Contribution of newly synthesized cholesterol to rat plasma and bile determined by mass isotopomer distribution analysis: bile-salt flux promotes secretion of newly synthesized cholesterol into bile

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To quantify the contribution of newly synthesized cholesterol to total plasma and biliary cholesterol under physiological conditions, unrestrained rats were infused intravenously with [1-^{13}C]acetate (0.6 mmol/h per kg) from 12:00 to 24:00 h, and fractional and absolute cholesterol-synthesis rates were determined by mass isotopomer distribution analysis (MIDA). As bile diversion leads to changes in cholesterol metabolism, rats were equipped with permanent catheters in the bile duct and duodenum, allowing sampling of small amounts of bile from an intact enterohepatic circulation. For comparison, rats with chronic bile diversion were also studied. Fractional synthesis of plasma cholesterol was $10.8 \pm 1.7\,\%$ (mean $\pm$ S.D.) after 12 h in rats with intact circulation. Fractional synthesis of biliary cholesterol was significantly higher than that of plasma cholesterol, i.e. $16.5 \pm 2.0\,\%$ ($P < 0.05$) after 12 h. In contrast, no differences between fractional synthesis of cholesterol in plasma and bile were found in bile-diverted animals (31.8 ± 2.1 and $33.1 \pm 3.3\,\%$ respectively after 12 h). The calculated absolute rate of cholesterol biosynthesis increased from 53 ± 10 to 221 ± 19 μmol/day per kg after bile diversion. A comparison of MIDA results with those obtained from balance studies indicated that MIDA does not assess total body synthesis in rats, presumably because of incomplete equilibration of newly synthesized molecules with cholesterol in the plasma compartment. These studies demonstrate that the contribution of newly synthesized cholesterol to biliary cholesterol is higher than to plasma cholesterol under physiological conditions, probably reflecting bile-salt-induced secretion of newly formed cholesterol by the periportal hepatocytes.

INTRODUCTION

Our understanding of the molecular events involved in the regulation of cholesterol biosynthesis has greatly expanded in the past few years, as the result of the isolation and purification of key regulatory proteins and the characterization of their genes. Much less information is available on the quantitative aspects of the process *in vivo*, in particular in humans, and on the relative importance of newly synthesized cholesterol compared with preexisting cholesterol for the various metabolic routes. Over the past few years, mass isotopomer distribution analysis (MIDA), a stable isotope procedure for measurement of synthesis and turnover of biological polymers including cholesterol, has been introduced [1,2]. This procedure allows calculation of the isotopic enrichment of precursor acetyl-CoA subunits that have entered newly synthesized cholesterol during administration of [1-^{13}C]acetate and subsequent calculation of fractional and absolute synthesis rates [2].

The biliary pathway constitutes the major route for removal of cholesterol from the body, either unchanged or after conversion into bile salts. Regulation of biliary cholesterol secretion is still incompletely understood and the relative contribution of preformed (lipoprotein-derived) and newly synthesized cholesterol to biliary cholesterol under different conditions remains speculative. Quantification of the fraction of newly synthesized cholesterol in bile has previously been performed by using radiolabelled precursors, mainly ^3H_2O, in a number of studies [3–7], in particular in rats. These studies yielded values ranging from 8 to 18 % newly synthesized cholesterol in bile. A fundamental problem encountered in these studies concerns the fact that the (prolonged) collection of bile needed for performing the measurements requires interruption of the enterohepatic circulation of bile salts. This sampling procedure, in itself, affects hepatic cholesterol synthesis and the process of biliary cholesterol secretion, because both processes are, in part, controlled by bile salts [8,9]. Interruption of their enterohepatic circulation leads to up-regulation of hepatic cholesterol synthesis and reduction of biliary cholesterol secretion. Furthermore, use of anaesthetized or restrained rats may affect the outcome of these studies, as both anaesthesia and the stress of restraint have been shown to interfere with cholesterol metabolism [10,11]. To circumvent these influences, we have made use of a rat model developed in our laboratory that allows collection of small-volume bile samples from an intact enterohepatic circulation in unrestrained freely moving animals with normal food intake [9,12]. In the present study, we have used MIDA to compare the contribution of *de novo* synthesis to plasma cholesterol and biliary cholesterol in rats under normal physiological conditions. For comparison, studies were also performed in rats with prolonged (> 8 days) bile diversion in which cholesterol and bile salt biosynthesis is

Abbreviations used: MIDA, mass isotopomer distribution analysis; GC/MS, gas chromatography/mass spectrometry; HMG, 3-hydroxy-3-methylglutaryl.

