Changes in biliary lipid secretion during normal development and diurnal cycling in the rat

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Abstract Biliary lipid secretion in conjunction with hepatic cholesterol synthesis was determined in normal male rats at 4, 5, 7, and 9 weeks of age, during a period of linear growth and a near fourfold increase in liver size. Studies were performed both at periods of low (mid-light cycle) and high (mid-dark cycle) hepatic cholesterol synthesis. Biliary bile salt, phospholipid, and cholesterol secretion (per g of liver) markedly decreased with an increase in liver size. Whereas the secretion of bile salts and phospholipid was not significantly different in mid-dark and mid-light periods for animals of the same age, cholesterol secretion was greater in the mid-dark than in the mid-light period at 5, 7, and 9 weeks of age. Relationships between biliary cholesterol secretion and bile salt and phospholipid secretion differed at mid-dark and mid-light periods, as follows: cholesterol secretion was not significantly related to bile salt secretion at mid-dark ($r = 0.49$, $P > 0.05$) but was related at mid-light ($r = 0.73$, $P = 0.003$); and although cholesterol secretion was significantly related to phospholipid secretion at mid-dark, this relationship was not nearly as strong as at mid-light ($P < 0.005$, comparing $r = 0.95$ at mid-light with $r = 0.53$ at mid-dark). In contrast, at mid-dark, biliary cholesterol secretion was strongly related to hepatic cholesterol synthesis ($r = 0.84$, $P < 0.0001$) whereas at mid-light the two were not significantly related ($r = 0.13$, $P > 0.05$). These results show that at mid-light, when hepatic cholesterol synthesis is at a low point, cholesterol secretion is more dependent on bile salt and phospholipid secretion than at mid-dark when cholesterol synthesis is high. As neither bile salt nor phospholipid secretion was increased at the mid-dark period, it is possible that increased cholesterol secretion at mid-dark occurred in response to increased hepatic cholesterol synthesis.—**Robins, S. J., J. M. Fasulo, P. D. Lessard, and G. M. Patton.** Changes in biliary lipid secretion during normal development and diurnal cycling in the rat. *J. Lipid Res.* 1993. **34:** 1445–1450.

Supplementary key words liver • bile salts • phospholipid • cholesterol • bile • cholesterol synthesis

Although the secretion of biliary cholesterol is to a great extent dependent on the secretion of bile salts (1–3) and phospholipid (4), there is great variability in cholesterol secretion that cannot be predicted just by changes in biliary bile salt and phospholipid secretion. To more fully define the process by which cholesterol is mobilized for biliary secretion, it has been suggested (5, 6) that bile cholesterol derives from a special metabolically active pool of cholesterol within the liver and that the availability of this precursor pool will also determine the magnitude of biliary cholesterol secretion. Our recent studies (7) are in accord with this concept and suggest that this precursor pool for bile cholesterol may be those periportal hepatocytes that ordinarily are the only hepatocytes that stain positive for 3-hydroxyl-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (8, 9).

A number of pharmacologic measures have been used to change the amount of cholesterol in this metabolically active precursor pool (5, 6, 10–13). In particular, studies that have been undertaken to decrease hepatic cholesterol synthesis with HMG-CoA reductase inhibitors have demonstrated that an acute decrease in hepatic cholesterol synthesis is associated with a decrease in biliary cholesterol secretion (14–17). However, HMG-CoA reductase inhibitors that decrease cholesterol synthesis have also been shown to decrease the rate of synthesis and secretion of bile salts (15–17) that may independently affect the biliary secretion of cholesterol. The link between hepatic cholesterol and bile salts may also explain why certain other pharmacologic measures that increase hepatic cholesterol synthesis but also increase bile acid synthesis (as the administration of bile acid sequestrants) do not result in an increase in biliary cholesterol secretion (7).

The present studies relating cholesterol synthesis to biliary secretion were undertaken to avoid the complex changes that may be associated with the use of pharmacologic agents to manipulate cholesterol synthesis. The present studies were performed in rats during normal growth. These studies were conducted during a period of large changes in liver size and biliary lipid secretion to re-

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl.

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late changes in hepatic cholesterol synthesis during the normal diurnal cycle to the secretion of biliary cholesterol.

