<sup>3</sup>H-Cholesterol, cholesteryl oleate ([1-<sup>14</sup>C]oleate), [1-<sup>14</sup>C]oleic acid, [1-<sup>14</sup>C]oleoyl CoA, and 2-<sup>14</sup>C-acetate were obtained from New England Nuclear (Boston, MA). Silicic acid was obtained from Mallinckrodt Chemical Works (St. Louis, MO). Celite was obtained from Johns-Manville Products Corp. (Denver, CO). Silica gel and all organic solvents were obtained from E. Merck (Darmstadt, Federal Republic of Germany). Polyethylene catheters were purchased from Clay Adams Inc. (Parsippany, NJ).

Male Wistar rats in the weight range 100–140 g were subjected to reversed light cycling for 2–3 wk before use. The mid-dark point was set at 10 a.m. The animals were fed ground chow that contained cholesterol 13–20 mg%. Some groups received ground chow that contained 0.25–1% (wt/vol) diosgenin for 6 d before the experiments. In another experiment, groups of rats received 1% (wt/vol) diosgenin for different periods of time, from two to six days. Diosgenin was dissolved in chloroform, mixed with the diet, and the solvent evaporated by heating 24 h at 37°C under a hood.

**Biliary secretion studies.** Initial and bile salt-depleted bile specimens were obtained as previously described (19). Values are presented as the nanomole of lipid secreted per gram of liver weight per minute of bile collection. In some experiments, sodium taurocholate dissolved in 0.9% NaCl was infused intravenously in different concentrations to increase the outputs of biliary lipids. The kinetic characteristics of

biliary cholesterol and phospholipid outputs were studied as a function of biliary bile salt output. Simulated curves were derived with the experimental data by standard computer procedures (B.M.D. PAR Subroutine, Biomedical Computer Programs P-Series, University of California, Los Angeles, CA) as previously described (19). As expected, the best curve fitting for the experimental data was a nonlinear regression of the form of the rectangular hyperbola  $y = ax/b + x$  for the relationship bile salts-cholesterol and bile salts-phospholipid. In this equation, the term  $a$  represents the maximal theoretical output of biliary lipid obtained with some experimental manipulation. This term is equivalent to the maximum velocity usually calculated in enzyme kinetics. The best mathematical expression for the relationship biliary cholesterol output as a function of biliary phospholipid output was the linear regression  $y = a + bx$ .

**Optical examination of bile samples.** A series of bile specimens from four control and four diosgenin-fed rats were immediately examined for the presence of solids, and microscopically (direct light and polarizing) for cholesterol crystals. This experiment was designed to determine whether diosgenin metabolites excreted into the bile (22) may have increased cholesterol solubility. Bile samples were maintained at 20°C in the darkness. Small aliquots (5  $\mu$ l) of bile were examined daily for 5 d. Because supersaturated bile specimens from diosgenin-fed rats remained isotropic, we disturbed the physicochemical equilibrium of bile specimens by freezing. After thawing, the specimens were examined again for the presence of crystals.

**Determination of ACAT.** Hepatic microsomal fractions were obtained and ACAT activity was measured as previously described (19, 20). The reaction was performed within the first 24 h after killing the animals between 10:00 and 14:00 h. The microsomes were preincubated for 5 min at 37°C and the reaction was initiated by the addition of oleoyl-CoA, specific activity 5,100 dpm/nmol, and a final concentration of 100  $\mu$ M in the assay. Initial rates of ACAT activity were measured by incubation for periods of 7 min. All experiments were performed in duplicate.

**Determination of hepatic cholesterol synthesis and esterification in isolated hepatocyte.** Isolated hepatocytes from normal and diosgenin-fed rats were prepared as described by Berry and Friend (23). These experiments were started between 08:30 and 10:00 in the morning and the incubations were performed usually between 11:00 and 13:00 h. The cells were resuspended in Krebs-Henseleit bicarbonate buffer pH 7.4, which contained 2.5 mM CaCl<sub>2</sub>. Between 87 and 90% of the cells excluded Trypan Blue. Control rats were always studied in parallel with the diosgenin-fed animals.

The incubations were started by addition of 0.2 ml of cell suspension (7–10 mg of protein) to 0.8 ml of buffer that contained either 0.5 mM bovine serum albumin (fatty acid free), 1 mM [1-<sup>14</sup>C]oleic acid (0.75  $\mu$ Ci/ml), or 1.8 mM 2-<sup>14</sup>C-acetate (1.6  $\mu$ Ci/ml). The rates of incorporation of oleic acid into cholesteryl oleate was linear as a function of time over the first 15 min, whereas linearity was observed over the first 90 min of incubation in the studies of acetate incorporation into cholesterol. Therefore, the incubation times for the experimental series were fixed in 10 and 60 min for the first and second reaction, respectively. Incubations were performed in 25-ml Erlenmeyer flasks oxygenated with 5% CO<sub>2</sub> in 95% O<sub>2</sub> at 37°C.

The reactions were stopped with 20 vol of chloroform-methanol 2:1 (24). After separating the two phases with 20% water, the lower phase was removed and evaporated at 45°C under nitrogen. In the studies of oleate incorporation into cholesterol ester, the residue was dissolved in chloroform and applied to thin layer chromatoplates. Lipids were separated using a solvent mixture of hexane:ethyl-acetate

1. *Abbreviations used in this paper:* ACAT, acyl-CoA:cholesterol acyltransferase; CoA, coenzyme A; EE, ethynyl estradiol.

9:1 (25). Cholesterol, cholesterol ester, and triglycerides were located by comparison with authentic standards. The spot corresponding to cholesterol ester was scrapped from the plates into counting vials for radioactivity determination. Recovery of known amounts of cholesterol oleate ( $[1-^{14}\text{C}]$ oleate) added to six samples of isolated hepatocytes was >87%.

In the studies of acetate incorporation into cholesterol, the residue of the chloroform extracts was subjected to alkaline hydrolysis under 0.625 N alcoholic KOH. Sterols were extracted with petroleum ether and precipitated with digitonin (26). The digitonides were quantitatively transferred to counting vials using methanol. Results of substrate incorporation into products were expressed as the picomole of acetate or oleate incorporated into cholesterol or cholesterol oleate, respectively, per milligram of hepatocytic protein per hour of incubation.