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expected to be maximally up-regulated and bile-salt flux across the liver is absent [8,9]. By comparing MIDA data with balance measurements (synthesis equals output minus intake) we also determined the part of total body cholesterol synthesis that is actually in equilibrium with plasma cholesterol and therefore is accessible for MIDA application in rats.

MATERIALS AND METHODS

Materials

[1-^{13}C]Acetate, sodium salt (99 %) was obtained from Isotec (Miamisburg, OH, U.S.A.), and reagents for derivatization from Pierce (Rockford, IL, U.S.A.). Solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Animals

Male Wistar rats (290–320 g) were maintained in a light- and temperature- (21 °C) controlled room (lights on 06:00 to 18:00 h) and fed on normal rat chow *ad libitum* (RMH-B; Hope Farms BV, Woerden, The Netherlands). The fat and cholesterol contents of this chow were 6.2 and 0.01 % (w/w) respectively. Control animals used for the stable-isotope-infusion experiment ($n = 7$) were equipped with permanent catheters in the bile duct and duodenum. The two catheters were connected to each other during the operation session to maintain an intact enterohepatic circulation. During the same session, animals were provided with a double heart catheter via the left jugular vein, one for repeated blood sampling and a second thinner one for continuous infusion. Details of the experimental model can be found in refs. [9] and [12]. In another group of rats the enterohepatic circulation was interrupted 1 day after surgery, with the same surgical procedure as the control rats, and the bile duct catheter was connected to a fraction collector. These rats were used for stable isotope experiments ($n = 5$) after 8 days, i.e. after regaining preoperative body weights and normal food intake. A second set of control and bile-diverted rats (both groups $n = 5$) were used for sterol balance experiments. Experimental procedures were approved by the Ethics Committee for Animal Experiments of the State University Groningen.

Experimental procedures

On the day of the experiment, the thinner heart catheter was connected to an infusion pump for continuous infusion of [1-^{13}C]acetate at a rate of approx. 0.6 mmol/h per kg body weight from 12:00 to 24:00 h. Blood samples of 0.25 ml were taken before and 6 and 12 h after the start of the infusion (incorporation phase), and subsequently at 1, 2, 3, 6, 12, 24, 36 and 48 h after termination of the infusion (decay phase). Small-volume bile samples (100 μl) were taken from rats with intact enterohepatic circulation during short-term (< 5 min) disconnection of bile duct and duodenal catheters before and at 6 and 12 h after the start of the infusion. Bile from rats with interrupted circulation was collected continuously in 1 h fractions by means of a fraction collector. Bile production was determined gravimetrically. Faeces were collected in 24 h samples from rats with intact enterohepatic circulation and from rats after 8 days of bile diversion.

Lipid analysis

Concentrations of bile salts and cholesterol in bile, plasma and faeces were determined as described previously [13,14]. Cholesterol and bile-salt secretion into bile was calculated by multiplying bile flow by cholesterol and bile-salt concentration in bile. Cholesterol for gas chromatography/mass spectrometry (GC/MS) analysis was extracted from 100 μl of heparin plasma with 2 ml of 95 % ethanol/acetone (1:1, v/v) and from 10–50 μl of bile. Free cholesterol was subsequently derivatized using *N*,*O*-bis-(trimethyl)trifluoroacetamide with 1 % trimethylchlorosilane at room temperature. Samples were dried under nitrogen and dissolved in 500 μl of hexane.

GC/MS analysis

Samples were analysed on a magnetic sector mass spectrometer (70-250S; VG, Manchester, U.K.), equipped with a Chrompack CP-Sil 19 column (Middelburg, The Netherlands), using splitless injection. Oven temperature increased from 120 to 260 °C at 20 °C/min, from 260 to 280 °C at 2.5 °C/min and finally from 280 to 300 °C at 20 °C/min. Ions at m/z 368 to 372 were measured under selected ion recording.

