Origin of biliary cholesterol and lecithin in the rat: contribution of new synthesis and preformed hepatic stores

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Abstract The contribution of de novo synthesis to the secretion of cholesterol and lecithin in bile was assessed in isolated rat livers, perfused with a lipid-free medium. Cholesterol and lecithin synthesis were measured by the incorporation of tritiated water and [^{14}C]choline, respectively. Taurocholate stimulated the secretion of biliary lipids to the same extent in perfused livers and in live rats. During the first hour of perfusion, and when hepatic synthesis was active, newly synthesized cholesterol accounted for about 10% of biliary cholesterol and newly synthesized lecithin for 3% of biliary lecithin. Fasting reduced the contribution of newly synthesized cholesterol in bile to less than 1% but did not change the rate of biliary cholesterol secretion. After 2 hours of perfusion, newly synthesized biliary cholesterol accounted for only 4% of total hepatic sterol synthesis. Biliary lecithin, synthesized by choline incorporation accounted for only 7% of newly synthesized hepatic lecithin. We conclude that new synthesis makes only a small contribution to biliary cholesterol and lecithin secretion, and that, in the absence of perfusate lipids, both biliary cholesterol and lecithin must be predominantly mobilized from a preformed hepatic pool.—**Robins, S. J., and H. Brunengraber.** Origin of biliary cholesterol and lecithin in the rat: contribution of new synthesis and preformed hepatic stores. *J. Lipid Res.* 1982. **23:** 604–608.

Supplementary key words bile • cholesterol synthesis • lecithin synthesis • perfused livers • bile lipid secretion

The secretion of cholesterol in bile depends on the coordinated and simultaneous secretion of lecithin and bile salts. The mechanisms that regulate the secretion of bile salts are now well understood (1). Bile salts undergo an efficient enterohepatic circulation, and the rate of bile salt secretion is almost entirely dependent on the recycling frequency of preformed bile salt from a largely intact pool. In contrast, neither cholesterol, which is absorbed to a limited extent via an enterolymphatic circulation (2), nor lecithin, which is extensively hydrolyzed during absorption (3), is likely to undergo appreciable recirculation. Thus, biliary cholesterol and lecithin must derive from de novo synthesis in the liver, circulating lipoproteins, or a preformed hepatic pool. Attempts to date to quantitate the contribution made to biliary cholesterol secretion by specific processes have suggested, both in the rat (4–6) and in humans (7, 8) that preformed rather than newly synthesized cholesterol is predominantly secreted in bile. However, this conclusion is largely inferential, and based in humans (8) on a mathematical model using compartmental analysis, and in the rat (6) on a failure to change biliary cholesterol secretion by changing hepatic cholesterol synthesis, the hepatic esterified cholesterol storage pool, and dietary cholesterol (provided in the form of infused chylomicrons). At this time, there appears to be no data that, in quantitative terms, would indicate to what extent biliary lecithin is derived from a specific source.

We have undertaken the present study, using the isolated perfused rat liver, to directly measure the contribution of new synthesis to biliary cholesterol and lecithin secretion. We present evidence that, in the absence of perfusate lipids, the bulk of cholesterol and lecithin that are secreted in bile derive from a preformed hepatic pool.

MATERIALS AND METHODS

Tritiated water (5 Ci/g), [1,2-^{14}C]choline chloride (5.4 μCi/μmol), and internal counting standards of [^{3}H] and [^{14}C]toluene were purchased from New England Nuclear. Enzymes and coenzymes were supplied by Boehringer. Choline chloride was obtained from Sigma and sodium taurochlorate (A grade) from Calbiochem.

