Hepatic cholesterol synthesis and the secretion of newly synthesized cholesterol in bile

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To determine the effect of increased hepatic cholesterol synthesis on the secretion of newly synthesized cholesterol in bile, rats were fed with cholestyramine, a bile-acid-binding resin that increases the number of hepatocytes that synthesize cholesterol. Cholesterol synthesis was measured 15 min after [^3H]water injection to avoid appreciable exchange between the liver and serum of newly synthesized cholesterol that accumulates in the serum in studies of several hours duration. At 15 min after [^3H]water injection, the specific radioactivity of cholesterol in the liver and hepatic microsomes was greatly increased in resin-fed animals compared with controls. However, with resin, the specific radioactivity of newly synthesized cholesterol that was secreted in bile was the same as for controls. At 15 min after [^3H]water injection the specific radioactivity of serum cholesterol

was minimally increased and not different in resin and control groups. In contrast, in studies that were longer than 60 min, newly synthesized cholesterol in serum was appreciably increased in resin-fed animals, and newly synthesized cholesterol in bile was also greatly increased compared with controls. Thus, when appreciable cholesterol exchange is avoided, an increase in hepatic cholesterol synthesis and the number of hepatocytes that synthesized cholesterol does not result in an increase in newly synthesized cholesterol in bile. Our results suggest that newly synthesized cholesterol is secreted in bile from a fixed subpopulation of hepatocytes. From a comparison of the specific radioactivity of newly synthesized cholesterol in whole liver and bile, it can be estimated that this subpopulation of hepatocytes represents about 20 % of the total hepatocyte mass.

INTRODUCTION

The movement of cholesterol from the liver into bile is a complex and poorly understood process. Bile cholesterol is entirely free cholesterol (FC) and derives in part from hepatic preformed stores and from new hepatic synthesis. We have previously shown in the rat [1,2] that radiolabelled FC that is taken up from lipoproteins by the liver rapidly equilibrates throughout the liver with all other hepatic FC. Thus, bile preformed cholesterol has the same specific radioactivity as preformed cholesterol throughout the liver. In contrast, cholesterol that is newly synthesized in the liver has a much higher specific radioactivity in bile than in the liver [3–5]. This has led to the speculation that newly synthesized cholesterol in bile may derive from a special pool of cholesterol within the liver or, alternatively, that newly synthesized cholesterol is preferentially secreted from the liver into bile.

Attempts to identify a precursor site for newly synthesized bile cholesterol have thus far been unsuccessful, isolating from whole liver by conventional techniques intracellular organelles in which cholesterol synthesis takes place. However, it has been demonstrated that hepatic cholesterol synthesis may ordinarily only take place in a subpopulation of hepatocytes that are localized to the periportal region of the liver lobule [6,7]. Thus it is possible that newly synthesized cholesterol may be directly secreted into bile from just a portion of the total hepatocyte mass. As a consequence, newly synthesized cholesterol in bile would have a relatively high specific radioactivity compared with newly synthesized cholesterol in the liver that is mixed with cholesterol from hepatocytes that are not synthesizing cholesterol.

It has been demonstrated [6] that, when rats are treated with a bile-acid-binding resin to increase hepatic cholesterol synthesis,

the number of hepatocytes that synthesize cholesterol will also increase. In the present study we have treated rats with a bile-acid-binding resin to determine the effect of an increase in the number of hepatocytes that synthesize cholesterol on the relative specific radioactivity of newly synthesized cholesterol in the liver and bile. We reasoned that, if the specific radioactivity of newly synthesized cholesterol in liver more closely resembled bile as a result of increased hepatic synthesis, then the disparity in specific radioactivities between liver and bile could be attributed to the size of the hepatic site of cholesterol synthesis rather than to the preferential secretion of newly synthesized cholesterol from the liver into bile.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY, U.S.A.) were maintained in a 12 h-light-cycled room and fed *ad libitum* for 14–21 days on either rat chow (Ralston Purina Co., St. Louis, MO, U.S.A.) or chow that was mixed with the bile-acid-binding resin cholestyramine [3% (w/w), provided in Cholybar, from Warner–Lambert Co., Morris Plains, NJ, U.S.A.). At the mid-point of the dark cycle, rats were anaesthetized with Nembutal (50 mg/kg), operated on to cannulate the bile duct, and rapidly injected in the femoral vein with a bolus of [^3H]water (NEN Research Products, Boston, MA, U.S.A.). The amount of [^3H]water injected was ~ 120 mCi for short-term experiments and in the range ~ 30–50 mCi for longer experiments.