Calculations

The MIDA method is described in detail in the original articles by Neese and Hellerstein [1,2]. MIDA makes it possible to determine the enrichment of the acetyl-CoA precursor units that enter newly synthesized cholesterol molecules during the [^{13}C]acetate infusion period, the so-called precursor pool enrichment. This is achieved by analysing the mass isotopomer pattern of the cholesterol molecules according to a combinatorial probability model of cholesterogenesis [1]. After determination of precursor pool enrichment, using two different isotopomers (M1 and M3), it is possible to calculate the relative amount of the individual isotopomers in newly synthesized cholesterol. Comparing this value with the isotopomer enrichment of cholesterol during the experiment reveals the fraction of newly synthesized cholesterol. After cessation of label incorporation, the rate constant of decay is determined by analysing enrichments of the higher isotopomers to exclude the chance of continuing label incorporation [2]. It then becomes possible to calculate absolute cholesterol-synthesis rates, using an estimate of body free cholesterol pool size [1]. This 'rapidly miscible' pool supposedly includes liver, intestine and plasma. The following non-steady-state equation is used: $K = k \times f \times \text{pool size}/(1-e^{-kt})$, where K (μmol/h) is the absolute rate of cholesterol synthesis, f (in %) is the fraction of newly synthesized cholesterol molecules and k (h^{-1}) is the rate constant of isotopic cholesterol removal from plasma.

Statistical analysis

All values are expressed as means $\pm$ S.D. Differences between groups were analysed using Student's t tests for paired or unpaired data.

RESULTS

The fractional synthesis of plasma cholesterol in control rats, representing the physiological situation, was 4.3 ± 2.4 and 10.8 ± 1.7 % after 6 and 12 h of [1-^{13}C]acetate infusion respectively. Fractional synthesis of plasma cholesterol was greatly increased by interruption of the enterohepatic circulation, i.e. 21.5 ± 2.9 and 31.8 ± 2.1 % at 6 and 12 h respectively. Analysis of decay curves of higher-mass isotopomers ($M+2$, $M+3$) after termination of the infusion, as shown in Figure 1 for a rat with interrupted enterohepatic circulation, yielded values for the decay rate constants of 0.05 ± 0.01 and 0.09 ± 0.01 h^{-1} in intact and bile-diverted animals respectively. Since no major differences in plasma and hepatic cholesterol content were observed between control and long-term bile-diverted rats in a previous study [8],

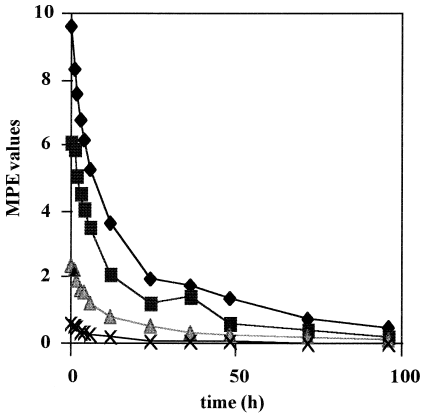

Figure 1 Decay of cholesterol isotopomers in plasma of a representative rat with interrupted enterohepatic circulation

[1-^{13}C]Acetate was infused from 12:00 to 24:00 h at a rate of 0.6 mmol/h. After cessation of [1-^{13}C]acetate administration, blood samples were collected at the indicated time points for determination of molar percentage excess (MPE) values of EM1 (◆), EM2 [■], EM3 [▲] and EM4 (×). Values of M2 and M3 were used for calculations of the rate constant of decay as described by Neese et al. [2].

Table 1 Diurnal rhythm in cholesterol synthesis rates in control and bile-diverted rats

Cholesterol synthesis rates were determined at the end of the light period, representing daytime, and at the middle of the dark period, representing night-time, during infusion of [1-^{13}C]acetate as described in the Materials and methods section. Values in μmol/h per kg represent the mean ± S.D. in seven control and five bile-diverted rats. *Significantly ($P < 0.05$) different from synthesis rates during daytime; †significantly ($P < 0.05$) different from synthesis in control rats.

	Cholesterol synthesis (μmol/h per kg)	
	Daytime	Night-time
Control	0.48 ± 0.28	0.82 ± 0.12*
Bile-diverted	2.98 ± 0.74†	2.63 ± 0.40†

we used an estimate of 130 mg/kg body weight for the total body cholesterol pool in both situations, as proposed by Neese and Hellerstein [1]. By doing so, we were able to calculate a total cholesterol synthesis rate of 53 ± 10 μmol/day per kg for control animals and 221 ± 19 μmol/day per kg in the bile-diverted animals. When absolute synthesis rates were calculated separately for day (12:00–18:00 h) and night (18:00–24:00 h), the absolute cholesterol synthesis in intact animals was almost twice as high during the night as during the day (Table 1), consistent with the often reported day/night rhythm of hydroxymethylglutaryl-(HMG)-CoA reductase activity in rat liver [15]. In contrast, no difference between day- and night-time synthesis was observed in the rats with interrupted enterohepatic circulation.