**Biliary secretion of newly synthesized cholesterol.** To measure the contribution of newly synthesized hepatic cholesterol to biliary cholesterol we used the method of Turley and Dietschy (9). Briefly, the rats (140–160 g, body weight) were anesthetized with diethyl ether and a PE-10 polyethylene catheter was placed in a tail vein. A bolus of 70 mCi of  $[\text{H}^3]$  water contained in 0.5 ml of 0.9% NaCl was infused at 08:30 under a hood, under negative pressure, and protected with a hygroscopic chamber in the outlet. The animals were left in this hood for 6 h to allow equilibration of the tritiated water in the body compartments of the animals. At the end of this period, a bile fistula was performed under pentobarbital anesthesia. Bile was collected for 1 h. At the end of this period, blood was collected by aortic puncture and plasma was obtained after centrifugation in sealed tubes for determination of water specific activity. Bile aliquots were saponified under 0.625 N alcoholic KOH at 80°C for 60 min. Sterols were extracted using petroleum ether and precipitated with digitonin (26). After washing and drying under vacuum, the precipitate was dissolved in pyridine. The free sterols were quantitatively extracted with diethyl ether (27). After evaporation, the extract was used for determinations of mass and radioactivity. The amount of newly synthesized cholesterol excreted into the bile was calculated according to the formula  $(\text{dpm } [\text{H}^3] \text{ sterol} \cdot (1.45)) / (\text{specific activity of plasma water} \cdot (18))$ . The number 1.45 represents the nanomole of acetyl CoA units incorporated into sterols for each nanomole of  $[\text{H}^3]$  water. Since 18 nmol of acetyl CoA are needed for the synthesis of 1 nmol of cholesterol, the number of acetyl CoA units incorporated into cholesterol was converted to nanomole of cholesterol synthesized by dividing the value by 18 (9).

**Chylomicron preparation and administration.** In one series of experiments, chylomicrons were administered to a group of rats. These chylomicrons were obtained from donor animals fitted with a PE-90 polyethylene catheter in the base of the mesentery as described by

Bollman, Cain, and Grindlay (28). Simultaneously, a liquid diet containing one egg yolk per 40 ml of a 8% aminoacid solution, 0.9% NaCl, and 0.2% KCl was infused through a gastric cannula at a rate of 1 ml/h by means of a Braun infusion pump (Braun Melsungen A.G., Federal Republic of Germany). Lymph was collected continuously for 2–4 d. The lymph from each animal was collected into sterilized Erlenmeyer flasks containing 150 mg sodium citrate and 1 mg of gentamicin. These flasks were changed every 24 h and were maintained in a cold room at 4°C. The lymph collected from several rats was pooled and centrifuged at 18,000 rpm  $\times$  18 h in a SW 27 swinging-bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 10°C. Chylomicrons were resuspended in 0.9% NaCl and dialyzed several times in a total volume of 5 l of 0.9 NaCl solution. Varying amounts of these chylomicrons were administered to diosgenin-fed animals intravenously as a bolus at 22:00 h. A 20-min bile sample was obtained 12 h later. The livers were then removed for cholesterol esters determination and preparation of microsomes.

**Analytical methods.** Bile salts were quantitated by the 3  $\alpha$ -hydroxy-steroid dehydrogenase method of Talalay (29). Phospholipids were measured in the chloroform phase of chloroform-methanol extracts by the colorimetric method of Baginski, Fos, and Zak (30). Free and esterified cholesterol were extracted and separated as previously described (20). Cholesterol was quantitated by the colorimetric method of Zak (31). Biliary cholesterol saturation was calculated according to the method of Carey (32). Biliary and microsomal protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (33). Biliary proteins were first precipitated with acetic acid and the precipitate was washed with diethyl ether before colour development.

**Statistics.** Results are presented as the mean  $\pm$  1 SD. The values of *a* and *b* of the mathematical formulas obtained from the kinetic studies of biliary lipid secretion, and the linear regressions of Fig. 8, represent the mean  $\pm$  1 SE. Differences in mean values were tested for significance using the unpaired *t* test (34).

## Results

**The effect of diosgenin feeding on body weight, bile secretion, and biliary cholesterol saturation.** The first series of experiments was undertaken to characterize bile secretion in the diosgenin-fed rat. Table I shows that feeding 1% diosgenin for 1 wk is well tolerated by the rat, since body and liver weights remained in the normal range. Similarly, bile flow, biliary bile salt, phospholipid, and protein outputs were normal. However, biliary cholesterol output increased >400% over the control

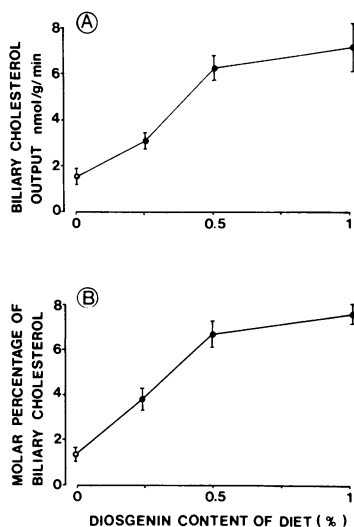
Table I. Effect of Diosgenin Feeding on Biliary Lipid and Protein Outputs

Experimental group	Body weight (g)		Liver weight	Bile flow	Biliary lipid outputs (nmol/g/min)			Biliary protein output	Biliary cholesterol molar percentage
	Initial	Final			Bile salts	Phospholipids	Cholesterol		
			g	$\mu\text{l/g/min}$				$\mu\text{g/min}$	%
Control (9)	213 $\pm$ 21	232 $\pm$ 26	11.4 $\pm$ 0.9	2.1 $\pm$ 0.3	102 $\pm$ 27	10.7 $\pm$ 2.9	1.6 $\pm$ 0.6	2.84 $\pm$ 1.1	1.4 $\pm$ 0.2
Diosgenin (9)	204 $\pm$ 17	226 $\pm$ 20	10.3 $\pm$ 1.3	2.0 $\pm$ 0.4	91 $\pm$ 28	10.5 $\pm$ 4.0	7.4 $\pm$ 2.8*	2.94 $\pm$ 0.7	7.0 $\pm$ 2.6

