Regulation of Rat Biliary Cholesterol Secretion by Agents That Alter Intrahepatic Cholesterol Metabolism

Evidence for a Distinct Biliary Precursor Pool

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Abstract

Propensity for cholesterol gallstone formation is determined in part by biliary cholesterol content relative to bile salts and phospholipid. We examined the hypothesis that the rate of biliary cholesterol secretion can be controlled by availability of an hepatic metabolically active free cholesterol pool whose size is determined in part by rates of sterol synthesis, as reflected by activity of the primary rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase and of sterol esterification, as reflected by the activity of the enzyme acyl coenzyme A/cholesterol acyltransferase (ACAT). Rats were prepared with biliary, venous, and duodenal catheters. The enterohepatic circulation of biliary lipids was maintained constant by infusion of a bile salt, lecithin, cholesterol replacement solution. Administration of 25-hydroxycholesterol decreased HMG CoA reductase activity, increased ACAT activity, and decreased biliary cholesterol output 26% by 1 h. By 2 h, ACAT activity and biliary cholesterol secretion were at control levels. Administration of mevinolin, a competitive inhibitor of HMG CoA reductase, had no effect on ACAT activity and decreased biliary cholesterol secretion 16%. Administration of progesterone, an inhibitor of ACAT, had no effect on HMG CoA reductase and increased biliary cholesterol output 32% at 1 h. By 2 h, all parameters were near control levels. None of these agents had any significant effect on biliary bile salt or phospholipid secretion.

Thus, acutely altering rates of esterification and/or synthesis can have profound effects on biliary cholesterol secretion independent of the other biliary lipids. These experiments suggest the existence of a metabolically active pool of free cholesterol that serves as a precursor pool for biliary cholesterol secretion. Furthermore, the size of this precursor pool is determined in part both by rates of cholesterol synthesis and esterification and is a key determinant of biliary cholesterol secretion.

Introduction

Cholesterol gallstone disease is common in Western populations. Normally the hydrophobic lipid, cholesterol, is maintained in solution in the bile through formation of mixed micelles composed of bile salt and lecithin (1). It is established that gallstones can form when the cholesterol content of the micelle relative to phospholipid and bile salt exceeds certain limits (2), and that such lithogenic bile is derived from the liver as opposed to forming in the gallbladder (3, 4). The rate of biliary lipid secretion is dependent in part on the rate of bile salt secretion (5, 6). However, this cannot account for all of the metabolic observations. Under different metabolic circumstances there can be either decreased bile sale secretion with a constant cholesterol output or increased cholesterol secretion with a maintained bile sale output (7–9). The physiologic basis for these abnormalities remains unknown. Cholesterol secreted into the bile can be derived from at least three sources: hepatic newly synthesized sterol, cholesterol derived from plasma lipoproteins, and sterol from preformed hepatic stores. Although all of these sources appear to be available for biliary secretion, the relative contributions of each and how they are regulated remain unclear.

One postulate was that the rate of cholesterol synethesis in the liver was an important determinant of biliary cholesterol secretion. This was based on the observations of several investigators (10–12) that patients with cholesterol gallstones had higher levels of the enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA)[1] reductase, than patients without gallstones. This enzyme catalyzes the rate-limiting step of cholesterol synthesis (13) and in most instances accurately reflects the rate of cholesterol synthesis (14). Moreover, feeding the bile salt chenodeoxycholate reduced the enzyme's activity and decreased biliary cholesterol excretion (11, 12). However, a thorough series of studies by Turley and Dietschy (6) in the rat suggested that the rate of hepatic cholesterol synthesis per se does not determine the rate of biliary cholesterol secretion. Moreover, it has been shown that chenodeoxycholate's mechanism of action is not through direct inhibition of HMG CoA reductase (15). Lastly, under normal circumstances, only 10–20% of biliary cholesterol is derived from new synthesis (16, 17).

Only free cholesterol is secreted in bile, but in the cell the sterol is in both the free and esterified form. The balance between the two is controlled by the rate of esterification of free cholesterol, catalyzed by the enzyme acyl coenzyme A/cholesterol acyltransferase (ACAT), and by the rate of hydrolysis of cholesterol esters both in the lysosomal and extralysosomal compartments. Nervi and colleagues (18) have investigated the relationship between the rate of cholesterol esterification via ACAT and biliary cholesterol secretion. They observed that decreased ACAT activity induced by chronic administration of progesterone to rats correlated with increased biliary cholesterol content.

Taken together, these observations suggested that there is a

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1. *Abbreviations used in this paper:* ACAT, acyl coenzyme A/cholesterol acyltransferase: DMSO, dimethylsulfoxide; GLC, gas-liquid chromatography; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

pool of free cholesterol that can serve as a precursor for biliary cholesterol. The existence of such a pool of cholesterol has been suggested by Schwartz et al. (19). However, the nature and regulation of this pool remain obscure. Synthesis and esterification might be important determinants of the biliary precursor pool. Previous work has relied on chronic administration of cholestyramine (6), cholesterol (6, 20), bile salts (6, 20), or progesterone (18) to uncover relationships between metabolic processes and biliary cholesterol secretion, making it difficult to distinguish between the direct effect of the agent and the role of compensatory changes. In the present study, a rat model was developed that allowed us to assess the effect of acute changes in cholesterol metabolism on biliary cholesterol secretion. Although there are numerous differences between human and rat sterol metabolism, the rat has been extensively studied and provides a model for elucidating principles that may be applicable to man. Specifically, the effect of acute alterations of hepatic cholesterol synthesis and esterification alone and together on biliary lipid secretion were studied.