METHODS

Animals

Male Sprague-Dawley rats (Taconic Animal Farms, Germantown, NY) were purchased in four lots of ~ 25 animals/lot over a 9-month period and maintained in a 12-h light-cycled room. Animals were delivered at 21-24 days of age, by estimate of the supplier. Animals were fed Purina Chow ad libitum. Studies were performed, beginning 9 days after arrival, at ~ 4, 5, 7, and 9 weeks of age. (In these animals, sexual maturity occurs at 6-8 weeks (18).) Studies were performed during the period of decreased hepatic cholesterol synthesis (mid-light period) and during the period of peak hepatic cholesterol synthesis (mid-dark period) of a 12-h cycle.

Study protocol

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and bile was collected from 0-30 min, immediately after cannulating the bile duct. Cholesterol synthesis was determined in half of the two groups of animals that were studied in each period of the light cycle. When cholesterol synthesis was determined, [^{3}H]water (25-60 mCi) (New England Nuclear, Boston, MA) was injected intravenously (by femoral vein injection) at 0 min. At the conclusion of bile collection, blood was obtained for determination of plasma [^{3}H]water specific activity and the liver was perfused free of blood and removed for analysis.

Analysis

Biliary bile salts and phospholipid were determined as previously described (4). Biliary cholesterol and hepatic cholesterol and cholesteryl esters were isolated from lipid extracts by high performance liquid chromatography and quantitated by integration in conjunction with internal stigmasterol standards, as described (19). Absolute amounts of newly synthesized cholesterol in bile and liver were measured as previously outlined (20), normalizing results to account for variations in the amount of [^{3}H]water that was given to individual animals. Statistical differences between groups of animals at different ages were determined by ANOVA at the 95% probability level with Fishers PLSD test. Statistical differences between groups at the mid-light and mid-dark periods of the light cycle were determined by Student's *t*-test.

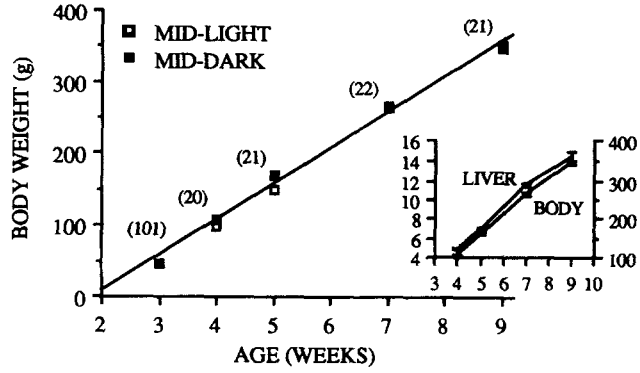

Fig. 1. Changes in body and liver weights with age. Weights of rats at delivery (3 weeks) and at four subsequent periods of study (at 4, 5, 7, and 9 weeks) are shown in mid-light and mid-dark periods, with total numbers of rats at each period in parentheses. The inset shows the change in liver weight in association with body weight at 4, 5, 7, and 9 weeks of age.

RESULTS

From delivery at ~ 3 weeks of age through periods of study at 4, 5, 7, and 9 weeks of age, rat weights increased 7-fold (from ~ 50 to 350 g). Weight gain was linear (r = 0.997) in animals studied in both the mid-light and mid-dark cycles (**Fig. 1**). Liver growth paralleled the increase in body weight (Fig. 1, inset).

Table 1 and **Table 2** show the changes in biliary bile salt, phospholipid, and cholesterol secretion with age

TABLE 1. Biliary lipid secretion at low point of hepatic cholesterol synthesis

Group – Age	Liver Weight	Bile Acids	Bile Phospholipid	Cholesterol
weeks[a]	*g*		*nmol/min/g liver*	
A – 4	4.6 ± 0.3	111 ± 21	20.5 ± 5.7	4.10 ± 2.35
B – 5	6.3 ± 0.5	105 ± 29	21.0 ± 4.6	3.14 ± 1.16
C – 7	12.3 ± 0.7	80 ± 25	14.1 ± 4.7	1.80 ± 0.56
D – 9	14.7 ± 1.8	73 ± 11	11.6 ± 2.9	1.14 ± 0.30
$P < 0.05$[b]	A vs. B,C,D B vs. C,D C vs. D	A vs. C,D B vs. C,D	A vs. C,D B vs. C,D	A vs. C,D B vs. C,D

Data shown as mean ± SD for 9-10 rats in each group. Bile was collected from 0-30 min after cannulating the bile duct. Rats were maintained in a 12-h light-cycled room and operated upon at the mid-point of the light cycle.
[a] Age in weeks, from birth date, based on estimate of supplier.
[b] Significant differences between groups A, B, C, and D at $P < 0.05$ were determined by ANOVA.