Short-term experiments were performed both to optimize a comparison of newly synthesized cholesterol between bile and liver samples and to minimize the consequences of transport of newly synthesized FC out of the liver (or other organs) into

blood and exchange back to the liver. In short-term experiments, newly synthesized cholesterol was measured in bile collected for just 5 min, from 10 to 15 min after [^3H]water injection. At 15 min rats were killed by exsanguination and livers were perfused free of blood, homogenized, and used to prepare microsomes [8].

Long-term experiments were performed to demonstrate that changes in the rate of secretion of newly synthesized FC in bile may depend not only on the rate of FC synthesis by the liver but also on the times (after the injection of a radiolabelled precursor) at which measurements of newly synthesized FC in bile are made. In these experiments, after injection of [^3H]water, newly synthesized cholesterol was measured in bile and serum at intervals for up to 120 or 240 min and in the liver that was obtained at the end of each experiment (half were concluded at 120 min and half concluded at 240 min). To minimize interruption of the entero-hepatic circulation, the bile-duct cannula was connected to a duodenal cannula and the connection was broken to obtain bile for 5 min periods. Serum was obtained at the mid-point of each bile collection from a femoral artery cannula.

Bile, serum, whole liver, and liver microsomes were extracted for lipids [9]. FC was isolated from cholesteryl esters and other neutral lipids by h.p.l.c., as we have previously described [10], and FC mass was determined by integration in conjunction with an internal stigmaterol standard. Bile acids were measured by an enzymic method [11] after purification on a Supelclean LC18 cartridge (Supelco, Bellefonte, PA, U.S.A.), by a previously described procedure [12]. The ^3H radioactivity in FC and in bile acids was determined by liquid-scintillation counting. The absolute amount of newly synthesized FC in bile was calculated as previously outlined [13], by using data showing that 21 H (measured as ^3H) atoms from water are incorporated into each newly synthesized molecule of cholesterol [14]. Serum [^3H]water has been shown to be fully equilibrated with tissue water by 5 min after intravenous injection of the radioisotope [15]. In the present experiments, the specific radioactivity of ^3H in serum water at 15 min (short-term experiments) and at 30 and 120 min (long-term experiments) was used as the precursor specific radioactivity to calculate newly synthesized cholesterol. (The specific radioactivities of [^3H]water at 30 and 120 min in individual animals differed by less than 3% and were averaged.) All results that were expressed in terms of specific radioactivity of newly synthesized FC (i.e. d.p.m./ μmol of FC) were normalized to account for variations in the amount of [^3H]water that was given to individual animals. Statistical differences between groups were determined by Student's *t* test for unpaired variables.

RESULTS

Newly synthesized cholesterol in bile in short-term experiments

Body weight and weight gain were not significantly different in resin-fed animals and in chow-fed controls (Table 1). However, resin-fed animals had smaller livers than controls and significantly lower rates of biliary cholesterol and bile salt secretion than controls (Table 1). In this group of animals, newly synthesized cholesterol was measured in bile from 10 to 15 min after the intravenous injection of [^3H]water, and in liver 15 min after the radioisotope had been injected.