Perfusions

Male Sprague-Dawley rats (Charles River) were kept for 3 weeks in a controlled environment (24°C; 12 hr of light from 6 AM to 6 PM each day). The animals were fed Charles River chow from 9 AM to 12 noon (9), and were used for perfusions when they weighed 200–250 g. The perfusion apparatus and the surgical procedure were described previously (9). Livers were perfused with recirculating Krebs-Ringer bicarbonate buffer contain-

ing 4% dialyzed bovine serum albumin (fraction V, fatty acid poor; Miles Biochemicals) and glucose (25 mM in livers from fed rats and 4 mM in livers from 2-day-starved rats). The rate of perfusion was 50 ml/min. Sodium taurocholate was infused directly into the portal cannula at 38 μmol/hr. The rate of taurocholate infusion was selected to approximate the normal return of bile salts to the liver in the rat (10). After 30 min of perfusion, measurement of rates of lipid synthesis was initiated by *i*) adding 50 mCi of ^3H_2O to the perfusate reservoir and *ii*) adding 5 μmol of [1,2-^{14}C]choline chloride (10^4 dpm/μmol) to the perfusate reservoir and at the same time establishing a constant portal venous infusion of [1,2-^{14}C]choline of the same specific activity at 15 μmol/hr. The additions of choline resulted in a constant perfusate concentration of about 35 μM (11). After addition of isotopes, the perfusion was continued for 2 hr. Rates of secretion of total and newly synthesized biliary lipids were determined at 30-min intervals. The amount of newly synthesized total hepatic sterols and lecithin was measured at the conclusion of the perfusion, i.e., after 2 hr. Perfusions were performed at periods of active hepatic cholesterol synthesis (i.e., immediately after rats were schedule-fed (9)) and after 2 days of fasting to suppress synthesis.

In vivo experiments

The secretion of bile lipids was measured in restrained, unanesthetized rats. The bile salt pool was initially depleted by 15 hr of biliary drainage. Thereafter, sodium taurocholate was infused into the portal vein at a rate of 38 μmol/hr for 150 min. Throughout the experiment, the rats were infused intravenously with a solution containing 145 mM NaCl and 5 mM KCl at a rate of 1 ml/hr, but they were not fed.

Lipid analysis

Lipids from liver and bile were extracted by the method of Folch, Lees, and Sloane Stanley (12). Incorporation of label into digitonin-precipitable sterols was determined as described previously (9), except that the sterols were recrystallized from the digitonides (13) as recommended by Andersen and Dietschy (14). In brief, the calculation of cholesterol synthesis was performed as follows. Dpm of cholesterol in a sample of bile or liver were divided by the specific activity of tritium determined in perfusate ^3H_2O (as dpm/μmol) to obtain the μmol of tritium incorporated. This value was then converted to μmol of cholesterol synthesized, dividing by 21. The value of 21 represents the μg-atoms of tritium incorporated per μmol of cholesterol synthesized and is the product of the ratio of H/C incorporated into rat liver digitonin-precipitable sterols (determined as 0.76 (15)) and the number of C atoms incorporated into each newly synthesized cholesterol molecule (which is 27). Cholesterol was assayed with cholesterol oxidase in a Beckman Analyzer.

Lecithin was isolated by two-dimensional chromatography on silica gel (16) and eluted with chloroform–methanol 2:1. Synthesis of lecithin was calculated by the rate of choline incorporation on an equimolar basis. That is, the dpm incorporated into lecithin in bile or in liver were divided by the dpm/μmol of infusate choline to obtain the μmol of newly synthesized lecithin. In three cases, after perfusion, the specific activity of hepatic choline was determined by a radioenzymatic procedure (11). The values measured differed by less than 9% from the specific activity of infusate choline. Amounts of lecithin were determined by phosphorus analysis and total bile salts by an enzymatic method, as described previously (3). All radioactivity was measured by liquid scintillation counting in Liquifuor (New England Nuclear), and quench corrections made by the addition of internal standards.

RESULTS

The viability of the isolated perfused livers was assessed by the ratios [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] in the perfusate. These ratios reflect the oxido-reduction status of the cytosolic and mitochondrial NADH/NAD$^+$ pools, respectively. The ratios were in the normal range for livers from fed and starved rats (9).