TABLE 2. Biliary lipid secretion at maximum hepatic cholesterol synthesis

Group–Age	Liver Weight	Bile Acids	Bile Phospholipid	Cholesterol
weeks[a]	*g*		*nmol/min/g liver*	
A–4	5.0 ± 0.6	108 ± 29	16.3 ± 4.5	4.30 ± 1.14
B–5	7.6 ± 0.3	96 ± 23	19.9 ± 4.4	5.28 ± 1.73
C–7	10.9 ± 1.1	69 ± 22	13.8 ± 4.1	2.30 ± 0.56
D–9	14.1 ± 1.9	65 ± 17	12.1 ± 3.4	1.87 ± 0.77
$P < 0.05$[b]	A vs. B,C,D B vs. C,D C vs. D	A vs. C,D B vs. C,D	A vs. D B vs. C,D	A vs. C,D B vs. C,D

Data shown as mean ± SD for 10–12 rats in each group. Bile was collected from 0–30 min after cannulating the bile duct. Rats were maintained in a 12-h light-cycled room and operated upon at the mid-point of the dark cycle.
[a]Age in weeks, from birth date, based on estimate of supplier.
[b]Significant differences between groups A, B, C, and D at $P < 0.05$ were determined by ANOVA.

when measured in the mid-light (during decreased hepatic cholesterol synthesis) and mid-dark periods (during peak hepatic cholesterol synthesis). As expressed per g of liver, the secretion of all three biliary lipids decreased with increased age and liver size. However, while there were no differences in bile salt or phospholipid secretion in mid-light and mid-dark periods in animals of the same age (**Fig. 2A** and **Fig. 2B**), biliary cholesterol secretion was significantly greater in the mid-dark than mid-light phase at three of the four periods of study ($P < 0.005$, < 0.05, and < 0.01 at 5, 7, and 9 weeks of age, respectively) (Fig. 2C). Hepatic cholesterol content, for both unesterified and esterified cholesterol, was generally higher in younger than older animals (**Table 3**). However, liver cholesterol was not significantly different at the mid-dark compared to the mid-light period at any one of the four ages of study.

Cholesterol synthesis was determined in half of each group of animals in which bile was collected in mid-light and mid-dark periods. In these animals the relation of the secretion of biliary lipids to each other and to hepatic cholesterol synthesis is shown in **Table 4** and **Fig. 3** in both mid-light and mid-dark periods. The secretion of bile salts to phospholipid and secretion of phospholipid to cholesterol were significantly related in mid-light and mid-dark groups combined and in each of these groups when separately analyzed (Table 4). The relationship of phospholipid secretion to cholesterol secretion was much stronger when lipid secretion was measured at the mid-light compared to the mid-dark period ($P < 0.005$, comparing $r = 0.95$ at mid-light with $r = 0.53$ at mid-dark). The relationship of bile salt secretion to cholesterol secretion was not significant for mid-light and mid-dark groups combined ($P = 0.066$, Table 4) and was not significant for the group at mid-dark when separately analyzed ($P = 0.063$, Table 4). However, the relationship of bile salt to cholesterol was significant for the group of animals that were studied at the mid-light period ($P = 0.003$, Table 4).

Biliary cholesterol secretion was highly correlated with hepatic cholesterol synthesis for mid-light and mid-dark groups combined ($r = 0.76$, $P = 0.0001$). However, when mid-light and mid-dark groups were separated, the correlation was significant only during the period of high cholesterol synthesis and not during the period of low synthesis (Fig. 3). Hepatic cholesterol synthesis did not significantly correlate with amounts of hepatic cholesterol (data not shown), nor was the amount of hepatic cholesterol significantly correlated with biliary cholesterol secretion (data not shown).

DISCUSSION

We have demonstrated in chow-fed rats that there are large differences in biliary lipid secretion during normal growth and that the relationship of cholesterol secretion to both bile salt and phospholipid secretion is different in the mid-dark and mid-light periods of a diurnal cycle. In particular, we have found at mid-dark, or at the high point of hepatic cholesterol synthesis, that biliary cholesterol secretion is not significantly related to bile salt secretion and is less strongly related to phospholipid secretion than at mid-light. However, when cholesterol synthesis is at its high point, we found a strong linear relationship between biliary cholesterol secretion and hepatic cholesterol synthesis.

