Evidence for a common biliary cholesterol and VLDL cholesterol precursor pool in rat liver[1]

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Abstract Hepatic free cholesterol levels are influenced by cholesterol synthesis and ester formation, which, in turn, might regulate cholesterol secretion into bile and plasma. We manipulated the rates of hepatic cholesterol synthesis and esterification and measured biliary and very low density lipoprotein (VLDL) cholesterol secretion, and bile acid synthesis. Mevalonate decreased HMG CoA reductase by 80%, increased acyl coenzyme A: cholesterol acyltransferase (ACAT) by 60% and increased [^{3}H]oleate incorporation into microsomal and VLDL cholesteryl esters by 174% and 122%, respectively. Microsomal and biliary free cholesterol remained constant at the expense of increased microsomal and VLDL cholesteryl ester content. Mevalonate did not change bile acid synthesis. 25-OH cholesterol decreased HMG-CoA reductase by 39%, increased ACAT by 24%, but did not effect 7α-hydroxylase. 25-OH cholesterol increased [^{3}H]oleate in microsomal and VLDL cholesterol esters by 71% and 120%. Biliary cholesterol decreased by 40% and VLDL cholesteryl esters increased by 83%. A small and unsustained decrease in bile acid synthesis ($^{14}CO_2$ release) occurred after 25-OH cholesterol. After orotic acid feeding, HMG-CoA reductase increased 352%, and [^{3}H]oleate in microsomal and VLDL cholesteryl esters decreased by 43% and 89%. Orotic acid decreased all VLDL components including free cholesterol (68%) and cholesteryl esters (55%), and increased biliary cholesterol by 160%. No change in bile acid synthesis occurred. Hepatic cholesterol synthesis and esterification appear to regulate a cholesterol pool available for both biliary and VLDL secretion. Changing cholesterol synthesis and esterification did not alter bile acid synthesis, suggesting that either this common bile/VLDL secretory pool is functionally distinct from the cholesterol pool used for bile salt synthesis, or that free cholesterol availability in this precursor pool is not a major determinant of bile acid synthesis.—**Stone, B. G., and C. D. Evans.** Evidence for a common biliary cholesterol and VLDL cholesterol precursor pool in rat liver. *J. Lipid Res.* 1992. **33:** 1665–1675.

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In humans and animals, the amount of cholesterol derived from de novo synthesis and dietary intake greatly exceeds the metabolic needs of the body, so that a mechanism for loss of cholesterol from the body must function to maintain cholesterol balance (1). In mammals, this is accomplished mainly through hepatic cholesterol secretion into the biliary system either as free cholesterol or after conversion to bile acids. In the liver, cholesterol exists both in the unesterified (free) form and as a fatty acid ester with the majority being free cholesterol.

The content of free cholesterol in the liver is the net result of all inputs and outputs. Input of free cholesterol to the liver is derived both from lipoprotein uptake and new cholesterol synthesis catalyzed by the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. While tissue culture studies have demonstrated the coordinate regulation of cholesterol synthesis and low density lipoprotein (LDL) receptor uptake, the primary response of the liver to changes in cholesterol availability in vivo is through the feedback regulation of new sterol synthesis (2–5).

The level of free cholesterol in the liver is also a function of the rate of cholesteryl ester formation. Hepatic cholesterol esterification is catalyzed by the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) and the newly formed cholesteryl esters are initially contained in the microsomal fraction (6–9). Prior work by several groups, including ours, has demonstrated that the level of ACAT activity is regulated by free cholesterol flux (6–10). In turn, the change in ACAT activity returns hepatic free cholesterol content toward normal, thus serving to maintain free cholesterol content within a very tight range in the cell.

Free cholesterol availability might also regulate the secretion of cholesterol from the liver into either bile or plasma. Hepatic cholesterol secretion into plasma occurs principally through the secretion of free cholesterol and cholesteryl esters contained in very low density lipopro-

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; HMG, 3-hydroxy-3-methylglutaryl; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HPLC, high performance liquid chromatography.