Methods

Materials

Chemicals. 4-^{14}C-Cholesteryl oleate (52.5 mCi/mmol), 1,2,6,7-^{3}H-progesterone (99 Ci/mmol), 1,2-^{3}H-cholesterol (40–60 Ci/mmol), 3-hydroxy-3-methyl-[3-^{14}C]glutaric acid (57.6 mCi/mmol), ^{3}H-water (100 mCi/g), 25-hydroxy 26,37-^{3}H-cholesterol (87 Ci/mmol), and D,L-5-^{3}H-mevalonic acid (dibenzylethylenediamine salt, 5 Ci/mmol) were obtained from New England Nuclear (Boston, MA). 1-^{14}C-Oleoyl coenzyme A (56–60 mCi/mmol) was from Amersham Corp. (Arlington Heights, IL). Cholesteryl oleate, oleic anhydride, oleoyl coenzyme A, β-NAD, β-NADP, glucose-6-phosphate, taurocholate (Na salt, 98% pure), α-phosphatidylcholine, cholesterol, 3-hydroxy-3-methylglutaryl coenzyme A, D,L-mevalonolactone, 3α-hydroxysteroid dehydrogenase, and glucose-6-phosphate dehydrogenase were from Sigma Chemical Co. (St. Louis, MO). 5α-Cholestane was from Applied Science Div., Milton Ray Co. Laboratory Group (State College, PA), 25-hydroxycholesterol and progesterone were from Steraloids, Inc. (Wilton, NH); Mylar-backed silica gel chromatography sheets were from Eastman Kodak Co. (Rochester, NY) and silica gel H was from E. Merck (Darmstadt, Federal Republic of Germany). Liquifluor and Aquasol were from New England Nuclear. Instagel was from Hewlett-Packard Co. (Downers Grove, IL). Polyethylene tubing was from Clay-Adams, Div. of Becton-Dickinson & Co. (Parsippany, NJ). Mevinolin was a gift of Mr. Alfred Alberts of the Merck Institute for Therapeutic Research (Rahway, NJ). All other chemicals were analytical grade.

Animals. Male Sprague Dawley rats (Simonsen, Gilroy, CA) were housed under normal lighting conditions (lights on 6 a.m.; lights off 6 p.m.) and fed standard rat chow ad lib. They weighted between 300 and 380 g at the time of use.

Methods

Preparation of animals and experimental protocol. Groups of age- and weight-matched animals were used in all experiments. Under ether anesthesia, polyethylene catheters (PE-10) were inserted into the bile duct and bile was allowed to drain by gravity. Polyethylene tubes (PE-100), through which a bile replacement solution was infused at a rate of 1.4 ml/h to maintain the enterohepatic circulation, were placed intraduodenally. In addition, they were fitted with either femoral vein catheters (PE-10) for 25-hydroxycholesterol or progesterone administration or intragastric tubes (PE-50) for mevalonalactone or mevinolin administration. After surgery the animals were placed in individual restraining cages and allowed free access to food and water. After a 21-h recovery period the protocol outlined in Fig. 1 was followed. First a 1-h bile sample was obtained, and then either the test agent dissolved in its vehicle or the vehicle alone was administered. Two 1-h bile samples were then collected. The animals were allowed to recover for another 21-h period, a 1-h bile sample was collected, and the protocol was repeated. At this time the solution not given the day before was administered and two subsequent 1-h samples were then obtained. The order of administration of the agent or vehicle alone was determined randomly, allowing each animal to serve as his own control.

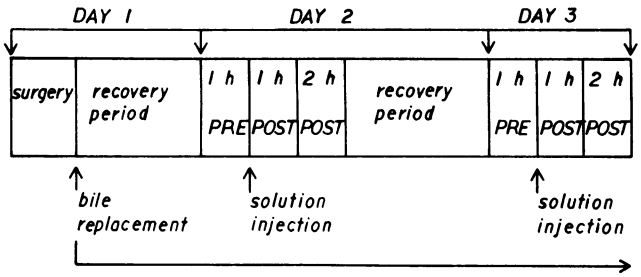

Figure 1. Experimental protocol for studies of biliary lipid secretion. Under ether anesthesia, each 300–380-g rat was fitted with a biliary drainage catheter. An intraduodenal tube was placed, through which a bile replacement solution of 24 mM taurocholate, 3 mM lecithin, and 0.45 mM cholesterol was infused at 1.4 ml h^{-1} to maintain the enterohepatic circulation. In addition, each rat was fitted with a femoral vein catheter for administration of 25-hydroxycholesterol or progesterone or with an intragastric tube for administration of mevinolin or mevalonate. After a 21-h recovery period, during which the animals were allowed free access to food and water, a 1-h bile collection was obtained. Then, either the test agent or the appropriate vehicle alone was administered and two consecutive 1-h bile samples collected. This was followed by a 21-h recovery period with bile replacement, and the sequence was repeated, except that the solution (agent or vehicle alone) that was not given the day before was administered. The order of administration of the test agent or vehicle alone was determined randomly; thus, each animal served as its own control.

Bile replacement solution. Sodium taurocholate (98% pure), 2.4 mmol, 0.3 mmol L-α-phosphatidyl-choline (derived from egg yolk, 60% pure), and 45.0 μmol cholesterol (recrystalized sequentially from glacial acetic acid, ethanol, and acetone) were dissolved in chloroform/methanol (2:1). The chloroform/methanol was removed under vacuum using a rotary evaporator, leaving a uniform layer of bile salt, cholesterol, and phospholipid. This was redissolved in 100 ml of normal saline, and formed an optically clear solution with the following concentrations: 24 mM taurocholate, 3 mM lecithin, and 0.45 mM cholesterol. This solution was stored at 15°C and used within 1 wk of preparation.

Test agent preparation and administration. 25-Hydroxycholesterol was dissolved in 100% ethanol at a final concentration of 20 mg/ml, and 6.25 mg/kg body weight was injected intravenously through the femoral catheter. Progesterone was dissolved in 100% ethanol at 100 mg/ml, and 31.25 mg/kg body weight was injected intravenously to each animal. Mevalonolactone (1 g/ml in normal saline) was administered at a dose of 200 mg/animal through an intragastric tube. Mevinolin (17.5 mg/ml in dimethylsulfoxide) was diluted with normal saline to a final concentration of 3.5 mg/ml, and a dose of 1 mg/kg body weight was administered intragastrically. The control solutions were the above mentioned vehicles alone administered in the same manner as the test solution.

Preparation of microsomes. Animals were killed by exsanguination, the livers removed, rinsed in iced normal saline, and microsomes prepared, as described previously (21). Samples of liver were homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.2, and the homogenate centrifuged at 10,000 *g* for 10 min. The resultant supernatant was centrifuged at 105,000 *g* for 60 min and washed by resuspension and recentrifugation. The final microsomal pellet was then resuspended in the same buffer at a final concentration of ~10 mg protein/ml.