In both live rats and perfused livers, the secretion of bile salt was constant, averaging 87 ± 3.4% (SEM; n = 5) and 92 ± 8% (n = 12) of the amount infused. **Table 1** shows that, in spite of the absence of lipids in the perfusate, the secretion of cholesterol in bile was the same in isolated livers as in live rats. Furthermore, the secretion of cholesterol in bile was not diminished even when cholesterol synthesis was decreased 15-fold by fasting. The secretion of lecithin in bile was also the same in live rats as in the perfused liver. Fasting did not result in a significant decrease in choline incorporation into lecithin in the perfused liver. As previously noted in live rats (6), fasting was accompanied by a small increase in biliary lecithin secretion (differences not significant).

As shown in **Fig. 1,** after a 30-min lag following the addition of isotopes to the perfusate, the specific activity of biliary cholesterol and lecithin increased linearly with time. Thus, new synthesis accounted for an increasing proportion of biliary cholesterol and lecithin during the course of these perfusions. However, as calculated during the linear phase of isotope incorporation, rates of synthesis were constant from one 30-min bile collection period to the next. In Table 1, rates of secretion and synthesis of biliary lipids are similarly expressed as μmol/

TABLE 1. Hepatic synthesis and biliary secretion of cholesterol and lecithin[a]

	Total Biliary Secretion		Biliary Secretion of Newly Synthesized Lipids[b]		Total Hepatic Synthesis[c]	
	Cholesterol	Lecithin	Cholesterol	Lecithin	Cholesterol	Lecithin
	μmol/hr		*μmol/hr*		*μmol/hr*	
Isolated livers						
From fed rats (n = 6)	0.504 ± 0.069	4.55 ± 0.51	0.048 ± 0.012	0.154 ± 0.019	1.29 ± 0.08	2.17 ± 0.12
From fasted rats (n = 6)	0.497 ± 0.055	5.45 ± 0.33	0.003 ± 0.001[d]	0.185 ± 0.008	0.07 ± 0.03[d]	1.75 ± 0.29
Live rats, fasted (n = 5)	0.478 ± 0.076	5.44 ± 0.47				

[a] All values are shown as mean ± SEM.
[b] Calculated during the linear period of isotope incorporation.
[c] Calculated as the sum of incorporation of isotopes in liver and bile lipids. Liver values were determined at 2 hr, but are shown as an hourly rate for ease of comparison with bile.
[d] Statistically different from corresponding value in livers from fed rats ($P < 0.01$).

Biliary secretion rates were compared in isolated livers and in live rats, infused with taurocholate via the portal vein. Rates of cholesterol synthesis were measured by the incorporation of tritium (specific activity 4.5×10^3 dpm/μmol) from ^3H_2O and rates of lecithin synthesis by the incorporation of (1,2-^{14}C)choline (specific activity 10^4 dpm/μmol). As described in Materials and Methods, μmol of newly synthesized cholesterol and lecithin were calculated from the dpm in a sample and the specific activity of precursor. For cholesterol, μmol newly synthesized = (dpm in sample) × $(4.5 \times 10^3$ dpm/μmol $H_2)^{-1} \times (0.76 \times 27)^{-1}$. For lecithin, μmol newly synthesized = (dpm in sample) × $(10^4$ dpm/μmol choline$)^{-1}$.