The effects of resin feeding on the relative amounts of newly synthesized cholesterol in bile, whole liver and liver microsomes are shown in Table 2. In controls the specific radioactivity of [^3H]FC in bile was about 4 times that in the whole liver and in liver microsomes. In contrast, in resin-fed animals, although the specific radioactivity of [^3H]FC in bile was

the same as in controls, the specific radioactivity of [^3H]FC was markedly increased in whole liver and even more so in liver microsomes. The absolute amount of newly synthesized cholesterol that was secreted in bile was 7.2 ± 2.9 nmol/h per g of liver in controls and 5.1 ± 2.3 nmol/h per g of liver in resin-fed animals. The percentage of cholesterol in bile that was newly synthesized was precisely the same in both groups of animals, 7.0 ± 2.1 (S.D.)% in controls and 7.0 ± 1.3 % in animals fed resin.

In these animals, at 15 min after injection of [^3H]water, the specific radioactivity of [^3H]FC in serum was not significantly different in resin-fed and control groups and was relatively low compared with liver, averaging just 14.1 ± 3.5 (S.D.)% of the radioactivity in liver (for control and resin-fed groups combined).

Newly synthesized cholesterol in bile in more prolonged experiments

In another group of resin-fed and control animals the specific radioactivity of [^3H]FC was determined in bile, serum and (whole) liver for up to 240 min after the administration of [^3H]water. Time-dependent changes in the specific radioactivity of [^3H]FC in bile are shown in Figure 1(a) and in serum and liver in Figure 1(b). The specific radioactivity of [^3H]FC in bile increased in a curvilinear manner. In sharp contrast with a comparison at 15 min (above), from 120 to 240 min after [^3H]water injection the specific radioactivity of [^3H]FC in bile was significantly higher in resin-fed animals than in controls ($P < 0.03$). The specific radioactivity of [^3H]FC in serum increased more linearly than in bile and was significantly higher in

Table 1 Animal weights and biliary lipid secretion

Data are shown as means $\pm$ S.D. Weights are final weights. The number of rats in each group is shown in parentheses. Rats were fed on either rat chow (Control) or rat chow to which was added 3% cholestyramine (Resin-fed). Rats were maintained in a 12 h-light-cycled environment, and after 14–21 days were operated on at the mid-point of the dark phase to collect bile from 10–15 min after [^3H]water injection (see the Materials and methods section). Abbreviation: NS, not significant.

Group	Body wt. (g)	Liver wt. (g)	Wt. gained (g/day)	Bile cholesterol (nmol/h per g of liver)	Bile bile salts (nmol/h per g of liver)
Control (9)	290 $\pm$ 21	12.0 $\pm$ 1.1	8.1 $\pm$ 1.7	102 $\pm$ 25	2763 $\pm$ 626
Resin-fed (8)	276 $\pm$ 20	10.8 $\pm$ 1.3	7.5 $\pm$ 1.3	70 $\pm$ 20	1675 $\pm$ 300
<i>P</i>	NS	0.03	NS	0.006	0.003

Table 2 Specific radioactivities of free cholesterol in bile, liver and hepatic microsomes

Data are means $\pm$ S.D. for rats fed on a chow diet alone or a chow diet that contained 3% cholestyramine (Resin-fed). The specific radioactivities of [^3H]FC in bile, whole liver and liver microsomes are shown after the administration of [^3H]water. Bile was collected from 10–15 min and liver was obtained 15 min after [^3H]water injection (see the Materials and methods section).