Assay of ACAT activity. ACAT activity was assayed as described previously (21). To the assay mixture containing 150 μg microsomal protein was added 5 nmol of ^{14}C-oleoyl coenzyme A (specific activity ~24,000 dpm/nmol) to initiate the reaction. The assay was terminated after 4 min by the addition of chloroform/methanol (2:1) followed by ^{3}H-cholesteryl oleate (8,000 dpm) as internal standard to estimate recovery. The product was isolated and the results calculated as described previously (21).

Assay of HMG CoA reductase activity. HMG CoA reductase activity was assayed as described previously (22). Microsomes prepared as above were suspended at a final concentration of 2.5 mg protein/ml in buffer containing 0.1 M sucrose, 50 mM KCl, 40 mM KH_2PO_4, and 30 mM EDTA, pH 7.4. The assay mixture contained 10 mmol dithiothreitol, 15 mmol EDTA, 35 mmol NaCl, 15 mmol $NADP^+$, 2 U glucose-6-phosphate dehydrogenase, and 500 μg microsomal protein. The reaction was initiated by the addition of 85 nmol of D,L-3-^{14}C-HMG CoA and terminated after 20 min with 10 N NaOH. D,L-^{3}H-mevalonolactone (90,000 dpm) was added to estimate recovery. The product was isolated, and results calculated as described previously (22).

Measurement of [^{3}H]OH incorporation into cholesterol in vivo. Animals were prepared as described above and constantly infused with the bile replacement solution. After a 21-h recovery period, the test agent or vehicle alone and 50 mCi of [^{3}H]OH adjusted to isotonicity were delivered through the appropriate catheters. After 2 h, the animals were exsanguinated, and the specific activity of plasma water was determined, accordingly to Jeske and Dietschy (23). The liver was rinsed with iced saline in situ and 400–800-mg samples were saponified in alcoholic KOH at 70°C. ^{14}C-Cholesteryl oleate was added to assess recovery. The nonsaponifiable lipids were extracted with 3 × 6 ml of petroleum ether, the ether extracts washed twice with water, and separated by thin-layer chromatography on silica gel H by developing with benzene/ethyl acetate 5:1 (24). The cholesterol bands were identified, scraped into scintillation vials and counted. The rate of ^{3}H-incorporation into cholesterol was then calculated by the method of Jeske and Dietschy (23).

Biliary lipid determination. Bile salts were measured according to Turley and Dietschy (25). Biliary cholesterol was determined colorimetrically according to Mann (26) after saponification of the bile samples (27). Phospholipid phosphorus was assayed according to the method of Bartlett (28) after total lipid extraction (29).

Other determinations. Microsomal protein was determined by the biuret method (30) using bovine serum albumin as a reference standard. Microsomal cholesterol was determined using gas-liquid chromatography (GLC), as described previously (22). For determination of free cholesterol, 0.25 ml of microsomes containing 2.5–5 mg protein and 37.5 mg 5-α-cholestane as an internal standard were extracted, according to Folch et al. (29). The chloroform layer was taken to dryness and dissolved in tetrachloroethylene. A 1-μl sample was analyzed by GLC. Total cholesterol was subsequently measured in the remainder of the sample by the method of Ishikawa et al. (31). The amounts of free and total cholesterol were quantitated by comparing the cholesterol peak areas to that of the added 5-α-cholestane. The cholesterol ester content was calculated as the difference between the total and free cholesterol in the same sample.

Statistical analyses. The significance of changes in biliary lipids was assessed using a paired *t* test. For each individual animal, changes between the 1-h sample and the first and second hour samples after administration of the test solution were compared with those changes evoked by the vehicle administration alone. All other statistical analyses used the group *t* test.

Results

The working hypothesis being tested in these studies is that there is an hepatic pool of free cholesterol from which biliary cholesterol is derived. Although it is established that the rate of cholesterol synthesis per se does not determine the rate of biliary cholesterol output under all circumstances (6), the size of this precursor pool could be regulated in part by the rate of cholesterol synthesis under certain conditions. Moreover, because only free cholesterol is secreted into the bile, the rate of cholesterol ester formation could be a determinant of the pool size. Changes in cholesterol input into this potential precursor pool by variations in synthesis rate could be balanced by changes in the rate of cholesterol ester formation, thus maintaining the free cholesterol pool and biliary cholesterol output constant. This would explain the observed lack of concordance between a change in any single metabolic parameter and the rate of biliary cholesterol secretion. To test this hypothesis, we used a number of compounds that are known to alter hepatic cholesterol metabolism.

Table I. Effect of Administration of the Various Agents on ACAT Activity

Agent	Vehicle control: pmol cholesteryl oleate mg protein^{-1} min^{-1}	Post 1 h: % of control activity	Post 2 h: % of control activity
25-Hydroxycholesterol	50.3±5.3 (9)	142.9* (5)	107.0 (4)
Mevalonate	90.3±6.9 (7)	145.4* (4)	159.5* (3)
Mevinolin	84.9±8.9 (7)	108.3 (5)	100.3 (5)

Liver microsomes were prepared and ACAT activity determined as described in Methods. The control values are the averages of the 1- and 2-h ACAT values in microsomes prepared from animals given the vehicle alone. The vehicles were as follows: intravenous ethanol (25-hydroxycholesterol); intragastric normal saline (mevalonate); intragastric DMSO/normal saline 1:10 (mevinolin). Mean±SE are given. The number of determinations are in parentheses.
* Different from control by grouped *t* test, $P < 0.05$.

Effect of 25-hydroxycholesterol on biliary cholesterol output. The compound 25-hydroxycholesterol has been shown to both increase cholesterol ester formation (32) and decrease hepatic HMG CoA reductase activity (33, 34), the rate-limiting step of cholesterol synthesis. These changes should deplete this biliary precursor pool, and if the size of this pool is a determinant, should cause a decrease in biliary cholesterol secretion.