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teins (VLDL) (11, 12). Nascent VLDL cholesteryl esters are derived from the intrahepatic esterification of free cholesterol catalyzed by the ACAT reaction, and in tissue culture the rate of cholesteryl ester formation by ACAT is a determinant of the amount of VLDL cholesterol secreted into the media (13). However, as suggested above, caution must be used in extrapolating tissue culture studies to the whole animal with an intact biliary system.

The rate of biliary cholesterol secretion is determined both by the specie and rate of bile acid secreted, and biliary phospholipid secretion (14–17). Since only free cholesterol is secreted in bile, we have proposed and presented data consistent with the concept that the intrahepatic availability of free cholesterol in a biliary precursor pool is also a determinant of biliary cholesterol secretion (18). Moreover, these studies demonstrated that intrahepatic cholesterol synthesis and esterification rates regulate this pool and therefore biliary cholesterol secretion. Other work has also demonstrated a role for hepatic ACAT activity in regulating the size of a biliary cholesterol precursor pool (19). However, the relationship of cholesterol synthesis and esterification to VLDL cholesterol secretion was not determined in either of these studies.

Finally, free cholesterol can be converted to bile salts with the rate-limiting step of bile salt synthesis catalyzed by the enzyme cholesterol 7α-hydroxylase. Bile acid synthesis appears to be under feedback regulation by bile acids returning to the liver (20). In addition, there is both evidence for and disputing the claim that bile salt synthesis in the rat is regulated by free cholesterol availability in some precursor pool (20–24). In turn, the conversion of free cholesterol to bile acids is a potential means of eliminating cholesterol from the body and thus maintaining cholesterol homeostasis.

Although the level of free cholesterol in a hepatic precursor pool could potentially determine biliary cholesterol and VLDL cholesterol (directly and through increasing cholesteryl ester formation) and bile acid synthesis, the evidence for this is inconsistent and incomplete. While the metabolic processes that potentially maintain hepatic free cholesterol levels relatively constant have been described, the time sequence and relative importance of the various responses to changes in hepatic free cholesterol have not been completely elucidated. Therefore, in this series of experiments we acutely regulated hepatic free cholesterol availability and simultaneously measured the various outputs of free cholesterol in vivo and in the isolated perfused liver. In addition, we manipulated the rate of cholesterol synthesis and cholesterol esterification and quantitated changes in biliary cholesterol secretion, VLDL cholesterol secretion, and bile acid synthesis to determine if the cholesterol available for secretion by these routes is contained within a common pool of free cholesterol that is regulated by the rate of cholesterol synthesis and esterification.

METHODS

Materials, animals, and agent administration

3-Hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (40–60 mCi/mmol), [26-^{14}C]cholesterol (59 mCi/mmol), [4-^{14}C]cholesterol, [1,2,6,7-^{3}H]cholesteryl oleate (80–90 Ci/mmol), [1-^{14}C]oleoyl coenzyme A (60 mCi/mmol), [9,10-^{3}H]oleic acid (9 Ci/mmol), and [5-^{3}H]mevalonolactone (13.8 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [3,4-^{13}C]Cholesterol was obtained from Cambridge Isotope Laboratories (Woburn, MA). 5-Cholesten-3β,25-diol (25-OH cholesterol) and 5-cholesten-3β, 7α-diol (7α-hydroxycholesterol) were from Steraloids (Wilton, NH). Essentially fatty acid-free albumin, orotic acid, mevalonolactone, and taurocholate were from Sigma (St. Louis, MO). Earle's balanced salt solution was from GIBCO (Grand Island, NY).