hr. Thus, from Table 1, one can readily determine the percentages of newly synthesized cholesterol and lecithin which are secreted in bile by dividing the μmol of biliary lipid that is newly synthesized by the total μmol of lipid secreted in bile. In a similar manner, one can readily determine the percentages of newly synthesized hepatic cholesterol and lecithin that is secreted in bile, by dividing the μmol of newly synthesized biliary lipid by the μmol of total newly synthesized lipid in the liver. In perfused livers from fed rats, newly synthesized cholesterol accounted for 9.5% of the cholesterol secreted in bile. In the same perfusions lecithin, which was newly synthesized via choline incorporation, accounted for 3.4% of biliary lecithin. In perfused livers from fasted rats, these percentages were 0.6% for cholesterol and, again, 3.4% for lecithin. When calculated at the end of perfusions, newly synthesized cholesterol and lecithin secreted in bile constituted small percentages of total hepatic sterols and lecithin that were newly synthesized. In fed rats, 3.7% of newly synthesized hepatic sterols and 7.4% of lecithin were secreted in bile after 2 hr. In fasted rats, 4.0% of newly synthesized hepatic sterols and 10.6% of lecithin were secreted.

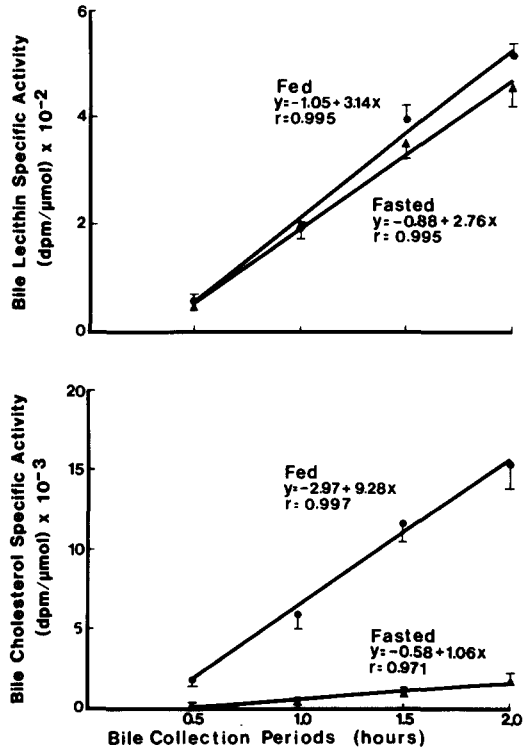

Fig. 1. Time course of incorporation of ^3H_2O into biliary cholesterol and of [1,2-^{14}C]choline into biliary lecithin by perfused livers from fed (○) and 2-day-fasted (△) rats. Isotopes were added to the perfusate at zero time. The data points represent mean ± SEM for six perfusions in each group. Regression lines were calculated by the method of least squares.

DISCUSSION

Using the isolated, perfused rat liver, we have directly determined the contribution made to biliary cholesterol and lecithin secretion by new synthesis during a 2-hr period. To our knowledge, this has not been previously accomplished, although there are several studies (4–8) which suggest by indirect measurement that the bulk of biliary lipids derive from preformed sources.

We have found in this system that newly synthesized cholesterol and lecithin constitute a relatively small proportion of total hepatic synthesis and, during the initial hour of perfusion, make only a small contribution to total biliary output. Our results further demonstrate that the

immediate secretion of both cholesterol and lecithin in bile in response to bile salt ordinarily does not require perfusate lipoproteins or any other extrahepatic source of lipids (such as red cell membrane lipids). Consequently, biliary cholesterol and lecithin must originate directly from some preformed hepatic pool(s) that can be rapidly mobilized. In this study, we have not identified the precise location of this pool, its size, or the mechanism by which pool repletion ordinarily takes place. However, our data (Table 1) show that hepatic synthesis can compensate for biliary cholesterol secretion only in livers from fed rats. Hepatic synthesis of lecithin is insufficient to compensate for biliary secretion in livers from either fed or fasted rats. Thus, it seems that for balance to be maintained, the biliary pool of lecithin and cholesterol must be eventually repleted by the uptake of exogenous lipids from the circulation.