First, to assess whether this compound rapidly affected these enzyme activities in vito, a 6.25-mg/kg bolus of 25-hydroxycholesterol was administered intravenously to paired groups of animals. The activities of ACAT and HMG CoA reductase were determined in hepatic microsomes 1 and 2 h after the bolus had been administered. There was a 42.9% increase in the activity

Table II. Effect of Administration of the Various Agents on HMG CoA Reductase Activity

Agent	Vehicle control: pmol mevalonate mg^{-1} min^{-1}	Post 1 h: % of control activity	Post 2 h: % of control activity
25-Hydroxycholesterol	145±19 (9)	41.9* (5)	66.0* (4)
Mevalonate	95±14 (7)	23.1* (4)	19.9* (3)
Progesterone	157±5 (5)	97.6 (4)	103.2 (3)

Microsomes were prepared and HMG CoA reductase activity measured as described in Methods. The control values are the averages of the 1 and 2 h reductase activity in microsomes prepared from animals given the vehicle alone. Mean±SE are given. The number of determinations are in parentheses.
* Different from control by grouped *t* test, $P < 0.01$.

of ACAT ($P < 0.05$) 1 h after the bolus was administered (Table I). By 2 h, the activity had returned to the control value. HMG CoA reductase activity decreased to 41.9% of control at 1 h ($P < 0.01$) with a return to 66% of control by 2 h (Table II). Thus, 25-hydroxycholesterol administration acutely elicited the metabolic responses in vivo that have previously been demonstrated in vitro.

The acute effects of the administration of this sterol on biliary lipid composition were examined using the experimental model and the protocol described in Methods and Fig. 1. There was no significant change in bile flow during the course of the experiment (Table III). A small and nonsignificant change in bile salt output (Fig. 2 *A*) occurred in the first hour after administration of 25-hydroxycholesterol compared with the preadministration value. A similar change was observed with administration of the vehicle alone, suggesting that this change was not due to administration of 25-hydroxycholesterol. A decrease in phospholipid secretion (Fig. 2 *B*) greater than that seen with vehicle administration alone was also observed within the first hour after the administration of 25-hydroxycholesterol. However, this effect was not statistically significant.

In contrast, there was a significant decrease in biliary cholesterol output ($P < 0.05$) in the first hour after 25-hydroxycholesterol administration (Fig. 2 *C*). This decrease in biliary cholesterol output was not seen with administration of the control vehicle alone and is, therefore, an effect of the 25-hydroxycholesterol. By the second hour, biliary cholesterol output had returned to the preadministration value. The values from the individual experiments are shown in the insert (Fig. 2 *C*) and demonstrate the consistency of this phenomenon. The fall in cholesterol output was seen in every animal and the return toward control in the second hour in all but one animal. The striking temporal relationship between the reduced biliary cholesterol output and the changes in intrahepatic enzyme activities responsible for modulating the free cholesterol concentration suggest that the observed decrease is due to diminished precursor pool size.

To demonstrate that the change in biliary cholesterol output was not due to substitution of 25-hydroxycholesterol or one of its metabolites for the cholesterol molecule in bile, radiolabeled 25-hydroxycholesterol was administered and the appearance of radiolabel in the bile monitored. The samples were also analyzed by GLC for the appearance of 25-hydroxycholesterol or metabolites. Neither a significant amount of 25-hydroxycholesterol nor nonpolar metabolites were detected (data not shown). Therefore, it is unlikely that 25-hydroxycholesterol or one of its

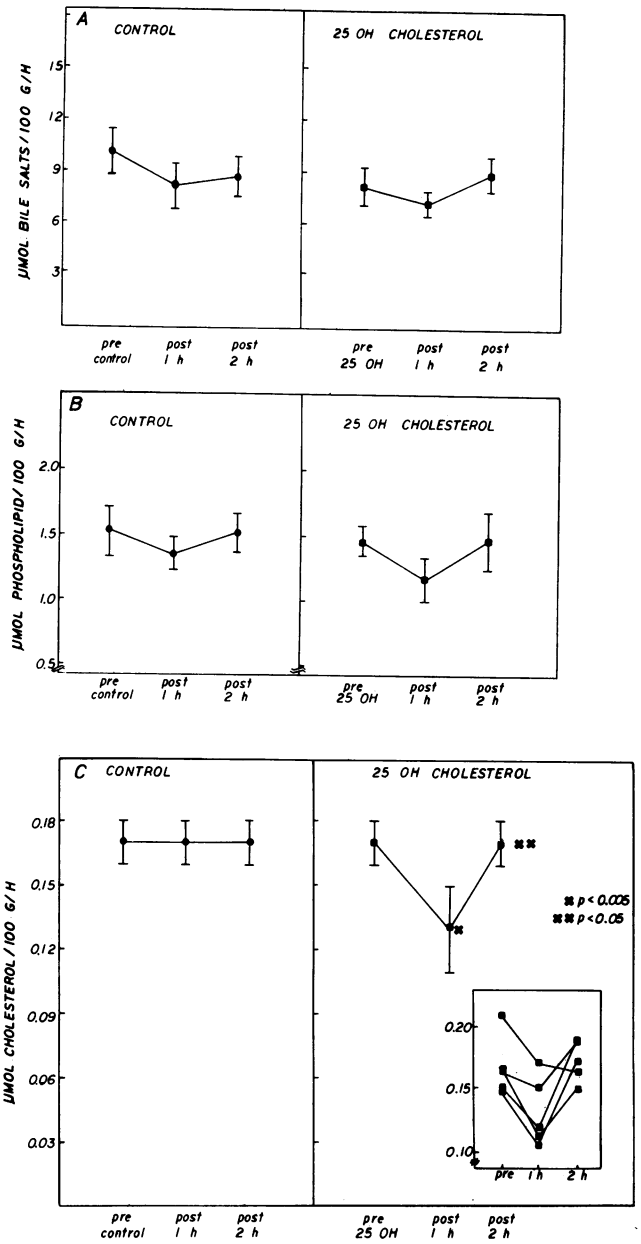

Figure 2. Effect of 25-hydroxycholesterol administration of biliary lipid secretion. The animals were prepared as described in the legend to Fig. 1. The bile samples were analyzed for the content of bile salt, cholesterol, and phospholipid. The effect of administration of ethanol alone is shown on the left of each pair. On the right, the effect of administration of a 6.25-mg/kg bolus of 25-hydroxycholesterol in ethanol is shown (*A*) Effect on bile salt secretion. (*B*) Effect on phospholipid secretion. (*C*) Effect on cholesterol secretion (*inset* shows individual experiments). Each point is the mean±SE of five determinations. *Different from control value by paired *t* test, $P < 0.005$.