Male Sprague-Dawley rats (Biolab, St. Paul, MN) were housed under normal lighting conditions and, in all but the orotic acid feeding experiments, were fed a standard pelleted diet. Mevalonate (1 g/ml) was administered as the lactone form and each rat received 200 mg by intragastric (i.g.) gavage under light ether anesthesia. Control rats were administered 0.2 ml of normal saline in an identical fashion. 25-OH cholesterol (20 mg dissolved in 1 ml ethanol) was administered either intravenously (i.v.) or added to the perfusate of the liver perfusion at a dose of 6.25 mg/kg body wt. In these experiments the control group received ethanol (0.3125 ml/kg body wt). To administer orotic acid, controls received a ground diet and the orotic acid-fed group received the identical ground diet supplemented with 2 g % orotic acid. Age- and weight-matched animals were used for all experiments and the animals weighed between 230 and 350 g at the time of use.

Microsomal preparation and enzyme measurements

After the stated times, the animals were killed and microsomes were prepared as previously described (18). One half of the microsomal pellet was resuspended in 0.25 M sucrose, 1 mM EDTA and assayed for HMG-CoA reductase and ACAT activities as previously described (18). The remaining microsomal pellet was resuspended and washed in sodium pyrophosphate buffer (0.1 M) containing 1 mM EDTA and then suspended 0.1 M K_2PO_4 buffer containing 10 mM β-mercaptoethylamine for the measurement of 7α-hydroxylase activity. Microsomal protein (1 mg) was preincubated with 2 mM $NADP^+$, and 20 mM glucose-6-phosphate for 5 min at 37°C after which 1 unit of glucose-6-phosphate dehydrogenase was added. The reaction mixture was incubated for 15 min at 37°C and stopped by the addition of choloroform–methanol 2:1. [^{13}C]Cholesterol (5 μg) was added to assess recovery and the sterols were extracted into chloroform by the method of Folch, Lees, and Sloane Stanley (25). The chlo-

roform was evaporated under a N_2 stream and the trimethylsilyl ether derivatives of the extracted sterols were formed by the addition of 50 μl of Tri-Sil (Pierce, Rockford, IL). Blanks consisted of the complete incubation mixture without added glucose-6-phosphate dehydrogenase. The mass of 7α-hydroxycholesterol was determined by gas chromatography–mass spectroscopy using selected ion monitoring, comparing the relative signal intensity to known standards, and correcting for the amount of 7α-hydroxycholesterol in the blanks (26). The 7α-hydroxycholesterol measurements were made by the University of Minnesota Research Core Center for the Study of Advanced Liver Disease. The rate of 7-hydroxycholesterol formation was linear for 30 min and up to 2 mg of microsomal protein (data not shown).

VLDL secretion and characterization

Nascent VLDL secretion was determined in the isolated perfused liver as we have previously described (27). It should be noted that in the isolated perfused liver reuptake of approximately one fourth of the secreted VLDL triglycerides occurs over a 3-h perfusion period (28). It is not known whether the re-uptake of other VLDL components occurs after initial secretion but the possibility exists. Therefore, the values given for VLDL secretion represent the net accumulation of the various VLDL components in the perfusion.

After bile duct cannulation, the donor liver was perfused in situ with perfusate consisting of 20–25% sheep red blood cells in Earle's balanced salt solution supplemented with 3 g/dl of fatty acid-free bovine serum albumin. The perfusate was collected from the hepatic vein, reoxygenated with 95% O_2/5% CO_2, and recirculated. To maintain bile flow, taurocholate coupled to fatty acid-free albumin was infused directly into the portal vein at the physiologic rate of 120 μmol/kg per h. The rate of triglyceride and cholesteryl ester synthesis in hepatic microsomes and secreted VLDL was quantitated by the initial addition to the perfusate and subsequent incorporation over the perfusion period of [^{3}H]oleate (4.6 μCi/nmol, 7.6 pmol/kg) into these lipids. At the end of the perfusion VLDL was harvested from the recirculating perfusate by ultracentrifugation. The donor liver was flushed with iced saline (100 ml) and homogenized, and microsomes were prepared as described above.