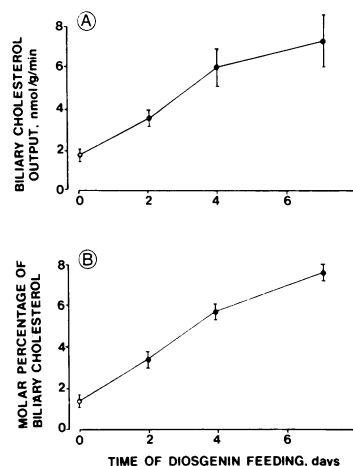
Both groups were fed ground chow for 1 wk before the experiment. One group received a 1% diosgenin diet. Values are the mean  $\pm$  1 SD. The asterisks indicate that the value is significantly different at the *P* < 0.05 level. The number of animals in each group are shown in parentheses.

value. Biliary cholesterol output and saturation as a function of the diosgenin content of the diet and the duration of diosgenin feeding are illustrated in Figs. 1 and 2, respectively. As seen in panel A of Fig. 1, the maximal rate of cholesterol output appeared with 0.5% diosgenin concentration in the diet. With this dose, the molar percentage of biliary cholesterol also increased from a normal value of  $\sim 1.4\%$  to  $>6\%$  (panel B of Fig. 1). We found significantly higher rates of biliary cholesterol output and concentration as early as 2 d of 1% diosgenin diet; rates reached the highest levels by the 7<sup>th</sup> d of observation (Fig. 2). These results demonstrated that diosgenin specifically increases biliary cholesterol output without modifying the rates of biliary bile salt nor phospholipid outputs. Thus, it is very unlikely that the effect of the drug may be related to a detergent effect on the membranes of the biliary excretory apparatus induced by hydroxylated diosgenin metabolites excreted into the bile (22).

To determine whether these diosgenin metabolites may have increased the solubility of biliary cholesterol through a bile salt-like effect, we observed the morphology of bile samples under polarized light microscopy. Sterile bile samples from diosgenin-fed rats with theoretical cholesterol saturations over 200% were maintained at 20°C in the darkness and were examined daily for a period of 5 d. No precipitates were found and bile samples remained isotropic. The absence of a solid phase in bile specimens from diosgenin-fed rats was expected, since supersaturated cholesterol solutions can remain isotropic for long periods of time (35). The physicochemical equilibrium of these biles was disturbed by freezing and thawing. After thawing, the bile samples from diosgenin-fed animals appeared turbid, and under polarized light microscopy the field was full with birefringent crystals in the form of parallelepipeds, as is commonly found in supersaturated bile from patients with cholesterol gallstones. In addition, we also found birefringent



**Figure 1.** Effect of the diosgenin content of the diet on biliary cholesterol output and the molar percentage of cholesterol in bile. Rats were fed different concentrations of the drug for 6 d before the experiments. There were four rats in each group. The open circle represents a control group fed ground chow. Values represent the mean  $\pm$  SD.

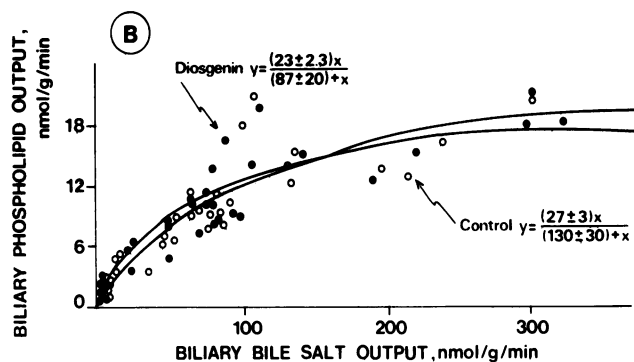
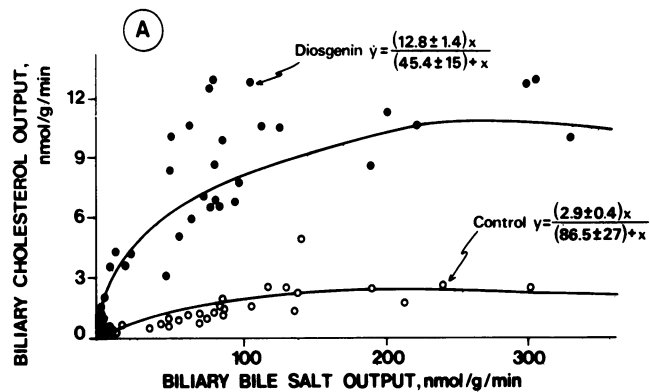


**Figure 2.** Relationship between biliary cholesterol output and the molar percentage of cholesterol in bile as a function of the time of 0.5% diosgenin feeding. There were four rats in each group. The open circle represents a control group fed ground chow. Values represent the mean  $\pm$  SD.

droplets that decreased in number on heating from room temperature to 40–45°C, as has been found in supersaturated human bile (36). No changes were observed in the bile samples from control animals. After centrifuging the turbid biles from diosgenin-fed rats, we found a white precipitate with the chromatographic and chemical characteristics of cholesterol. Thus, these results were inconsistent with the possibility that diosgenin metabolites may have solubilized biliary cholesterol through a bile salt-like effect. Although this observation does not rule out the possibility that diosgenin metabolites could solubilize hepatocytic cholesterol and carry it into bile through a different, unknown mechanism, it is more likely that the high rate of biliary cholesterol output induced by the drug is the consequence of a specific expansion of the biliary cholesterol precursor pool.