Table III. Effect of Administration of the Various Agents on Bile Flow

Agent	Preadministration	Post 1 h	Post 2 h
	ml × 100 g body wt^{-1} × h^{-1}		
Control (all vehicles) (22)	0.33±0.01	0.33±0.02	0.33±0.01
25-Hydroxycholesterol (5)	0.34±0.02	0.34±0.02	0.34±0.02
Mevalonate (6)	0.32±0.03	0.36±0.04	0.34±0.04
Mevinolin (6)	0.35±0.03	0.33±0.03	0.34±0.03
Progesterone (5)	0.38±0.02	0.43±0.02*	0.41±0.03

The experimental protocol was that described in the legend to Fig. 1. The bile flow rate for the hour before administration of the agent (preadministration) is compared with the first and second hour after the agent was given. The control values are the flow rates associated with administration of the vehicle alone and represent the average of all the individual controls 1 h before and 1 and 2 h after the appropriate vehicle was administered. Each animal received the agent or the vehicle alone and the order of administration was random. Therefore, changes in flow rates due to the agent administration are compared with flow rate changes induced in the same animal by administration of the vehicle. Mean±SE is shown. The number of determinations are in parentheses.
* Different from control by paired *t* test, $P < 0.05$.

Table IV. Effect of the Various Agents on Liver Microsomal Cholesterol Content

Agent	Total	Free	Ester
	g cholesterol mg protein^{-1}		
Control (19)	22.20±0.59	20.07±0.77	2.67±0.39
25-Hydroxycholesterol			
1 h (5)	20.95±0.55	17.93±0.75*	3.02±0.39
2 h (5)	19.49±1.08*	16.80±1.07*	2.69±0.40
Mevalonate			
1 h (4)	22.62±2.02	19.30±0.82	2.95±1.26
2 h (4)	26.92±1.42*	21.17±0.26	5.74±1.46*
Progesterone			
1 h (3)	22.05±4.33	22.17±4.43	0.10±0.10‡
2 h (3)	25.30±1.62	24.23±1.48	1.18±0.76

Microsomes were prepared and total, free, and ester cholesterol contents were determined as described in Methods. The control values are the averages of all the microsomal cholesterol contents measured after administration of the various control vehicles at 1 and 2 h. The total and free cholesterol contents were measured by GLC as described in Methods; ester was calculated as the difference. All values are the mean±SE. The number of animals are given in parentheses.

* Different from control by grouped t test, $P < 0.05$.

‡ Different from control by grouped t test, $P < 0.001$.

metabolites replaced the cholesterol in bile. These data demonstrate that 25-hydroxycholesterol, which both stimulates cholesterol esterification and concomitantly blocks cholesterol synthesis, induced a decrease in biliary cholesterol output without altering biliary bile salt or phospholipid secretion.

The microsomal free cholesterol content was slightly but significantly decreased in the treated group as compared with control at both 1 and 2 h after treatment (Table IV). This decrease suggests that there may be depletion of a small biliary precursor pool recovered in the microsomal fraction that is, however, overshadowed by a large structural cholesterol pool.

Taken together, these results suggest that 25-hydroxycholesterol, by increasing cholesterol ester formation while concomitantly decreasing cholesterol synthesis, caused depletion of the free cholesterol pool available for biliary secretion.

Effect of mevalonate on biliary lipid output. It has been suggested that the activities of HMG CoA reductase (35) or ACAT (36) may determine the rate of biliary cholesterol output. To demonstrate that it is the balance between cholesterol synthesis and esterification that controls the size of the precursor pool and not the activities of the enzymes per se, mevalonate was administered. Prior work from this laboratory (21, 34) has demonstrated that a 200-mg intragastric bolus of mevalonate decreases hepatic HMG CoA reductase activity and stimulates ACAT activity. Thus, administration of mevalonate evokes the same enzyme activity changes as 25-hydroxycholesterol. Unlike 25-hydroxycholesterol, however, mevalonate administration should not deplete the metabolically active free cholesterol pool because mevalonate itself is converted to cholesterol.

After intragastric administration of a 200-mg bolus of mevalonate, the changes in ACAT activity (Table I) and HMG CoA reductase of activity (Table II) were comparable to those induced by 25-hydroxycholesterol administration. There were no significant changes in bile flow, bile salt, or phospholipid output, nor were there any changes in biliary cholesterol output 1 or 2 h after the administration of mevalonate (Table III and Fig. 3). There was a statistically significant increase in microsomal cholesterol ester content 2 h after the administration of mevalonate (Table IV) with no change in microsomal free cholesterol.

Concurrent administration of 6.25 mg/kg 25-hydroxycholesterol and 200 mg of mevalonate resulted in a constant amount of biliary cholesterol secretion over the 2-h collection period (0.22±0.03) μmol cholesterol secreted h^{-1} 100 g^{-1} vs. 0.22±0.22 μmol cholesterol secreted h^{-1} 100 g^{-1}, $n = 4$). Thus, the decreased free cholesterol pool induced by 25-hydroxycholesterol admin-