Measurement of bile acid synthesis in vivo

In addition to the 7α-hydroxylase activity, a second in vivo measure of the rate of bile acid synthesis was used. Rats were injected with [26-^{14}C]cholesterol (5 μCi, i.p.) and after a 5-day equilibration period, bile acid synthesis was determined by the timed release of expired $^{14}CO_2$ from [26-^{14}C]cholesterol (29). In order to quantitatively trap the expired $^{14}CO_2$, the animal was placed in a Plexiglass box equipped with a water seal and air was pulled from the box through a series of traps containing phenethylamine–methanol–toluene–Liquiflor 27:27:41:5. The rate of $^{14}CO_2$ released was determined by counting the radioactivity contained in a aliquot from each scintillant trap over a 10-min timed collection. The bile acid synthesis rate was calculated as the rate of cholesterol converted to bile salts using the specific activity of biliary [26-^{14}C]cholesterol obtained by bile duct cannulation at the end of the experiment. The bile cholesterol specific activity was chosen for calculating bile acid synthesis because the precursor pool of cholesterol utilized for bile acid synthesis and for biliary cholesterol appear to be in near equilibrium (30, 31). In a separate selected group of animals, the microsomal and/or biliary cholesterol specific activity was determined 5 days after the administration of [4-^{14}C]cholesterol (5 μCi). The animal was anesthetized, an acute bile fistula was placed, and a bile sample and small lobe of liver were obtained before agent administration. Then either 25-OH cholesterol or mevalonate was administered and 15-min bile collections were obtained for a 2- to 3-h period. The animal was killed and an additional liver sample was harvested. Microsomes were prepared by the standard method, and the specific activity of the bile or microsomal cholesterol was determined by a mass measurement and liquid scintillation counting of the [^{14}C]cholesterol separated by HPLC (10).

Sample analysis

Free cholesterol and cholesteryl ester contents in microsomes and VLDL were measured by HPLC (10). The incorporation of [^{3}H]oleate into triglycerides and cholesteryl esters was determined by liquid scintillation counting of the individual lipid fractions after HPLC separation (32). VLDL triglyceride, phospholipid, and protein contents were measured as previously described (27). Bile cholesterol was routinely measured by gas chromatography, bile salts were quantitated by the 3α-hydroxysteroid method, and biliary lecithin was measured by the choline oxidase method, all as previously described (27).

Statistical analysis

Where possible, each animal served as its own control and in these circumstances a paired t-test was used to assess significance. In some experiments groups were compared by Student's t-test using a $P < 0.05$ as significant.

RESULTS

Effect of mevalonate on hepatic cholesterol metabolism, biliary cholesterol secretion, and VLDL cholesterol secretion

To effect an increase in hepatic cholesterol availability derived from new synthesis, we administered the cholesterol precursor mevalonate (200 mg i.g.). At 1 h, mevalonate

TABLE 1. Effect of mevalonate administration on HMG-CoA reductase, ACAT, 7α-hydroxylase activity, and microsomal cholesterol content

Variable	Mevalonate	Control
HMG-CoA reductase activity		
(pmol mevalonate/mg/min)	16 ± 3[a]	80 ± 7
ACAT activity		
(pmol cholesterol ester/mg/min)	181 ± 17[a]	113 ± 6
7α-Hydroxylase activity		
(pmol 7α-hydroxycholesterol/mg/min)	35 ± 4	30 ± 3
Microsomal free cholesterol		
(μg cholesterol/mg protein)		
Post 1 h	22.91 ± 0.51	21.72 ± 0.51
Post 2 h	23.82 ± 0.60	23.32 ± 0.67
Microsomal cholesteryl esters		
(μg cholesterol/mg protein)		
Post 1 h	1.48 ± 0.10	1.41 ± 0.07
Post 2 h	2.88 ± 0.27[b]	1.97 ± 0.29

Rats were gavaged with mevalonate (200 mg in the lactone form) and microsomes were prepared as described in the Methods. HMG-CoA reductase, ACAT, and 7α-hydroxylase activities were measured 1 h after gavage and the microsomal cholesterol (free and ester) contents were determined both 1 and 2 h after mevalonate administration. The values are expressed as the mean ± SE of eight determinations in each group.