Group	Sp. radioactivity (d.p.m./ μmol)		
	Bile	Liver	Microsomes
Control (9)	18305 $\pm$ 3425	3535 $\pm$ 501	3960 $\pm$ 660
Resin-fed (8)	18318 $\pm$ 5461	9733 $\pm$ 1780	12466 $\pm$ 2358
<i>P</i>	NS	< 0.0001	< 0.0001

Figure 1 Changes in the specific radioactivity of biliary FC (a) and serum and liver FC (b) after intravenous injection of [³H]water

Data are means $\pm$ S.E.M. for rats fed on a chow diet alone (Control; $n = 4$) or a chow diet that contained 3% cholestyramine (Resin; $n = 5$). Bile was collected for 5 min periods by interrupting a biliary–duodenal cannula. Serum was obtained at the mid-point of each bile collection. Liver was obtained for analysis after either 120 min (in two control and three resin-fed animals) or 240 min (in two control and two resin-fed animals). Differences between Control and Resin groups were significant as follows. For bile: at 120 min ($P < 0.03$), 180 min ($P < 0.02$) and 240 min ($P < 0.03$). For serum: at 30 and 240 min ($P < 0.01$); at 45 and 60 min ($P < 0.001$); at 120 min ($P < 0.0001$). For liver: at 120 min ($P < 0.002$); at 240 min ($P < 0.02$).

Figure 2 Changes in the specific radioactivity of biliary bile salts after intravenous injection of [³H]water in the groups of animals shown in Figure 1

Data are means $\pm$ S.E.M. for rats fed on a chow diet alone (Control; $n = 4$) or a chow diet that contained 3% cholestyramine (Resin; $n = 5$). At all time points differences between Control and Resin groups were significant (as shown below): at 15, 180 and 240 min ($P < 0.02$); at 30 min ($P < 0.0003$); at 45, 60 and 120 min ($P < 0.0001$).

resin-fed animals than in controls at 30 min after [³H]water injection ($P < 0.01$) and at all time points thereafter ($P < 0.01$ – 0.001). At 120 and 240 min after [³H]water injection,

the specific radioactivity of serum [³H]FC ranged from 70 to 88 % of the specific radioactivity of [³H]FC in the liver.

Biliary secretion of newly synthesized bile salts

As a result of resin feeding and a decrease in bile salt secretion (Table 1), the synthesis of bile salts was significantly increased (Figure 2). In contrast with the secretion of newly synthesized FC in bile, the secretion of newly synthesized bile salts was 3–4-fold greater in resin-fed animals than in controls at every time period at which bile was sampled ($P < 0.02$ – 0.001).

DISCUSSION

Results demonstrate that the large difference in specific radioactivity of newly synthesized cholesterol in bile and liver can be decreased to a considerable extent by stimulating hepatic cholesterol synthesis with a bile-acid binding resin. Treatment of rats with a resin has been shown to increase the number of liver cells that synthesize cholesterol [6]. We found that with resin treatment hepatic cholesterol synthesis was markedly increased, but was not accompanied by increased secretion of newly synthesized cholesterol in bile. It can thus be inferred that the specific radioactivity of newly synthesized cholesterol in bile is greater than in the liver, not because there is 'preferential' secretion of newly synthesized cholesterol in bile, but because newly synthesized cholesterol is secreted in bile from just a portion of the total hepatocyte mass. That is, although all hepatocytes may have the potential to synthesize cholesterol, only a select population of these cells will provide newly synthesized cholesterol for bile.

Our findings with regard to the secretion of newly synthesized cholesterol in bile are strikingly different from the results of others, who have found that resin treatment not only increases hepatic cholesterol synthesis but also increases the secretion of newly synthesized cholesterol in bile [4,16]. We believe that the finding of an increase in the specific radioactivity of bile cholesterol with resin treatment does not truly reflect an increase in newly synthesized bile cholesterol and can most probably be attributed to FC exchange between the liver and blood during the course of an experiment that takes place over a relatively prolonged period. It is possible for FC exchange to confound the results of a study in the following manner: when a prolonged time (of the order of several hours) is allowed to elapse after the administration of a radioisotopic precursor of cholesterol, appreciable amounts of newly synthesized FC are transported from the liver into the blood and result in an increasing specific radioactivity of serum FC. When this radiolabelled FC exchanges back into the liver, it has the specific radioactivity of serum FC. Thus, in reality, in this type of experiment the source of newly synthesized FC in bile may be viewed as not just the newly synthesized FC that is directly derived from its site of synthesis in the liver, but also the newly synthesized FC (with a different specific radioactivity) that is derived from the serum. Our previous studies [1] suggested that because of the rapidity of serum FC exchange (with a $t_{1/2}$ of ~ 4 min) and transport into bile, in studies of many hours duration the specific radioactivity of bile cholesterol will always closely correspond to the specific radioactivity of FC in the serum. Our present study demonstrates that, although by 4 h after administration of [³H]water the specific radioactivity of bile FC still clearly exceeded the specific radioactivity of FC in serum (cf. Figures 1a and 1b), the specific radioactivity of bile FC had reached a plateau, whereas the specific radioactivity of serum (and liver) FC was linearly increasing. Furthermore, the specific radioactivity of