To be able to relate our data obtained with MIDA to whole-body sterol balance, we measured dietary intake and faecal neutral and acidic sterol excretion in control and bile-diverted rats. Dietary intake was small (of the order of 5 μmol/day per kg). Bile diversion surprisingly increased the total excretion of neutral sterols into the faeces from 157 to 348 μmol/day per kg. Faecal acidic sterol loss was 65 μmol/day per kg in control rats. Diversion of bile resulted in a direct loss of 26 μmol/day per kg cholesterol and 1070 μmol/day per kg acidic sterols (i.e. bile salts) from the body.

Table 2 Fractional cholesterol synthesis (%) in plasma and bile of control and bile-diverted rats

Fractions of newly synthesized cholesterol were determined at 6 and 12 h during infusion of [1-^{13}C]acetate as described in the Materials and methods section. Values represent the mean ± S.D. in seven control and five bile-diverted rats. *Significantly ($P < 0.05$) different from plasma fractional synthesis values; †significantly ($P < 0.05$) different from fractional synthesis in control rats measured in the same compartment.

		Fractional cholesterol synthesis (%)	
	Time (h)	Plasma	Bile
Control	6	4.34 ± 2.36	7.39 ± 2.23*
	12	10.75 ± 1.74	16.48 ± 1.97*
Bile-diverted	6	21.48 ± 2.86†	18.86 ± 3.79†
	12	31.81 ± 2.07†	33.06 ± 3.33†

In the intact situation, fractional synthesis of biliary cholesterol was significantly higher than that of plasma cholesterol (Table 2); this difference was not observed in the bile-diverted animals. No differences in precursor pool enrichments between plasma and biliary cholesterol were found in intact and bile-diverted animals (Table 3). Precursor pool enrichment values were calculated using two different isotopomers (M1 and M3), with similar results. At 6 h after the start of the infusion, however, control rats showed a small but significant difference with the 12 h time point as well as with the 6 and 12 h points of the bile-diverted rats. These differences are in all probability not biologically relevant and are presumably due to a greater variability in the results of the 6 h samples of the control rats, caused by the relatively small amount of newly synthesized cholesterol molecules present in these samples.

DISCUSSION

In this study we have used MIDA to quantify biosynthesis of cholesterol and to investigate the contribution of *de novo* synthesis to biliary cholesterol in rats under various conditions. In the past, cholesterol synthesis, derived from the isotopomer pattern of plasma cholesterol, was proposed to be similar to whole-body synthesis [1]. Cholesterol synthesis was 53 μmol/day per kg body weight in chow-fed rats, a value comparable with that found in rats by Neese et al. [1] using MIDA (23 μmol/day per kg). These values are substantially lower than those from our sterol-balance study, which gave an average value of 217 μmol/day per kg. The values are also lower than those reported by Spady and Dietschy [16] for chow-fed rats, making use of the tritiated water approach (259 μmol/day per kg), in which cholesterol synthesis is measured in all contributing tissues individually. Earlier studies, using balance methodology, yielded values of the order of 200 μmol/day per kg [17,18]. Evidently MIDA only measures cholesterol synthesis that contributes to the rapidly exchangeable cholesterol pool including plasma. Synthesized cholesterol that does not enter this pool during the time of the experiment is not measured with MIDA and thus leads to an underestimation of MIDA result. For instance, intestinal cholesterol synthesis has been found to contribute very significantly to total cholesterol synthesis in rats [19,20], whereas Turley et al. [20] showed that cholesterol synthesis of small bowel contributes very little to the blood compartment in rats over 1 h after administration of ^3H_2O. If this is also true for more prolonged periods of time, this

Table 3 Precursor pool enrichments (values of P) of control and bile-diverted rats

Precursor pool enrichments were determined at 6 and 12 h during infusion of [1-^{13}C]acetate as described in the Materials and methods section. Results were tested for significant differences between plasma and bile, P(EM1) and P(EM3), control and bile-diverted and $t = 6$ h and $t = 12$ h. *Significantly ($P < 0.05$) different from values of P of control rats at 6 h after start of infusion.