[a]Different from control, $P < 0.01$.

[b]Different from control, $P < 0.05$.

administration resulted in a decreased HMG-CoA reductase activity and increased ACAT activity (**Table 1**). The increased ACAT activity resulted in the accumulation of cholesteryl esters in microsomes over the next 2 h without changing free cholesterol content. In agreement with the measured changes in microsomal ACAT activity, there was a significant increase in [^{3}H]oleate incorporation into microsomal and VLDL cholesteryl esters in the perfused liver (**Table 2**). VLDL cholesteryl ester secretion increased with mevalonate administration without changing the secretion of the other VLDL components (**Table 3**). Over the same 2-h perfusion period there was no significant change in biliary free cholesterol secretion (Table 2). By the end of the perfusion there was no significant difference in hepatic free cholesterol (2.6 ± 0.1 mg/g liver vs. 2.4 ± 0.2 mg/g liver, control) or cholesteryl ester content (0.11 ± 0.02 mg/g liver vs. 0.09 ± 0.02 mg/g liver, control) between the mevalonate-treated and control livers.

Effect of mevalonate administration bile acid synthesis

Another potential fate of hepatic free cholesterol is the conversion to bile salts. With mevalonate administration, there was no significant change in bile acid synthesis as measured by 7α-hydroxylase activity (Table 1). For the 3 h after mevalonate administration, bile acid synthesis, as measured by $^{14}CO_2$ release from [26-^{14}C]cholesterol, averaged 98 ± 6% (mean ± SEM, n = 3) of the pre-administration value. As the cholesterol derived from mevalonate would not be radiolabeled, a change in bile acid synthesis by the release of $^{14}CO_2$ from [26-^{14}C]cholesterol might be missed, as the radiolabeled precursor pool is diluted by unlabeled newly synthesized cholesterol. However, dilution of the radiolabeled pool by unlabeled free cholesterol should be reflected in a decrease in the bile free cholesterol specific activity. To test for this, we collected a bile sample promptly after cannulation of the bile ducts of two rats administered [4-^{14}C]cholesterol 5 days earlier to prelabel the hepatic free cholesterol pool. We compared the cholesterol specific activity of these bile samples (13.71 and 11.32 dpm/μg cholesterol) to that measured in bile collected every 15 min for a 2-h period after mevalonate administration. There was no change in

TABLE 2. Effect of mevalonate on [^{3}H]oleate incorporation into lipids and biliary lipid secretion in the isolated perfused liver

Variable	Mevalonate	Control
Microsomes (dpm/mg/h)		
Cholesteryl esters	175 ± 26[a]	66 ± 8
Triglycerides	2723 ± 252[b]	3736 ± 289
VLDL (× 10^3 dpm/g liver/h)		
Cholesteryl esters	10.0 ± 0.9[a]	4.5 ± 0.7
Triglycerides	296 ± 42	361 ± 36
Biliary lipids (μmol/kg body wt/h)		
Bile salt	94 ± 4	103 ± 5
Cholesterol	1.3 ± 0.1	1.5 ± 0.1
Phospholipid	9.1 ± 0.6	10.1 ± 0.5

Rats were gavaged with 200 mg of mevalonate in the lactone form. After 1 h the livers were harvested and perfused in situ with recirculating perfusate supplemented with [^{3}H]oleate (7.6 pmol/kg body wt, 4.6 μCi/nmol) and a constant infusion of bile salt directly into the portal vein as described in Methods. After a 2-h perfusion period the liver was flushed with iced saline and homogenized, and microsomes were prepared. The perfusate was harvested and the newly secreted VLDL was isolated by ultracentrifugation. The amount of [^{3}H]oleate incorporated into microsomal and VLDL lipids was quantitated by HPLC. The values are the mean ± SEM of nine perfusions in each group.