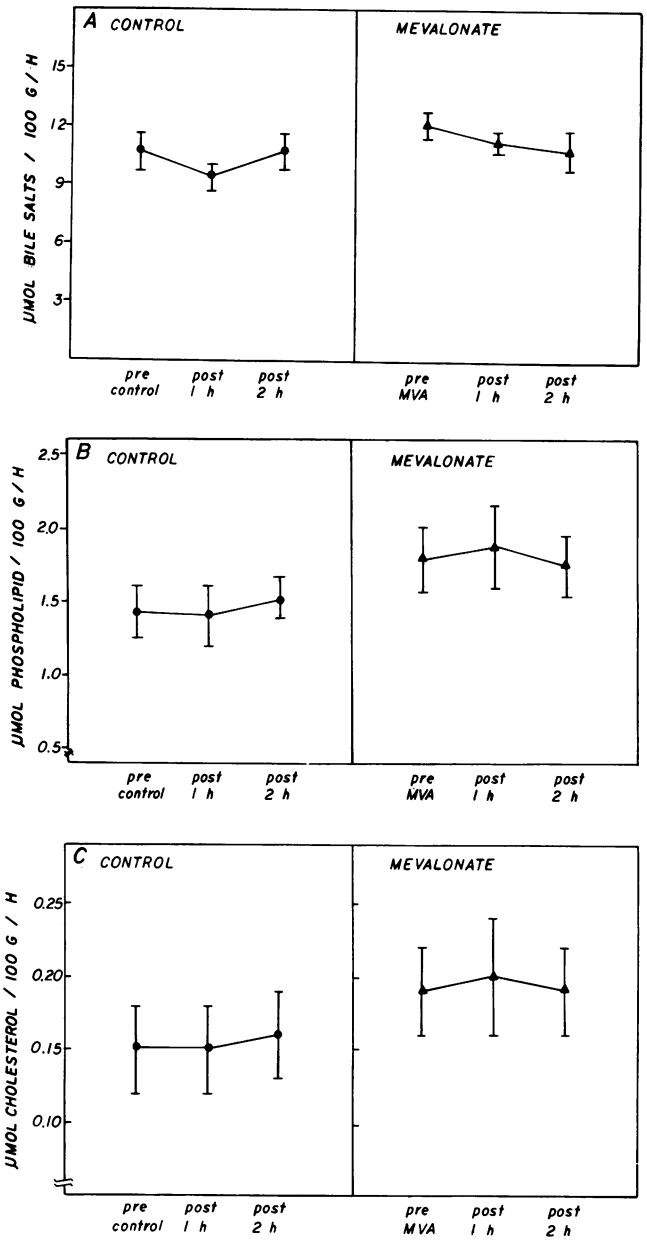

Figure 3. Effect of mevalonate on biliary lipid secretion. The same protocol as in the legend to Fig. 2 was used, except that a 200-mg bolus of mevalonolactone in normal saline was administered through an indwelling gastric catheter. The vehicle was normal saline. (*A*) Effect on bile salt secretion. (*B*) Effect on phospholipid secretion. (*C*) Effect on biliary lipid secretion. Each point is the mean±SE of six experiments.

istration can be repleted by mevalonate, whose conversion to cholesterol is not affected by the sterol, with concomitant restoration of biliary cholesterol secretion.

These results demonstrate that the activities of the enzymes per se do not regulate biliary cholesterol content. Rather, they suggest that it is the net effect of changes in the various processes that regulate intrahepatic cholesterol metabolism, which determines the size of the biliary cholesterol precursor pool itself which appears to be a determinant of biliary cholesterol output.

Effect of mevinolin on biliary lipid output. To determine whether biliary cholesterol output could be acutely altered by change in the rate of cholesterol synthesis alone, the compound mevinolin was administered. Mevinolin is a competitive inhibitor of HMG CoA reductase, and is reported to inhibit cholesterol synthesis in rat liver within 1 h of administration at a dose of 1 mg/kg body weight (37). The effect of mevinolin administration on cholesterol esterification rate has not been reported. If the rate of ester formation is not affected, then this mode of egress of free cholesterol from the biliary precursor pool will be maintained. The decreased input into the precursor pool, as a result of decreased synthesis, would cause a net decrease in cholesterol pool size and thus in biliary cholesterol output. Mevinolin, when administered at the above dose, did not alter ACAT activity (Table I). HMG CoA reductase activity was not determined because, although mevinolin inhibits sterol synthesis in vivo, HMG CoA reductase activity when assayed in vitro is higher than in controls (38). Mevinolin had a marked inhibitory effect on cholesterol synthesis as measured by [^{3}H]OH incorporation. The rate of cholesterol synthesis was depressed in the mevinolin-treated animals to 24% that of matched controls (303 nmol [^{3}H]OH incorporated g^{-1} h^{-1} in the treated compared with 1,277±348 in the controls, $P < 0.01$). In addition, there was a decrease in the amount of radiolabeled cholesterol excreted in the bile over the same 2-h period (11.3±0.8 nmol ^{3}H-cholesterol/g liver h^{-1} vs. 28.9±6.9 in the controls, $n = 3$, $P < 0.05$). Interestingly, the ratio of radiolabeled cholesterol excreted in the bile to the amount of radiolabeled in the liver was the same (3.4%) in both the control and treated animals. That the percentage of newly synthesized cholesterol that is excreted as biliary cholesterol is constant despite varying synthetic rates has also been demonstrated by others (16, 17).

No significant change in bile flow was observed after administration of mevinolin (Table III), nor was there a significant change in bile salt or phospholipid output (Fig. 4, *A* and *B*). However, total biliary cholesterol mass output decreased 16% during the 2 h after the administration of mevinolin compared with the preadministration value ($P < 0.05$, Fig. 4 *C*). Thus, a 76% decrease in cholesterol synthesis resulted in a 16% decrease in biliary cholesterol output. Based on this, it can be calculated that 21% of the biliary cholesterol output in the control was newly synthesized sterol, which was rapidly excreted. This estimate is in good agreement with previously reported values for this parameter for rats in the basal state (16, 17).

Microsomal cholesterol contents were not significantly changed (data not shown). Thus, an acute decrease in the rate of cholesterol synthesis without a compensatory change in esterification rate appears to decrease the size of the metabolically active biliary precursor pool, resulting in a decrease in biliary cholesterol output.

Effect of progesterone on biliary lipid output. To investigate whether a change in the rate of cholesterol ester formation alone could affect biliary cholesterol secretion, the compound progesterone was used. Chronic progesterone treatment in vivo increases biliary cholesterol output and decreases the activity of hepatic ACAT (18). ACAT activity is also decreased in vitro by the addition of progesterone to hepatic microsomes (21, 39), or by inclusion in the culture medium of human skin fibroblasts (40). Thus, progesterone administration in our in vivo model might be expected to acutely depress the rate of cholesterol ester formation without affecting the rate of cholesterol synthesis. The net result should be an expansion of the biliary free cholesterol