The relation between cholesterol synthesis and biliary cholesterol secretion confirms a previous study in rats (21) in which an increase in biliary cholesterol secretion was paralleled by hepatic cholesterol synthesis and shown to be related to food intake. Rats are nocturnal feeders and in our study, with food always available, it can be assumed that an increase in cholesterol synthesis and secretion also coincided with feeding, i.e., during the dark period of a 12-h cycle. With the data available, however, it is not possible to conclude that increased synthesis results in increased secretion or, alternatively, occurs in response to increased secretion. Our results are, however, consistent

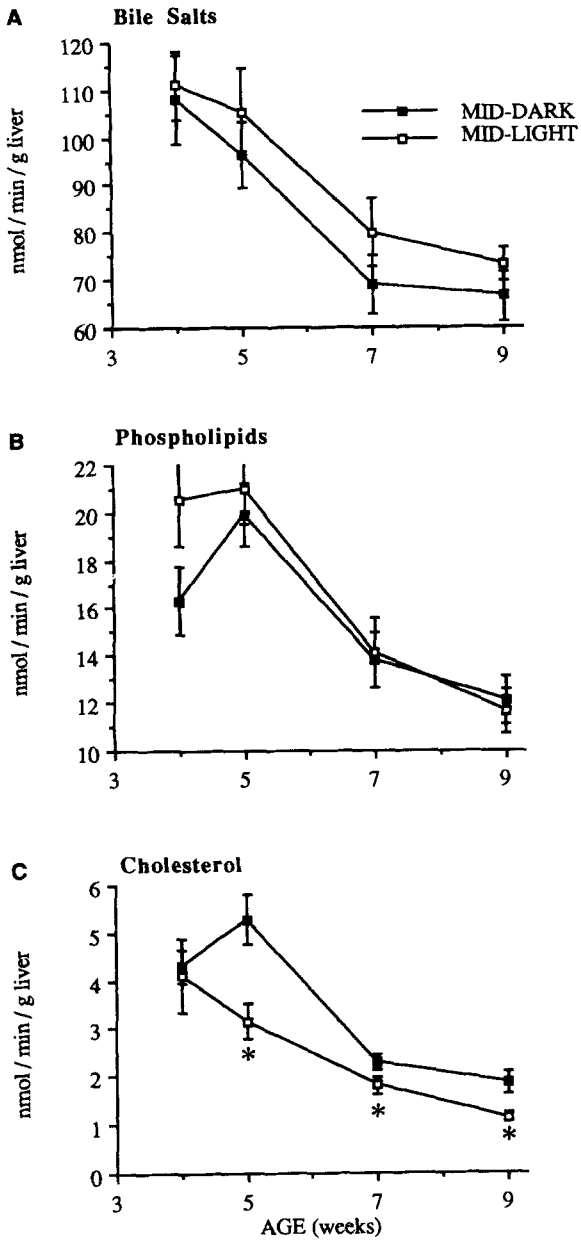

Fig. 2. Biliary secretion of bile salts (A), phospholipid (B), and cholesterol (C) in groups of rats at 4, 5, 7, and 9 weeks of age, studied in the mid-dark and mid-light periods of 12-h cycles. Bile was collected for 30 min after cannulating the bile duct. Data are shown as mean ± SEM for 10–12 animals at each phase of the light cycle and at each age. Significant differences between measurements made at mid-light and mid-dark are indicated by an asterisk.

with the first possibility since changes in cholesterol secretion occurred in the absence of changes in biliary bile salt or phospholipid secretion (Figs. 2A and 2B) and recent studies in humans (14–17) have demonstrated that biliary cholesterol secretion can be markedly decreased by drugs inhibiting HMG-CoA reductase, the rate-limiting enzyme in hepatic cholesterol synthesis.