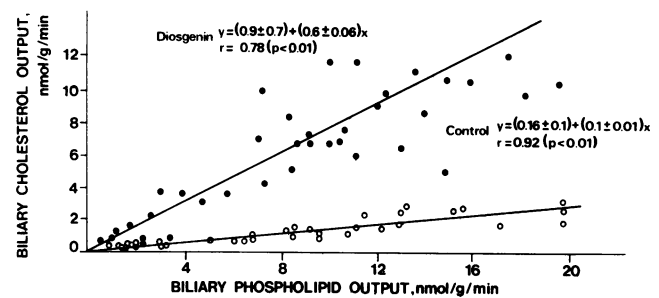
**Kinetic characteristics of biliary lipid outputs in the diosgenin-fed rat.** The relationship between cholesterol and phospholipid outputs as a function of bile salt output is shown in Fig. 3. The secretion of both lipids related to bile salt output by an ascending curve that appeared to pass through the origin in control and diosgenin-fed rats. Although the kinetic characteristics of biliary phospholipid as a function of bile salt output were similar in both groups of rats (panel B, Fig. 3), it was apparent that the amount of biliary cholesterol secreted per unit of bile salt was considerably higher in the diosgenin-fed animal (panel A, Fig. 3). The calculated maximal cholesterol output of the diosgenin group was  $>400\%$  higher than the maximal output of control rats. The relationship between cholesterol output as a function of phospholipid output was linear, as shown in Fig. 4. This experiment demonstrated that per mole of phospholipid secreted into the bile, almost 450% more biliary cholesterol was secreted in the diosgenin-fed rats.

To study the functional integrity of the biliary apparatus in the diosgenin-fed animals we stimulated bile secretion by overloading the liver cells with exogenous taurocholate (Fig. 5). After the intravenous infusion of taurocholate at a rate of 3.3  $\mu\text{mol/min}$  for a period of 20 min, biliary bile salt output

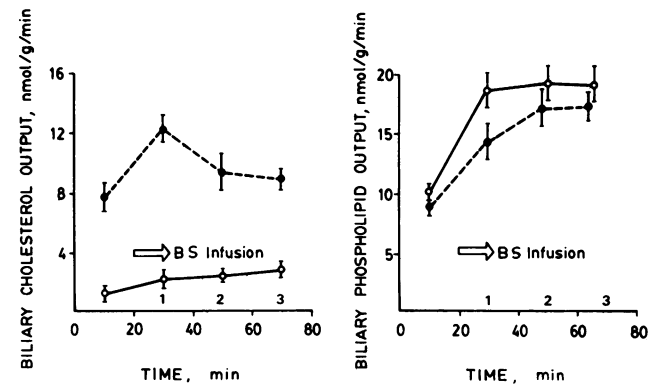
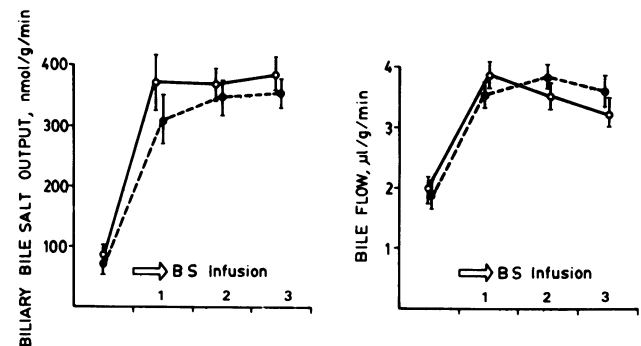


**Figure 3.** Relationship between biliary cholesterol (○), biliary phospholipid (●), and bile salt outputs. The control bile samples are represented by open circles. The bile samples from diosgenin-fed rats are represented by solid circles. Each group was formed with the nine rats of Table I, three rats infused intravenously with 45 mM taurocholate in 0.9% NaCl at a rate of 0.075 ml/min for a period of 20 min (equivalent to the period of bile collection), and a third group of three rats subjected to acute depletion of the bile acid pool as previously described (19). 5–6 bile samples were obtained from each depleted animal. The best curve fitting of the experimental values was represented by the rectangular hyperbola  $y = ax/b + x$ . The term  $a$  represents the maximal theoretical output of  $y$ , and the term  $b$  represents the bile salt output at which the half maximal cholesterol output is obtained. Maximal cholesterol output (○) of diosgenin-fed animals was increased 440% ( $P < 0.001$ ).

increased 400% in control and diosgenin-fed rats. When taurocholate was infused at a rate of 5  $\mu$ mol/min, biliary bile salt output reached a maximum with no further increments. Bile flow and biliary phospholipid output were similar in both groups of animals, as shown in Fig. 5. The pattern of biliary cholesterol output under taurocholate infusion was different



**Figure 4.** Linear regression between biliary cholesterol and biliary phospholipid outputs. The animal preparations were identical to those described in Fig. 3. The slope  $b$  of the diosgenin group was significantly increased ( $P < 0.001$ ).



**Figure 5.** Biliary secretory function under high intravenous taurocholate infusion in diosgenin-fed animals. The control bile samples are represented by open circles and the diosgenin bile samples by solid circles. There were 3–4 rats in each group. The arrow indicates the commencement of taurocholate infusion. During the first period, the animals received an intravenous infusion of 45 mM taurocholate at a rate of 0.075 ml/min for a period of 20 min. The second and third periods correspond to bile samples obtained under infusion of 50 mM taurocholate at a rate of 0.1 ml/min for a period of 40 min. BS, bile salt.

in the diosgenin-fed rats. In these animals, the maximal cholesterol output, 70% over the initial values, occurred during the first period of taurocholate infusion, whereas in the control group, cholesterol output reached a 100% increment during the last period of taurocholate infusion.

Taken together, these data permitted the conclusion that the biliary apparatus of the diosgenin-fed animal maintains its functional integrity, in spite of the massive excretion of cholesterol through the canalicular membrane. A toxic effect of diosgenin on the excretory function of the hepatocyte appeared very unlikely as a possible mechanism of the diosgenin-induced biliary cholesterol output.