[a]Different from control, $P < 0.01$.

[b]Different from control, $P < 0.05$.

TABLE 3. Effect of mevalonate on VLDL secretion and composition in the isolated perfused liver

Content	Output		Composition	
	Mevalonate	Control	Mevalonate	Control
	μg/g liver/h		*% by wt*	
Triglycerides	398 ± 64	480 ± 56	66.6 ± 1.4	69.8 ± 2.0
Free cholesterol	16 ± 3	16 ± 2	2.7 ± 0.2	2.2 ± 0.2
Cholesteryl ester	9 ± 1[a]	5 ± 1	1.6 ± 0.2[b]	0.7 ± 0.1
Phospholipid	91 ± 15	108 ± 22	15.3 ± 1.0	14.8 ± 1.4
Protein	82 ± 12	88 ± 13	13.9 ± 0.4	12.5 ± 0.7

One hour after the administration of mevalonate, donor livers were perfused in situ with Earle's balanced salt solution containing 20–25% red blood cells for 2 h. At the end of the perfusion period, the perfusate was collected and the red blood cells were sedimented by low speed centrifugation. VLDL was then isolated from the perfusate by ultracentrifugation and analyzed for lipid and protein content as described in the Methods. The values are expressed as the mean ± SEM of nine determinations in each group.

[a]Different from control, $P < 0.05$.

[b]Different from control, $P < 0.01$.

biliary free cholesterol specific activity (14.6 ± 0.4 and 12.2 ± 0.6, mean ± SEM of eight determinations in each of two rats) over a 2-h period after mevalonate compared to the bile cholesterol specific activity before administration. We also examined the microsomal free cholesterol specific activity before and after mevalonate administration as the cholesterol used for bile acid synthesis is thought to be derived from a pool of microsomal free cholesterol (22, 33). The specific activity of the microsomal free cholesterol after mevalonate (5.6 and 6.0 dpm/μg cholesterol) in each rat was virtually the same as that before its administration (6.2 and 5.8 dpm/μg cholesterol).

Together these results demonstrate that an acute increase in hepatic free cholesterol is promptly counteracted both by feedback inhibition of HMG-CoA reductase and an increase in cholesteryl ester formation and VLDL cholesteryl ester secretion. In turn, these changes serve to maintain constant hepatic free cholesterol content, biliary cholesterol secretion, and bile acid synthesis.

Effect of 25-OH cholesterol administration on hepatic cholesterol metabolism, biliary cholesterol secretion, and VLDL cholesterol secretion

Based on previous work, we have proposed the existence of a precursor pool of biliary cholesterol that is regulated by the rate of cholesterol ester formation and whose size is one determinant of biliary cholesterol secretion (18). The above data and our previous work suggest that biliary cholesterol and VLDL cholesteryl ester might be derived from a common precursor pool of cholesterol. In this series of experiments we administered 25-OH cholesterol to stimulate cholesterol esterification and perturb this pool of cholesterol to better define the relationship between the cholesterol secreted in bile and that secreted in VLDL.

At 1 hr 25-OH cholesterol (6.25 mg/kg body wt i.v.) decreased HMG-CoA reductase activity and increased ACAT activity compared to the vehicle control (**Table 4**).