During the 5-week period of study, the biliary secretion of lipids, expressed per gram of liver, decreased with an increase in liver size (Tables 1 and 2). However, with an increase in liver size, the total amounts of bile salt and phospholipid that were secreted in bile were actually increased (by 89% for bile salt and by 92% for phospholipid, for mid-light and mid-dark groups combined, from week 4 to 9). In contrast, the total amount of cholesterol secreted was not dependent on liver size and did not change significantly from week 4 to 9 for either mid-light or mid-dark groups. To our knowledge, in no other study has biliary lipid secretion been measured at several different periods during the post-weaning period of normal growth. Although there have been a number of studies of bile acid uptake and secretion in the perinatal period (22), in no study has the relationship of bile acid secretion to the secretion of phospholipid and cholesterol been determined in animals after weaning and during large changes in body weight and liver size. Furthermore, there are no data relating to biliary lipid secretion in normal humans during growth.

We have no explanation for the finding that in younger rats biliary lipid secretion is greater per gram of liver than in older rats. Much of this change might be secondary to changes in the enterohepatic cycling of bile salts and thus relate to changes not only in the bile acid pool size with growth but to changes in intestinal motility and absorption. Although the explanation for these changes has not been determined, it is clear from the present study that comparisons of biliary lipid secretion between animals of different weights (and ages) may not be justified.

Our results are contrary to a previous study in rats (11) in which large changes in hepatic cholesterol synthesis were not found to be associated with changes in biliary cholesterol secretion. This difference in results might be attributed to a number of methodologic differences between our present and the previous study, in which bile was collected for longer periods from chronically restrained rats with prolonged interruption of the enterohepatic circulation. However, this difference in results may also be related to the specific kinds of manipulations used to produce changes in hepatic cholesterol synthesis. For example, administration of a bile acid-binding resin was used to increase hepatic cholesterol synthesis but, as we have previously shown (7), bile acid-binding resins also increase bile acid synthesis and do not result in either an increase in newly synthesized cholesterol or total cholesterol in bile. Conversely, infusion of lipoprotein-cholesterol was used to suppress hepatic cholesterol synthesis and although synthesis was suppressed, an increase in hepatic cholesterol promotes cholesteryl ester formation (23) and may not result in an increase in the hepatic pool of unesterified cholesterol that is utilized for biliary secretion.

TABLE 3. Hepatic cholesterol at low and peak periods of hepatic cholesterol synthesis

Group – Age	Unesterified Cholesterol			Cholesteryl Esters		
	Mid-Light	Mid-Dark	$P < 0.05$	Mid-Light	Mid-Dark	$P < 0.05$
weeks	*μmol/g liver*			*μmol/g liver*		
A – 4	4.96 ± 0.69	5.19 ± 0.56	NS	1.46 ± 0.47	1.45 ± 0.42	NS
B – 5	5.41 ± 0.44	5.34 ± 0.31	NS	2.05 ± 0.76	1.45 ± 0.57	NS
C – 7	4.76 ± 0.34	4.89 ± 0.40	NS	1.15 ± 0.37	1.24 ± 0.51	NS
D – 9	4.68 ± 0.32	4.82 ± 0.28	NS	0.89 ± 0.50	1.12 ± 0.30	NS
$P < 0.05$	A vs. B B vs. C,D	A vs. D B vs. C,D		A vs. B,D B vs. C,D		

Data shown as mean ± SD for 10–12 rats in each group. Livers were obtained for analysis 30 min after cannulating the bile duct (Methods). Significant differences at $P < 0.05$ between groups of different ages (Groups A, B, C and D) are shown by ANOVA and between mid-light and mid-dark cycled animals by Student's *t*-test.

We (7, 20) and others (24, 25) have previously reported that the contribution that newly synthesized cholesterol makes to biliary cholesterol secretion in rats and hamsters is relatively small, i.e., on the order of 2–16%. We found this again to be the case in the present study. Throughout a sixfold range of biliary cholesterol secretion, the contribution of newly synthesized to total biliary cholesterol in animals studied at low and high periods of hepatic cholesterol synthesis averaged 3.0 and 5.4%, respectively. The question that might be then asked is how this small amount of newly synthesized cholesterol in bile can be reconciled with our finding that biliary cholesterol secretion is strongly correlated with the rate of hepatic cholesterol synthesis. Previous work has shown that HMG-CoA reductase is ordinarily restricted to just periportal hepatocytes that comprise ~ 20% of the total hepatocyte mass (8), that hepatic HMG-CoA reductase activity correlates with the number of cells that stain positive for HMG-CoA reductase (8), and that with an increase in hepatic cholesterol synthesis, the specific activity of cholesterol in bile remains constant (7). This suggests that all bile cholesterol may derive from just those periportal hepatocytes that ordinarily are HMG-CoA reductase positive. Variable amounts of newly synthesized cholesterol in bile (in different individual animals and at different ages) might reflect the presence of variable numbers of periportal cells, each, however, with the same specific activity of cholesterol. Thus, the specific activity of cholesterol in bile may be viewed as a "marker" of the