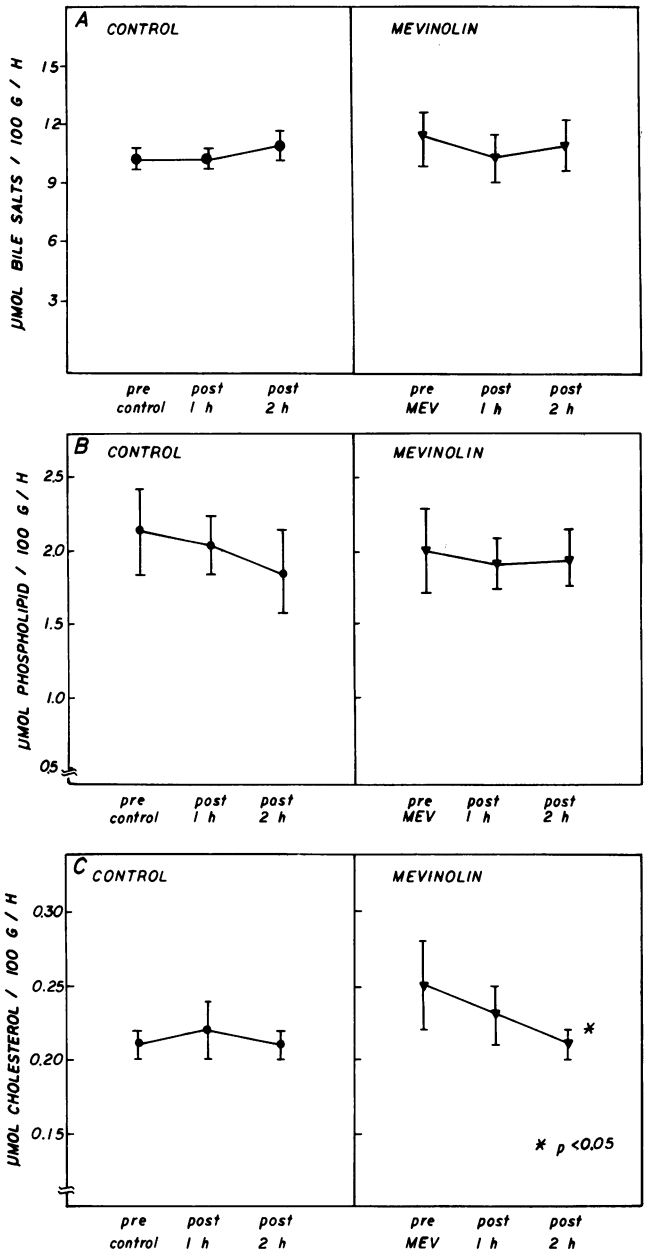

Figure 4. Effect of mevinolin on biliary lipid secretion. The same protocol as in the legend to Fig. 2 was used except that 1 mg/kg of mevinolin in a dimethylsulfoxide (DMSO)/saline (1:10) solution or the DMSO/saline vehicle alone was administered intragastrically. (*A*) Effect on bile sale secretion. (*B*) Effect on phospholipid secretion. (*C*) Effect on cholesterol secretion. Each point is the mean±SE of six determinations. *Different from control value by paired *t* test, $P < 0.05$.

pool with an increase in biliary cholesterol output. Progesterone administration did not change HMG CoA reductase activity (Table II). ACAT activity was difficult to measure. The effect of progesterone in vitro is reversed by washing the microsomes (41). Progesterone administered in vivo was lost from microsomes during preparation (data not shown). Moreover, the compound is rapidly metabolized. 1 h after injection of 10 mg of progesterone, only 22 ng/mg protein was recovered in the microsomal fraction. However, cholesterol esters were substantially reduced in microsomes 1 h after progesterone administration (Table IV), which suggests cholesterol esterification was inhibited as expected.

Bile flow was significantly increased 1 h after intravenous administration of progesterone as compared with the ethanol control (Table III). This effect appears to be bile salt independent, since no significant change in bile salt secretion was found (Fig. 5 *A*). Such a choleretic effect has been reported previously for the progesterone analogue pregnenolone-16-α-carbonitrile (42). No significant change in phospholipid output was seen with either progesterone or the control vehicle alone (Fig. 5 *B*). A marked increase ($P < 0.05$) in biliary cholesterol output was observed in the first hour after progesterone administration (Fig. 5 *C*). By 2 h, biliary cholesterol secretion had returned to normal. The changes in biliary cholesterol secretion paralleled the changes in microsomal cholesterol ester content. These results suggest that a change in intrahepatic cholesterol esterification without a compensatory change in cholesterol synthesis can affect biliary cholesterol secretion independently of bile salt secretion and support the hypothesis that esterification rates are an important determinant of the biliary cholesterol precursor pool.

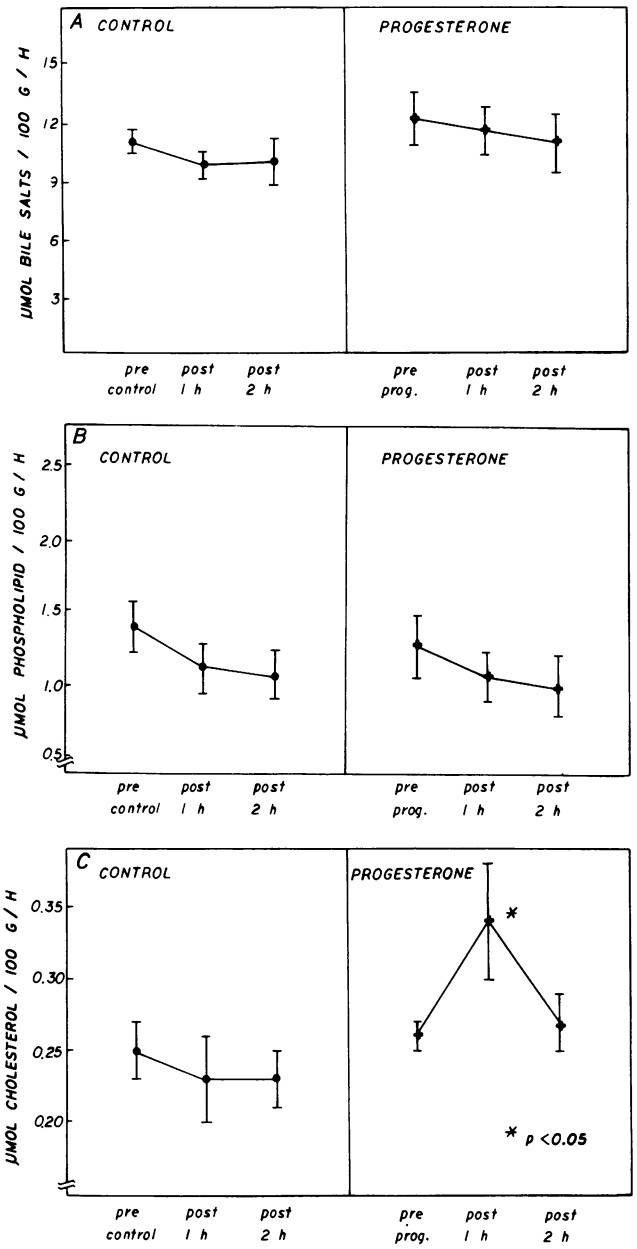

Figure 5. Effect of progesterone on biliary lipid secretion. The same protocol as in the legend to Fig. 2 was used, except that 31.25 mg/kg progesterone in ethanol or ethanol alone was administered intravenously. (*A*) Effect on bile sale secretion. (*B*) Effect on phospholipid secretion. (*C*) Effect on cholesterol secretion. Each point is the mean±SE of five determinations. *Different from control value by paired *t* test, $P < 0.05$.