TABLE 4. Effect of 25-OH cholesterol on HMG-CoA reductase, ACAT, and 7α-hydroxylase activities and microsomal cholesterol content

Variable	25-OH Cholesterol	Control
HMG-CoA reductase activity (pmol mevalonate/mg/min)	86 ± 9[a]	142 ± 26
ACAT activity (pmol cholesteryl ester/mg/min)	207 ± 12[a]	167 ± 13
7α-Hydroxylase activity (pmol 7α-hydroxycholesterol/mg/min)	30 ± 2	36 ± 5
Microsomal free cholesterol (μg/mg protein)	22.9 ± 1.0	21.6 ± 0.6
Microsomal cholesteryl ester (μg/mg protein)	1.3 ± 0.1	1.5 ± 0.3

One hour after the intravenous administration of 25-OH cholesterol (6.25 mg/kg dissolved in ethanol) or the ethanol vehicle control (0.3125 ml/kg body wt), rats were killed, microsomes were prepared, and the above measurements were determined as described in Methods. The values are the mean ± SEM of eight determinations in each group.

[a]Different from control, $P < 0.01$.

In agreement with the increased microsomal ACAT activity, 25-OH cholesterol added to the perfusate of the isolated perfused liver stimulated [^{3}H]oleate incorporation into microsomal and VLDL cholesteryl esters (**Table 5**). No difference in [^{3}H]oleate incorporation into VLDL or microsomal triglycerides occurred, demonstrating that the effect of 25-OH cholesterol was specific for fatty acid incorporation into newly formed cholesteryl esters and therefore mediated by the increased ACAT activity. Biliary cholesterol secretion decreased in the second 1-h bile collection after addition of 25-OH cholesterol to the perfusate, compared to both the ethanol vehicle and the 0.5-h perfusion period before administration of the agent (**Fig. 1**). No change in bile salt or biliary phospholipid secretion was appreciated (data not shown).

25-OH cholesterol stimulated VLDL cholesteryl ester secretion in the isolated perfused liver (**Table 6**). No other VLDL components were affected, and the percent of VLDL that was composed of cholesteryl esters more than doubled. The increase in VLDL cholesterol (approximately 0.43 μmol/kg body weight per h) almost exactly balanced the observed decrease in biliary cholesterol secretion (0.48 μmol/kg body weight per h, Fig. 1). There was no difference in the specific activity of the VLDL cholesteryl esters between the 25-OH cholesterol (4.7 ± 0.3 dpm ^{3}H/μmol cholesteryl ester secreted) and control perfusions (3.7 ± 0.9 dpms ^{3}H/μmol cholesteryl ester secreted, n = 7), suggesting that newly formed cholesteryl esters are not preferentially secreted in VLDL. These results demonstrate the reciprocal relationship between the secretion rate of free cholesterol into bile and cholesteryl esters in VLDL. Furthermore, biliary cholesterol and VLDL cholesteryl ester appear to be derived from a common intrahepatic pool that is regulated by ACAT activity.

TABLE 5. Effect of 25-OH cholesterol on [^{3}H]oleate incorporation into lipids in the isolated perfused liver

Content	25-OH Cholesterol	Control
Microsomes (dpm/mg/h)		
Cholesteryl esters	168 ± 14[a]	98 ± 21
Triglycerides	774 ± 130	715 ± 178
VLDL (× 10^3 dpm/g liver/h)		
Cholesteryl esters	15.2 ± 0.6[b]	6.9 ± 1.6
Triglycerides	423 ± 67	577 ± 130

In these studies donor livers were harvested and perfused in situ as described in Methods. After an initial 0.5 h, 25-OH cholesterol or the ethanol vehicle and [^{3}H]oleate (7.6 pmol/kg body weight, 4.6 μCi/nmol) were added to the perfusate and allowed to recirculate for an additional 2 h. At the end of the perfusion period, the liver was flushed with iced saline and homogenized, and microsomes were prepared. The perfusate was harvested and the newly secreted VLDL were isolated by ultracentrifugation. The amount of [^{3}H]oleate incorporated into microsomal and VLDL lipids was quantitated by HPLC. The values are the mean ± SEM of seven perfusions in each group.

[a]Different from control, $P < 0.02$.