*The effect of EE and chylomicron cholesterol on biliary lipid outputs in diosgenin-fed rats.* Two series of rats were fed diosgenin for 1 wk and were injected with different daily doses of EE 2 d before the experiments. As shown in Table II, the hormone induced a cholestatic effect reflected in the significantly lower bile flows. Biliary bile salt and phospholipid outputs remained in the normal range in the injected rats with the lower dose of EE. However, these rats (group C) presented an impressive decrement of biliary cholesterol output compared with the rats injected with corn oil (group B). The kinetic characteristics of biliary cholesterol and phospholipid outputs as a function of bile salt output in the EE-injected animal maintained the hyperbolic relationship, as shown in Fig. 6. However, the calculated maximal cholesterol output of this group decreased from 16 to 3 nmol/g per min (panel A, Fig. 6). This last figure was similar to the maximal cholesterol output calculated for the control rats (panel A, Fig. 3).

To study the effect of dietary cholesterol on biliary cholesterol secretion induced by diosgenin feeding, we injected intravenously a series of diosgenin-fed rats with different doses of chylomicron cholesterol. After 12 h of the injection we studied biliary cholesterol output (Fig. 7). We chose this preparation because diosgenin feeding interferes with dietary

cholesterol absorption (21, 37). The doses of chylomicron cholesterol administered varied approximately between 10 and 300% of the daily basal intestinal cholesterol absorption in the rat. Cholesterol output decreased as a function of the dose of chylomicron cholesterol infused.

These experiments suggested that biliary cholesterol output driven by diosgenin feeding could be modulated by intrahepatic effectors independently of the rate of biliary bile salt output. These effectors, EE and dietary cholesterol, both have the capacity to overload the hepatocyte with cholesterol; nevertheless, biliary cholesterol output paradoxically decreased.

*The relationships between biliary cholesterol output induced by diosgenin feeding and hepatic cholesterol metabolism.* The third major group of studies was designed specifically to quantitate several parameters of hepatic cholesterol metabolism under the experimental maneuvers employed to modulate biliary cholesterol output. In this manner, specific metabolic processes related to the regulation of hepatic cholesterol metabolism could be correlated with the changes observed in biliary cholesterol output.

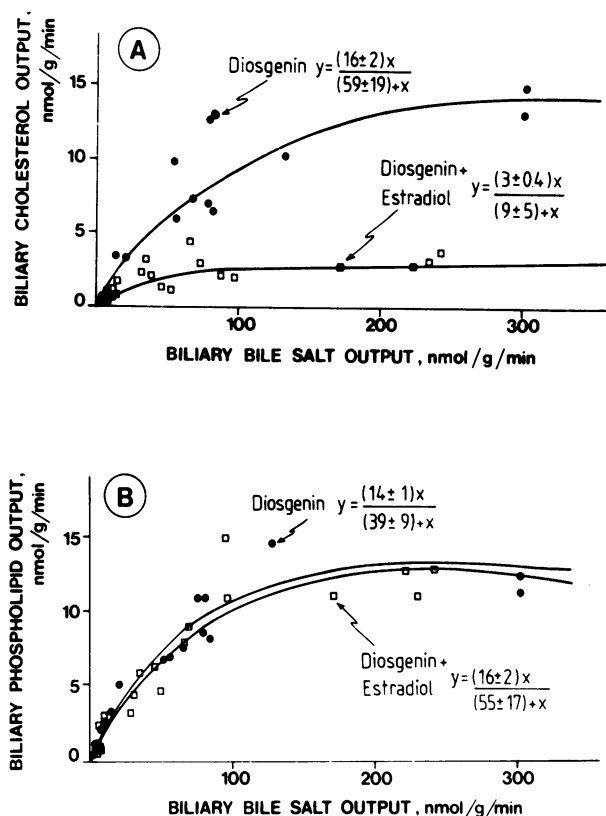
Relative rates of hepatic cholesterologenesis were measured in isolated hepatocytes obtained at the mid-dark point of the diurnal cycle, as shown in Table III. Diosgenin feeding increased the rate of acetate incorporation into hepatic sterols by >300%. Hepatic cholesterol concentration remained in the normal range. When the diosgenin-fed animal received EE then, as expected, hepatic cholesterol esters increased by 500% and cholesterol synthesis decreased, which indicated that in these animals the feed-back regulatory mechanism of hepatic cholesterologenesis was functioning. The high rates of hepatic cholesterologenesis induced by diosgenin are probably the result of its capacity to block dietary cholesterol absorption (21) and to increase the loss of cellular cholesterol through the canalicular membrane.

To investigate whether the high rate of biliary cholesterol

Table II. Effect of the Dose of EE on Biliary Lipid Outputs in Diosgenin-fed Rats

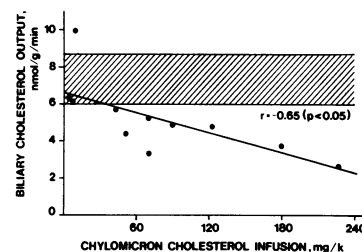
Experimental group		Body weight	Liver weight	Bile flow	Biliary lipid outputs (nmol/g/min)			Biliary cholesterol molar percentage
					Bile salts	Phospholipid	Cholesterol	
		g	g	$\mu\text{l/g/min}$				%
A Control	(6)	237 $\pm$ 28	11.1 $\pm$ 1.7	2.1 $\pm$ 0.4	93 $\pm$ 32	10.3 $\pm$ 2.8	1.4 $\pm$ 0.5	1.3 $\pm$ 0.2
B Diosgenin	(4)	229 $\pm$ 31	10.4 $\pm$ 1.8	2.1 $\pm$ 0.5	92 $\pm$ 34	12.5 $\pm$ 3.5	8.3 $\pm$ 2.9	8.3 $\pm$ 3.0
C Diosgenin + EE								
(2 mg/kg)	(5)	225 $\pm$ 30	11.5 $\pm$ 1.8	1.5 $\pm$ 0.3*	73 $\pm$ 17	8.6 $\pm$ 4.1	2.7 $\pm$ 1.3*	2.7 $\pm$ 0.9*
D Diosgenin + EE								
(5 mg/kg)	(4)	221 $\pm$ 32	12.1 $\pm$ 2.3	1.0 $\pm$ 0.3*	45 $\pm$ 11*	6.7 $\pm$ 2.4*	0.7 $\pm$ 0.2*	1.9 $\pm$ 0.3*

Groups B, C, and D were fed a 1% diosgenin diet for 6 d before the experiments. Groups A and B received daily subcutaneous injections of corn oil and groups C and D EE dissolved in corn oil (2–5 mg/kg of body weight) for 2 d before the experiments. 20 h after the last injection, a bile fistula was performed, and bile was collected for a period of 20 min. Values represent the mean $\pm$ SD. The number of rats in each group is shown in parentheses. The asterisks indicate that the value is significantly different from the group B value at the  $p < 0.05$  level.