serum FC in the resin-fed group was increasing in a considerably greater rate than in controls.

In the present study, we avoided the problem of cholesterol exchange by measuring bile cholesterol specific radioactivities very early after the administration of a cholesterol precursor (10–15 min after administration of [^3H]water) and before any significant increase in newly synthesized cholesterol in serum. It is possible to measure cholesterol synthesis this early after [^3H]water administration, since [^3H]water has been found to be fully equilibrated with the water in a variety of body tissues by 5 min after its intravenous injection [15]. We found that newly synthesized cholesterol accounted for just 7.0% of the cholesterol secreted in bile (calculated for 1 h of bile output) in both controls and resin-fed rats. We have previously reported [13] that 9.5% bile cholesterol was newly synthesized in studies using the isolated perfused rat liver, in a serum-free system in which exchange is negligible.

It is not possible to calculate the absolute rate of synthesis of bile salts from the incorporation of ^3H atoms from water without knowing the extent to which newly synthesized and preformed cholesterol in the liver serve as substrates for bile salt synthesis (i.e. ^3H labelling). However, it is clear from a comparison of bile-salt specific radioactivities (Figure 2) that the biliary secretion of newly synthesized bile salts in resin-fed animals greatly exceeded that of controls at every time point and, in contrast with cholesterol, the difference in synthesis between the two groups remained relatively constant over the 4 h of study (the ratio of d.p.m./ μmol of bile salt in controls to that in resin-fed animals ranged from 0.23 to 0.30 throughout the period of study).

With immunofluorescence microscopy, it has been observed [6] that about 20% of liver cells in untreated rats stain positive for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis. This estimate of the percentage of liver cells that are actively synthesizing cholesterol closely corresponds to the (percentage) difference between liver and bile specific radioactivities of newly synthesized cholesterol that we observed in the control group of rats. That is, in controls the specific radioactivity of cholesterol in the liver was 20% of the specific radioactivity of cholesterol in bile (Table 2). This would be expected if only 20% of hepatocytes synthesize cholesterol for bile and the specific radioactivity of newly synthesized cholesterol in the liver is decreased by the (preformed) cholesterol in the remaining 80% of hepatocytes that are not synthesizing cholesterol.

The population of hepatocytes that ordinarily is positive for HMG-CoA reductase is sharply localized to the periportal region of the liver [6,7]. With the induction of cholesterol synthesis by drug treatment (with a resin, a HMG-CoA reductase inhibitor, or both in combination), many more liver cells, which are widely distributed throughout the liver, stain positive for HMG-CoA reductase [6,7]. If the assumption is made that the amount of cholesterol synthesized by each 'synthesizing' liver cell is the same, then it is possible that all of bile cholesterol originates from a subpopulation of hepatocytes that constitutes about 20% of the total hepatocyte mass. This would explain why the specific radioactivity of FC in liver is ordinarily about 20% of the specific radioactivity of FC in the bile and why, with an increase in liver cholesterol synthesis, there is no increase in the specific radioactivity of newly synthesized cholesterol in bile.

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