Discussion

There is considerable uncertainty regarding the mechanism and regulation of cholesterol secretion into the bile. It is generally accepted that the rate of bile salt secretion determined in part the rate of biliary cholesterol secretion. Under most circumstances, as bile salt secretion increases, cholesterol secretion also increases. However, more cholesterol is secreted per molecule of bile salt at lower secretory rates than at higher rates (5). Moreover, when different bile salt species are secreted at the same rate they induce secretion of different amounts of cholesterol (43). Thus, in addition to the rate of bile salt secretion, intrahepatic metabolic factors seem to play an important role in determining the rate of cholesterol secretion into bile.

It has been suggested that the rates of cholesterol synthesis and esterification in the liver are important determinants of the rate of biliary cholesterol secretion. The activity of the enzyme HMG CoA reductase, which catalyzes the rate-limiting step of cholesterol synthesis, is elevated in patients with increased biliary cholesterol secretion and cholesterol gallstone disease (10–12). This led to the hypothesis that the rate of cholesterol synthesis determined the rate of biliary cholesterol secretion. It has been shown that under basal conditions, 10–20% of secreted cholesterol is derived within 30 min from newly synthesized sterol (16), and that synthesis and secretion varied together under some circumstances. Turley and Dietschy (6) demonstrated in the rat that the two are not necessarily related. However, this may not apply to man. Since 80% of biliary cholesterol is derived from preformed sources, factors other than the rate of cholesterol synthesis must be important in determining biliary secretion rate. However, it should be born in mind that ultimately all cholesterol is derived from either *de novo* synthesis or the diet.

It was suggested that the rate of cholesterol esterification could affect cholesterol secretion because chronic progesterone administration inhibited hepatic cholesterol esterification and increased biliary cholesterol secretion (18). Other factors that may be important include plasma lipoprotein cholesterol uptake (44) and intracellular transport of cholesterol, about which little is known. Thus, biliary cholesterol secretion is likely to be regulated by the net effect of a variety of interrelated processes

influencing the rates of entry and exit of cholesterol into the presecretory pool.

In the present work, we used an acute rat model to test the hypothesis that there is an active metabolic pool of cholesterol in the liver which, when altered, can affect biliary cholesterol secretion directly. The findings reported here and summarized in Table V are entirely consistent with this concept. The enterohepatic circulation of bile salt, cholesterol, and lecithin was rigidly maintained by using a constant infusion of these compounds into the duodenum of bile-diverted animals. This minimized any possible effects of changes in bile salt synthesis on these processes. In the face of this fixed bile salt return to the liver, none of the agents administered significantly altered bile salt secretion. Thus, changes in bile salt-coupled cholesterol secretion could not account for the changes in cholesterol secretion evoked by the agents 25-hydroxycholesterol, mevinolin, or progesterone. The lack of statistically significant changes in phospholipid secretion is also consistent with these effects, being mediated by changes in intrahepatic cholesterol metabolism alone. The time course of changes in secretion closely paralleled those of the changes in the metabolic parameters. Thus, with 25-hydroxycholesterol, HMG CoA reductase activity decreased, ACAT activity increased, and cholesterol secretion decreased within 1 h of drug administration.

That enzyme activities per se are not the determinant of secretion is demonstrated by the experiment with mevalonate, where the enzyme activities are altered but the free cholesterol pool apparently is maintained with no change in biliary cholesterol secretion. The observation that changing either of two enzymes involved in intrahepatic cholesterol metabolism (ACAT or HMG CoA reductase) independently affects biliary cholesterol secretion suggests that the metabolic regulation of secretion is likely to be subject to a variety of interrelated processes influencing the rates of free cholesterol entry to or exit from the proposed precursor pool. No data is available concerning the size or location of this pool. That the biliary cholesterol changes occurred within 1 h of pharmacologic manipulation suggests it is small. That microsomal free and ester cholesterol contents in some cases mirrored the changes in biliary cholesterol output suggests that the proposed precursor pool is a small subset of microsomal cholesterol. However, no data presented here allow further speculation on it location.

In addition to synthesis and esterification, other processes may play a role in determining the size of this pool. It has been suggested that lipoproteins, especially high density lipoproteins, are an important source of biliary cholesterol (44). Others have found that chylomicron cholesterol is not an important source of biliary cholesterol in the rat (6). Intracellular cholesterol ester hydrolysis does not seem to be a regulated process (45, 46) (Stone, B. S., unpublished observations). Thus, its role is likely to be passive.

Table V. Summary of Effect of Alteration of Cholesterol Homeostasis on Biliary Cholesterol Secretion

Agent	Synthesis	Esterification	Biliary cholesterol
25-Hydroxycholesterol	↓	↑	↓
Mevalonate	↑	↑	→
Mevinolin	↓	→	↓
Progesterone	→	↓	↑

The existence of a tightly regulated metabolically active free cholesterol pool which serves as the precursor for biliary cholesterol may clarify some of the confusing species differences in the response of biliary lipid output to a cholesterol-rich diet. For example, the rat that is relatively resistant to changes in biliary cholesterol saturation has a relatively high level of hepatic ACAT activity, and thus may rapidly esterify even large amounts of influxing dietary cholesterol, thus maintaining a constant pool size and biliary cholesterol secretion rate. In contrast, humans who are more prone to cholesterol gallstone formation and who normally have a higher mole percentage of cholesterol in the bile (47) have lower levels of this enzyme (39, 48). This may allow the biliary precursor pool to expand in response to a rapid influx of cholesterol, which would result in increased cholesterol secretion and supersaturation of the bile. However, before an extrapolation of this data, which was obtained from the rats, can be made to man, further experimentation in humans will be necessary.

The results of this study, together with evidence from other laboratories, support the conclusion that the rate of cholesterol secretion into bile is determined not only by the rate of bile salt secretion, but also by intrahepatic factors including rates of cholesterol synthesis and esterification. What other factors are involved, where the precursor biliary cholesterol pool is located, and how cholesterol secretion is coupled to bile salt secretion remain important areas for future research.

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