**Figure 6.** Kinetic characteristics of biliary cholesterol and phospholipid as a function of biliary bile salt outputs (○ and ●, respectively) in diosgenin-fed rats injected with EE. The animals were fed 1% diosgenin for 6 d. In addition, a group received two daily doses of 2 mg/kg body weight of EE before the experiments. The diosgenin group injected with corn oil was formed with the four animals of group B from Table II, which after the initial bile sample were intravenously infused with 45 mM taurocholate at a rate of 0.075 ml/min for a period of 20 min, and two rats were subjected to acute depletion of the bile acid pool (12 bile samples). The diosgenin group injected with EE was formed with the five animals of group C from Table II, which were also infused with taurocholate, and two rats subjected to acute depletion of the bile acid pool. The terms *a* and *b* of the EE-injected rats (●) were significantly decreased ( $P < 0.001$ ) compared with the diosgenin-fed rats animals injected with corn oil.

output found in the diosgenin-fed animal was the result of the increased hepatic cholesterologenesis induced by the drug, we quantitated the contribution of newly synthesized cholesterol to biliary cholesterol by using the tritiated water method of Turley and Dietsch (9). Although the diosgenin-fed rat secreted more newly synthesized cholesterol than the control animal by a factor of 5, it was apparent, as shown in panel B of Fig. 8, that the relative contribution of newly synthesized cholesterol to the absolute biliary cholesterol output was similar in the control, diosgenin-fed, and diosgenin-fed and EE-injected group of animals. This contribution varied between 12% in the



**Figure 7.** The effect of chylomicron cholesterol on the diosgenin-induced biliary cholesterol output. The hatched area represents the mean output with its 95% confidence level. These values were obtained from 22 1% diosgenin-fed rats. In this group, five rats were

intravenously infused with 5 ml of 0.9% NaCl in 30 min, at the mid-light phase of the diurnal cycle. Black circles correspond to diosgenin-fed rats intravenously injected with different doses of chylomicron cholesterol (5–230 mg/kg body weight) at the mid-light phase of the diurnal cycle. Bile was collected 12 h later.

control and 19% in the diosgenin-fed rat; however, the difference was not statistically significant. These percentages of contribution of newly synthesized hepatic cholesterol to biliary cholesterol are in the range of those previously communicated (9).

These experiments demonstrated that the absolute increment of biliary cholesterol output induced by diosgenin was not the result of its capacity to increase hepatic cholesterologenesis. It was apparent that the drug specifically increased the size of the precursor pool of biliary cholesterol through a different mechanism.

The final series of experiments was performed to study the role of hepatic cholesterol esterification as a major determinant of the size of the hepatic free cholesterol precursor pool, from which biliary cholesterol originates. The rate of oleate incorporation into cholesterol esters was significantly decreased in hepatocytes isolated from diosgenin-fed rats, whereas EE stimulated esterification by 300% (Table III). Whether the inhibition of hepatic cholesterol esterification induced by diosgenin was the result of a direct inhibitory effect of the drug on the microsomal enzyme ACAT or a secondary deficiency of metabolically active free cholesterol as substrate for the enzyme cannot be determined with our data. The concentration of free cholesterol in the environment of the enzyme is one of the major determinants of its activity (38–42). Although the microsomal free cholesterol concentration was normal in diosgenin-fed rats (Table IV), this may not necessarily indicate the true concentration of free cholesterol that has access to the active site of the ACAT (38). The stimulation of this enzyme by EE is probably related to the enhanced uptake of serum low density lipoproteins (LDL) through the increment of LDL receptors at the sinusoidal membranes (43, 44). In this manner, the hormone stimulates cholesterol esterification through the overloading of the microsomal membranes with higher amounts of free cholesterol (Table IV). EE appears to be ineffective as a direct stimulator of the ACAT reaction (20, 45).

To examine the relationship between hepatic cholesterol esterification and biliary cholesterol output we correlated both parameters in a series of 21 diosgenin-fed rats. Six of them

Table III. Effect of Diosgenin Feeding and EE Injection on 2-<sup>14</sup>C-Acetate Incorporation into Digitonin Precipitable Sterols, [<sup>14</sup>C]Oleate Incorporation into Cholesterol Esters and Cholesterol Concentration in Isolated Hepatocytes

Experimental group		Plasma cholesterol	Hepatic cholesterol (mg/g liver wt)		<sup>14</sup> C-Acetate incorporation into cholesterol	[ <sup>14</sup> C]Oleate incorporation into cholesterol esters
			Free	Ester		
		mg/dl			pmol/mg/h	pmol/mg/h
A Control	(5)	79±10	1.76±0.2	0.24±0.09	24±3	48±7.6
B Diosgenin	(5)	64±9	1.74±0.1	0.22±0.08	83±17*	34±10*
C Diosgenin + EE (2 mg/kg)	(4)	27±5*	2.10±0.3	1.07±0.11*	46±13*	116±16*

Hepatocytes were isolated at the mid-dark point of the diurnal cycle. The cells were resuspended in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 2.5 mM CaCl<sub>2</sub>. Each incubation flask contained 0.2 ml of cell suspension (7–10 mg of protein), 0.5 mM bovine albumin, 1 mM [1-<sup>14</sup>C]oleic acid or 1.8 mM 2-<sup>14</sup>C-acetate in a total volume of 1 ml. The incubations times were 60 min for acetate incorporation into cholesterol and 10 min per oleate incorporation into cholesterol esters. The flasks were incubated at 37°C in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The reactions were stopped with 20 ml of chloroform-methanol (2:1). Group C was treated as described in Table II. Results of substrate incorporation are expressed as the picomole incorporated into product per milligram of protein per hour. The asterisks indicate a significant difference at the *P* < 0.05 level. The number of rats in each group are shown in parentheses.

received in addition daily doses of 2 mg/k EE 2 d before the experiments; 10 were intravenously injected with different doses of chylomicron cholesterol and 5 received daily subcutaneous injections of corn oil. As shown in Fig. 9, biliary cholesterol output significantly decreased as a function of ACAT activity (panel A, Fig. 9) and the concentration of hepatic cholesterol esters (panel B, Fig. 9). The correlation coefficients between ACAT activity and hepatic cholesterol ester concentration was 0.91 (*P* < 0.001). There was no correlation between biliary cholesterol output and hepatic or microsomal free cholesterol concentrations.