TABLE 4. Relationships of biliary lipids determined at mid-light and mid-dark periods of the diurnal cycle

Biliary Lipid Relationships	Mid-Light + Mid-Dark (n = 29)		Mid-Light (n = 14)		Mid-Dark (n = 15)	
	r	*P*	*r*	*P*	*r*	*P*
Bile salt vs. phospholipid	0.64	0.0002	0.83	0.0003	0.67	0.006
Phospholipid vs. cholesterol	0.67	0.0001	0.95	0.0001	0.53	0.038
Bile salt vs. cholesterol	0.35	0.066	0.73	0.003	0.49	0.063

Correlation coefficients (*r*) and probability (*P*) values are shown for linear regression lines fitted to the biliary lipid data in studies of animals at 4, 5, 7, and 9 weeks of age. Biliary secretion was measured at the mid-light phase (or at the low point of hepatic cholesterol synthesis) and at the mid-dark phase (or high point of cholesterol synthesis) (Methods). Values for all data points (both light-cycled groups combined) are shown in the first column.

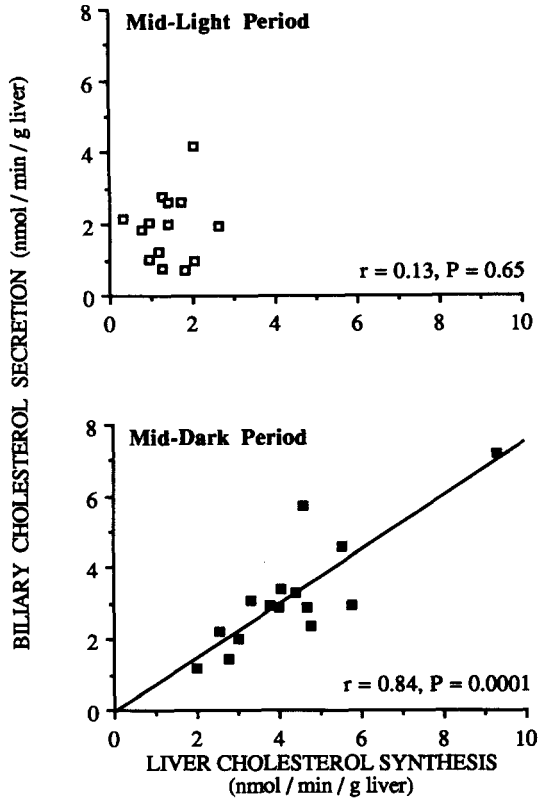

Fig. 3. Relationship of hepatic cholesterol synthesis to biliary cholesterol secretion in the mid-light and mid-dark periods of 12-h cycles. Values are for the same animals that are shown in Table 4, with studies conducted at 4, 5, 7, and 9 weeks of age. Synthesis was determined measuring [^{3}H]water incorporation into hepatic cholesterol, 30 min after injection of the isotope (Methods).

specific activity of cholesterol in the hepatic cell that is participating in biliary cholesterol secretion and the amount of newly synthesized and total cholesterol in bile may reflect the number of biliary cholesterol-secreting cells. It is clear that this concept will need to be verified by isolating periportal cells and demonstrating that the specific activity of cholesterol in these cells is the same as the specific activity of cholesterol in bile.

In conclusion, there are a great variety of metabolic changes that can influence biliary cholesterol secretion and there have been many previous studies in which animals have been manipulated to gain insight into the process by which cholesterol secretion is regulated. In the present study, we have sought to maintain animals in as normal a physiologic setting as possible and during a period of rapid growth have observed large changes in the interrelations of biliary lipids that were clearly affected by the changes that occur during the normal diurnal cycle. Most notably, our studies show that biliary cholesterol secretion is more highly variable than either bile salt or phospholipid secretion and that this variability is strongly linked to variability in hepatic cholesterol synthesis. Further studies will, however, be required to determine whether changes in hepatic cholesterol synthesis are responsible for the changes in biliary cholesterol secretion.

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