	Plasma		Bile	
	P(EM1)	P(EM3)	P(EM1)	P(EM3)
Control				
$t = 6$ h ($n = 7$)	0.063 ± 0.012	0.062 ± 0.013	0.064 ± 0.013	0.065 ± 0.010
$t = 12$ h ($n = 7$)	0.062 ± 0.003	0.064 ± 0.002	0.059 ± 0.008	0.061 ± 0.005*
Bile-diverted				
$t = 6$ h ($n = 5$)	0.055 ± 0.011	0.057 ± 0.011	0.056 ± 0.010	0.056 ± 0.010*
$t = 12$ h ($n = 5$)	0.057 ± 0.006	0.061 ± 0.007	0.057 ± 0.010	0.059 ± 0.009*

would lead to a significant underestimation of whole-body cholesterol synthesis measured with MIDA. Another factor contributing to the discrepancies between MIDA and the other two methods is that the cholesterol pool used for bile-salt synthesis in the liver may not be in equilibrium with the pool used for excretion of cholesterol into plasma and bile. If cholesterol used for bile-salt syntheses is transformed directly into bile salts at the endoplasmic reticulum, thereby by-passing the rapidly exchangeable intracellular cholesterol pool that equilibrates with plasma and bile, the results would also underestimate total cholesterol synthesis. Evidence for this possibility comes from the observation that the fraction of bile salts made from newly synthesized cholesterol is considerably higher than the fraction of newly synthesized biliary cholesterol [5]. Recently, we have obtained evidence, by the MIDA approach, supporting this possibility [21]. Finally, the estimate of the free cholesterol pool size, which is derived from a value calculated for humans [1], may lead to underestimation of absolute cholesterogenesis. In humans on the other hand, cholesterogenesis measured with MIDA has been found to be in the range reported using sterol-balance techniques [1,22,23]. The key difference between rats and humans in this respect probably lies in the relatively low cholesterogenesis in humans, which have a much higher cholesterol intake, making the body less dependent on *de novo* synthesis. In addition, bile-salt synthesis in humans is about 20 μmol/day per kg [24] and thus at least an order of magnitude lower than in rats [9]. A direct comparison between MIDA and sterol balance is being carried out in our laboratory.

The difference between cholesterogenesis measured with MIDA and sterol balance was even more pronounced in bile-diverted animals. Long-term bile diversion resulted in an increase in cholesterogenesis determined by MIDA from 53 to 221 μmol/day per kg, in line with the well-established up-regulation of HMG-CoA reductase gene expression and enzyme activity [8,16,25,26] during interruption of the enterohepatic circulation of bile salts. The increase in enzyme activity during feeding of bile-salt-binding resins [27] and complete bile diversion [9] is typically of the order of 6–8 times when compared with the control situation.

Faecal neutral-sterol-output measurements surprisingly showed an increased loss of neutral sterols in the bile-diverted rats, i.e. 348 μmol/day per kg compared with 157 μmol/day per kg in control rats. Several studies have shown a substantial increase in intestinal HMG-CoA-reductase activity after cholestyramine treatment in rats [28,29]. Increased neutral-sterol output during bile diversion thus presumably represents desquamation of intestinal cells with high cholesterol biosynthesis. Bile diversion leads to an additional loss of biliary cholesterol from the body and greatly increased conversion of cholesterol into bile salts. In addition to the increased faecal sterol loss, depressed bile-salt synthesis may also lead to an even larger proportion of newly synthesized cholesterol that does not contribute to the plasma cholesterol pool in the diverted animals. It is well established that increases in HMG-CoA reductase activity are parallelled by increases in cholesterol 7α-hydroxylase activity during bile diversion [8,30]. In addition, it is known from studies with radiolabelled precursors that the fraction of bile salts derived from *de novo* synthesized cholesterol may increase to 60–70 % during prolonged bile diversion [5,21], which is twofold higher than the fraction of newly synthesized biliary cholesterol.