These experiments demonstrated that the size of the biliary cholesterol precursor pool could be rapidly modulated by the activity of the ACAT reaction. Although both experimental maneuvers, EE and chylomicron cholesterol injections, overloaded the hepatocyte with extrahepatic cholesterol, it is apparent that this cholesterol in excess is not immediately accessible to the biliary precursor pool. Nevertheless, this precursor pool appears to be closely connected with the subcellular organelles where the esterification of hepatic cholesterol takes place.

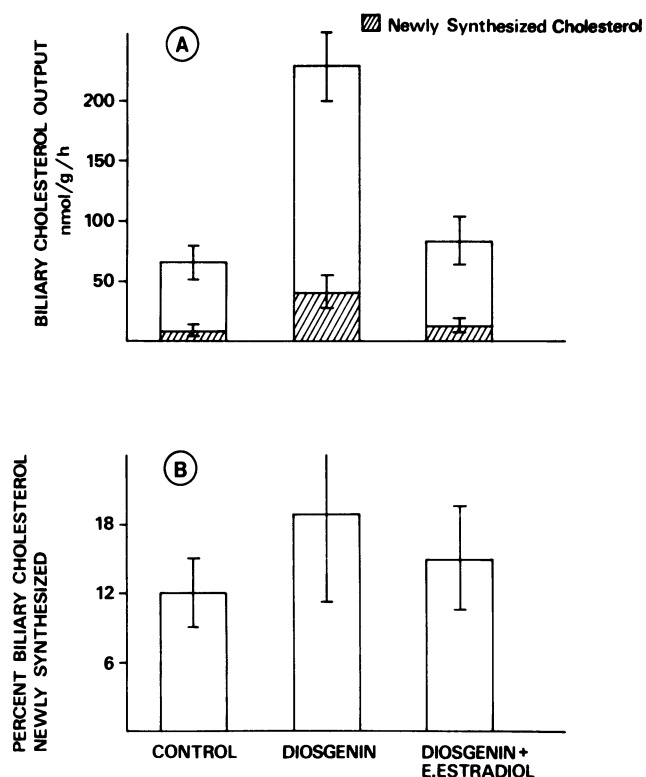
## Discussion

The present results demonstrate that diosgenin when fed to rats induces biliary cholesterol supersaturation by increasing biliary cholesterol secretion. This effect was rapidly reversed by either EE or chylomicron cholesterol. The contribution of newly synthesized hepatic cholesterol to biliary cholesterol output increased 500% in the diosgenin-fed rat. However, its relative contribution to the absolute rate of biliary cholesterol output remained in the range found in control animals. When we increased the size of the hepatic cholesterol ester pool by

the administration of EE or chylomicron cholesterol to diosgenin-fed rats, we found a negative reciprocal correlation between both parameters of hepatic cholesterol metabolism and the rate of biliary cholesterol output.

A remarkable observation of this study is the selective effect of diosgenin on biliary cholesterol output. The precise mechanism, however, has not been clearly elucidated by our experiments. A direct detergent effect of diosgenin or some of its hydroxylated metabolites (22) on the structural integrity of the biliary apparatus seems very unlikely, because bile flow, biliary phospholipid and protein outputs, and the biliary secretory function under high taurocholate infusion remained unchanged in the diosgenin-fed animal. A phospholipid- or bile salt-like effect of diosgenin metabolites in enhancing solubilization of biliary cholesterol is inconsistent with our observations. The appearance of cholesterol nucleation in bile specimens from diosgenin-fed rats after freezing and thawing was a strong evidence against this possibility. The third more likely interpretation relates to some effect of diosgenin on the intrahepatocytic regulatory mechanisms of biliary cholesterol output. Hepatic cholesterol esterification decreased by 30% in the isolated hepatocyte from diosgenin-fed animals. If the drug directly inhibits cholesterol esterification, then the availability of free cholesterol may have increased in the hepatocytic biliary precursor pool, as we previously postulated for the progesterone-induced biliary cholesterol output (19, 20). However, the lower hepatic esterification rate of diosgenin-fed rats may also be related to a decreased availability of microsomal free cholesterol, which acts as substrate for the ACAT reaction. It is known that this pool is small (38) and that the major proportion of free cholesterol present in the microsomal fraction represents cholesterol from plasma and Golgi membrane (46, 47), and, therefore, is not available as a substrate pool for





**Figure 8.** The contribution of newly synthesized cholesterol to biliary cholesterol in diosgenin-fed rats. ⊙ represents biliary cholesterol output and ⊙ represents the percentage of biliary cholesterol newly synthesized. Diosgenin-fed and EE-injected rats were prepared as described in Fig. 6. Body weights of these animals were between 135 and 150 g. They received an intravenous dose of 70 mCi of tritiated water in 0.5 ml of 0.9% NaCl at 08:30 under light ether anesthesia. 6 h later the animals were anesthetized with pentobarbital and bile was collected for a period of 60 min. Each bar represents the mean  $\pm$  1 SD of four rats in each group.

cholesterol esterification, a metabolic process located in the endoplasmic reticulum membranes (48). If diosgenin decreases the availability of free cholesterol in the environment of the ACAT as a secondary phenomenon determined by the high biliary cholesterol output, then a different, unknown mechanism responsible for the regulation of the size of the biliary cholesterol precursor pool should be invoked as an explanation for the diosgenin-induced biliary cholesterol output.