Cholesterol synthesis was measured by the incorporation of tritium from ^3H_2O. Because ^3H_2O rapidly and completely equilibrates with cell water (17) and is incorporated in a definable proportion to carbon atoms, this isotope appears to be an ideal precursor to determine cholesterol synthesis in a variety of tissues (14). Rates of synthesis that we determined for liver are similar to those previously reported using ^3H_2O in vivo (17). In particular, the rate of sterol synthesis which we measured in perfused livers from schedule-fed rats (alternatively calculated as 13.0 ± 0.9 (SEM) μmol of ^3H_2O incorporated into digitonin-precipitable sterols/hr $\times$ whole liver) is not significantly different than the rate determined in ad libitum-fed live rats at the midpoint of the dark cycle, i.e., at the period of maximum diurnal synthesis (16.1 ± 4.2) (17). As a result of fasting, hepatic sterol synthesis was markedly reduced and the contribution of newly synthesized biliary cholesterol to total cholesterol secretion fell to less than 1%. However, as previously observed in live rats (6), this marked decrease in synthesis was not associated with a reduction in biliary cholesterol secretion.

In the present study, new lecithin synthesis was measured by the incorporation of choline into lecithin, using the specific activity of infusate choline as a measure of the specific activity of the hepatic precursor pool. We have previously demonstrated that during the course of a 2-hr perfusion, the rat liver produces no free choline (11). In present experiments, the specific activity of infusate choline was kept constant, and was found to closely approximate the specific activity of liver choline. Furthermore, although lecithin synthesis can also occur by sequential methylations of phosphatidylethanolamine, bile lecithin does not appear to be synthesized by this pathway. Bile lecithin is predominantly composed of palmitoyl-linoleyl and -oleyl lecithins (18) which are preferentially synthesized via the incorporation of free choline (19). We found that newly synthesized biliary lecithin constitutes only a small fraction of total hepatic lecithin synthesis. We further found that choline incorporation into lecithin is unchanged by diet and was comparable to our measurements in vivo (20) which were done using a wide range of choline concentrations.

The isolated perfused liver is only viable for a relatively limited period of study. Thus, it seems highly unlikely that it would be possible using this technique to deplete and directly calculate the size of a preformed biliary lipid pool. However, since newly synthesized cholesterol constitutes a linearly increasing fraction of the total amount of cholesterol secreted, it is possible to estimate, at least for perfusions of livers from fed animals, the size of a biliary cholesterol pool within the liver. In this calculation it must be presumed that *i*) the specific activity of cholesterol in bile and in its immediate hepatic precursor site are the same, and *ii*) that no shrinkage of the hepatic biliary pool will occur. This can only be the case if hepatic synthesis is in excess of the amount of cholesterol secreted into bile. In this case, input of newly synthesized cholesterol into this hepatic pool should exactly balance the output of cholesterol in bile. Using the values obtained in livers from fed animals, the following data can be derived: if, after the first hour of biliary secretion, 9.5% of the total cholesterol in bile is newly synthesized, then, after the second hour, 19% of the total cholesterol will be newly synthesized. Extrapolating this initial rate, we can calculate that at 10.5 hr, 100% of biliary cholesterol would be newly synthesized. In this 10.5-hr period, 5.3 μmol of cholesterol (10.5 hr $\times$ 0.504 μmol/hr) will be secreted in bile. This represents 15.6% of the total liver cholesterol mass (measured at the end of these perfusions as 33.9 ± 1.0 μmol).

This calculation cannot be as readily applied to estimate the size of a biliary lecithin pool. The molecular composition of lecithins in bile and in liver are markedly different (21). In addition, lecithins of different compositions are synthesized at different rates (19).

Finally, it should be recognized that our studies have been conducted with a single bile salt at a single rate of infusion. Biliary secretion of cholesterol and lecithin may vary depending on the specific bile salt administered (22). Indeed, differences in cholesterol secretion provide the basis for current trials of certain bile salts in studies of gallstone dissolution (23, 24). In future studies, using the technique of isolated liver perfusion, it may be possible to precisely define the influence of individual bile salts on biliary lipid secretion and to relate changes in secretion to changes in lipid synthesis.

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