One of the advantages of the MIDA technique is that it enables measurement of short-term changes in synthesis rates, allowing us to examine the effect of bile diversion on the day/night rhythm of cholesterol synthesis. As expected, synthesis rates were higher during the night than during the day in control animals, probably reflecting the day/night cycle in HMG-CoA reductase activity [15]. Such a diurnal rhythm was not present, however, in the bile-diverted rats. The most likely explanation is that cholesterol synthesis is already maximally up-regulated during the daytime in bile-diverted rats, and that no further up-regulation at night can take place in these animals because of limited capacity of the synthetic cascade.

Fractional synthesis values of biliary cholesterol were significantly higher than those of plasma cholesterol in rats with an intact enterohepatic circulation. Similar results were recently reported for humans using MIDA [31]. A number of studies, using radioactive procedures, have reported a higher specific radioactivity of newly synthesized cholesterol in bile compared with liver [3,4,7]. Turley and Dietschy [3] hypothesized that this is due to subcellular compartmentalization with preferential channelling of newly synthesized cholesterol into bile. Using a tritiated water method, these authors found the specific radioactivity of free cholesterol to be higher in bile than in liver and plasma. An alternative hypothesis was proposed by Robins et al. [7] based on the reported localization of HMG-CoA reductase in the liver. Normally only 20 % of the hepatocytes, located in the periportal region, contain HMG-CoA reductase protein [27,32]. Thus Robins et al. [7] proposed that newly synthesized cholesterol is secreted into bile from this subpopulation of hepatocytes. They found a five times higher specific radioactivity of biliary cholesterol than liver or plasma cholesterol. If hepatocytes from the periportal subpopulation are indeed mainly involved in secretion of newly synthesized cholesterol into the bile, this could account for the plasma/bile difference observed in our and earlier studies.

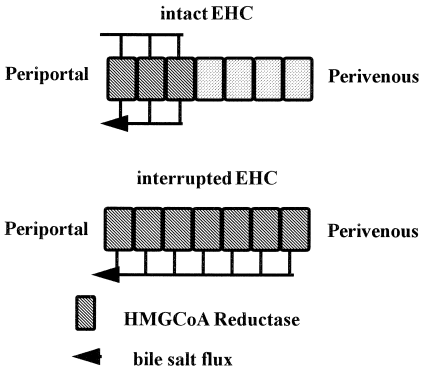

Figure 2 Model for cholesterol and bile-salt synthesis and secretion in the liver in control and bile-diverted rats

In this model bile-salt flux through the cholesterol-synthesizing hepatocytes is proposed to stimulate secretion of newly synthesized cholesterol into bile in the physiological situation only. EHC, enterohepatic circulation.

We observed that during chronic interruption of the enterohepatic circulation the fraction of newly synthesized cholesterol became similar for plasma and bile. Robins et al. [4] found that after cholestyramine treatment, specific radioactivity of biliary cholesterol was still higher than that of liver and plasma cholesterol. Cholestyramine is not as efficient as bile diversion in removing bile salts from the enterohepatic circulation [33] and thus may not affect cholesterol synthesis as strongly as complete bile diversion. In addition, transhepatic bile-salt flux is still present during cholestyramine feeding [33]. During up-regulation of cholesterol synthesis, more hepatocytes apart from those located around the periportal area show HMG-CoA reductase activity [32]. If all hepatocytes participate in cholesterol synthesis during maximal up-regulation, in our case through bile diversion, no dilution of newly synthesized cholesterol in the liver by preformed cholesterol from the non-synthesizing hepatocytes would occur. This could give rise to an equal relative amount of newly synthesized cholesterol in the plasma, liver and biliary compartment. We found similar precursor pool enrichments in plasma and bile in both experimental groups. The lack of a plasma/bile difference in general for precursor pool enrichments in both groups provides additional support for the hypothesis proposed by Robins et al. [7] and does not support the theory of the existence of different pools of newly synthesized cholesterol for secretion into bile and plasma.

With this information available we propose a role for bile salts in the regulation of the fraction of newly synthesized cholesterol into bile, as shown in Figure 2. In the physiological situation, the flux of bile salts goes through the hepatocytes of the periportal region, as shown by the arrows. This flux stimulates the secretion of newly synthesized cholesterol into bile specifically in this region. This phenomenon gives rise to the higher fraction of newly synthesized cholesterol found in bile. The disappearance of the bile-salt flux, by bile-diversion, triggers all hepatocytes to participate in cholesterol synthesis as well as its secretion into plasma and bile. In this situation, cholesterol synthesis measured in plasma and bile must, by definition, be identical.

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