The reversal of diosgenin-induced biliary cholesterol output by two known stimulators of the ACAT reaction, EE (20, 49) and dietary cholesterol (20, 38), is a strong evidence favoring the thesis that postulates a functional relationship between biliary cholesterol output and hepatic cholesterol esterification. The activity of the ACAT in the endoplasmic reticulum of the hepatocyte would be a major determinant of the size of the

**Table IV.** Effects of Diosgenin Feeding and EE Injection on Microsomal Cholesterol and on ACAT Activity

Experimental group	Microsomal cholesterol ( $\mu$ g/mg protein)		Microsomal protein $\mu$ g/g/liver wt	ACAT activity nmol/mg/min
	Free	Ester		
Control (9)	20.5 $\pm$ 2.3	1.8 $\pm$ 0.5	17.5 $\pm$ 1.9	103 $\pm$ 24
Diosgenin (6)	18.5 $\pm$ 2.2	1.7 $\pm$ 0.5	18.7 $\pm$ 2.1	62 $\pm$ 23
Diosgenin + EE (2 mg/kg) (5)	27.0 $\pm$ 4.0*	6.0 $\pm$ 1.5*	16.4 $\pm$ 1.8	171 $\pm$ 10*

All experiments were performed as described in Table II. All assays of ACAT activity were performed in the first 24 h after killing the rats. Maximal rates of esterification were obtained at oleoyl-CoA concentration of 100  $\mu$ M and initial rates were measured incubating the reaction for periods of 7 min and 0.15–0.35 mg of protein per assay. The asterisk indicates a significant difference from the control value at the  $P < 0.05$  level. The number of rats in each group are shown in parentheses.

biliary free cholesterol precursor pool and, therefore, a driving force for biliary cholesterol output. A further implication of these experiments is that the endoplasmic reticulum membranes and the biliary excretory apparatus are functionally and probably structurally related.

The microsomal enzyme ACAT appears to play a critical role in determining the fluxes of free cholesterol from the metabolically active pool to the different excretory pathways of the hepatocyte: biliary cholesterol; bile acid synthesis; very low density lipoprotein (VLDL); and high density lipoprotein synthesis. Several studies in recent years have demonstrated that cholesterol feeding (50–52) or EE injection (53) can markedly increase the cholesterol ester content of newly-secreted VLDL, and that both experimental manipulations can induce the ACAT activity (20). These observations strongly support the concept that the different excretory pathways of hepatic cholesterol are functionally interrelated. The present results are consistent with a model of hepatic cholesterol metabolism in which biliary cholesterol output and cholesterol secreted in lipoprotein particles through the sinusoidal membrane can be functionally related in a reciprocal manner.

Although there are remarkable differences between species in the rates of biliary cholesterol output, particularly between rat and man, their major regulatory mechanism is related to the canalicular transport of bile salts in all species so far studied (11–16). It is not known which intrahepatocytic determinants of the rate of biliary cholesterol output are functioning in other species besides the rat. If the intrahepatocytic mechanism described in this study also operates in man, it may be possible that abnormally high rates of biliary cholesterol output, as found in a significant proportion of patients with cholesterol gallstones, are the consequence of a low hepatic cholesterol esterification rate. If the activity of the ACAT reaction proves

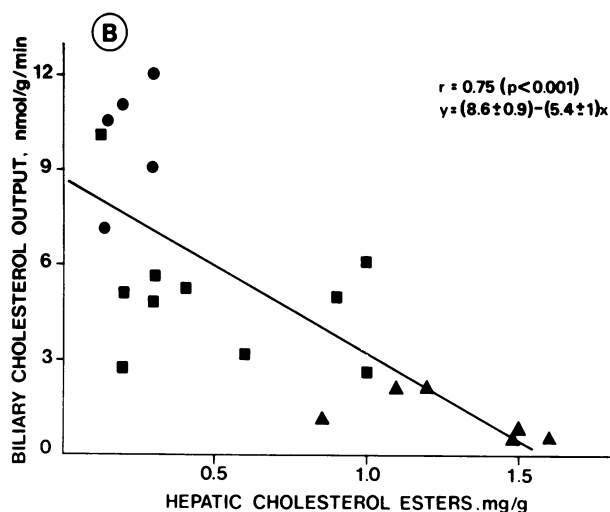
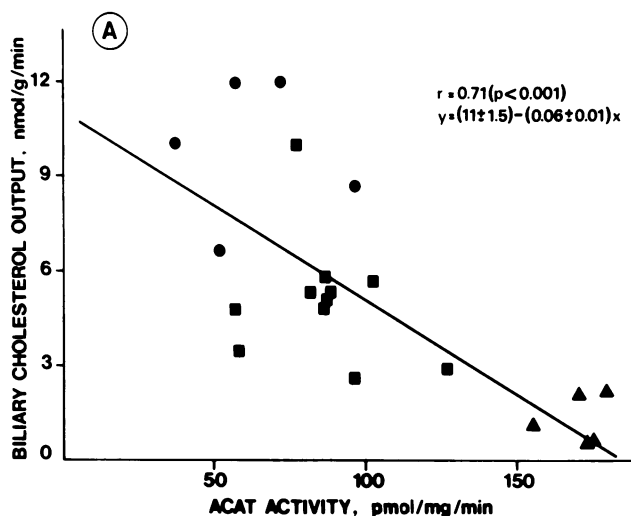


Figure 9. Relationship between biliary cholesterol output, ACAT activity, and hepatic cholesterol ester concentration. All animals were fed 1% diosgenin for 1 wk before the experiments. The triangles represent six rats injected with daily doses of EE (2 mg/kg) for 3 d before the experiments. The squares correspond to 10 rats injected with different doses of chylomicron cholesterol 12 h before the experiment. The circles represent five animals intravenously injected with 5 ml of 0.9% NaCl 12 h before the experiment. Initial bile samples were collected for a period of 20 min.

to be a major determinant of biliary cholesterol output in man, then the pharmacological manipulation of this enzyme may constitute an important achievement in the therapy of high biliary cholesterol secretory states.

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