[b]Different from control, $P < 0.001$.

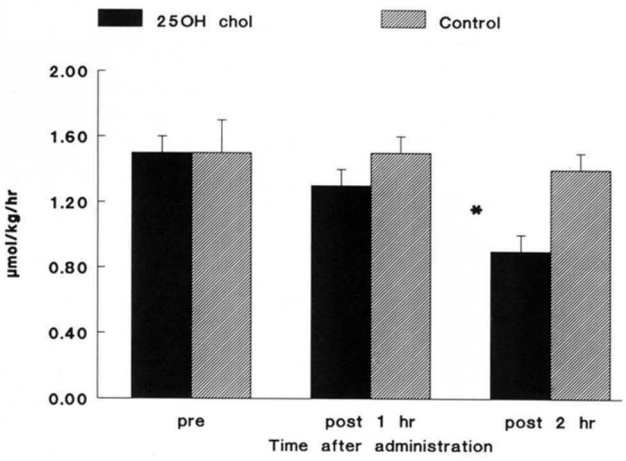

Fig. 1. Effect of 25-OH cholesterol on biliary cholesterol secretion in the isolated perfused liver. Donor livers were perfused in situ with constant bile salt replacement as described in Methods. After a 0.5-h bile collection (pre), either 25-OH cholesterol (6.25 mg/kg body wt, solid bars) or the ethanol vehicle (0.3125 ml/kg body wt, hatched bars) was added to the recirculating perfusate and hourly bile samples were collected for 2 additional hours. A statistically significant decrease in biliary cholesterol secretion occurred in the second 1-h bile collection (post 2 h) after 25-OH cholesterol compared to both the ethanol vehicle (post 2 h) and the preadministration value (pre). No significant change in bile salt or phospholipid secretion occurred over the same period (data not shown). The values are expressed as μmol/kg body weight per h and represent the mean ± SEM of 10 determinations in each group. *Different from pre and ethanol control, $P < 0.05$.

Effect of 25-OH cholesterol on bile acid synthesis

There was no difference in microsomal 7α-hydroxylase activity in rats treated with 25-OH cholesterol and control rats receiving the vehicle alone, either 0.5 h (data not shown) or 1 h after administration (Table 4). However, a small but significant decrease in bile acid synthesis as measured by $^{14}CO_2$ release from [26-^{14}C]cholesterol occurred within the first hour after an i.v. bolus of 25-OH cholesterol when compared in a paired fashion to the animals measured baseline (**Fig. 2**). No significant decrease was observed with the ethanol vehicle alone. By the second hour the effect of 25-OH cholesterol on $^{14}CO_2$ release was no longer present.

Again, to exclude changes in the specific activity of the [26-^{14}C]cholesterol precursor pool used to measure bile acid synthesis, we administered 25-OH cholesterol promptly after cannulation of the bile ducts of two rats with the cholesterol pool prelabeled with [4-^{14}C]cholesterol and determined the biliary cholesterol secretion rate and specific activity. Although the biliary cholesterol output decreased by 33% and 43% in these two animals over the next 2 h compared to that measured immediately before 25-OH cholesterol, the specific activity of the biliary cholesterol remained constant (data not shown). Finally, we measured the effect of 25-OH cholesterol in three rats with bile fistulae for more than 72 h, comparing this to the changes evoked with the ethanol vehicle alone. With com-

TABLE 6. Effect of 25-OH cholesterol on VLDL secretion and composition in the isolated perfused liver

Content	Output		Composition	
	25-OH Chol	Control	25-OH Chol	Control
	μg/g liver/h		*% by wt*	
Triglycerides	340 ± 65	390 ± 50	68.7 ± 2.8	72.4 ± 0.8
Free Cholesterol	13 ± 2	15 ± 2	3.1 ± 0.3	2.9 ± 0.1
Cholesteryl ester	11 ± 1[a]	6 ± 1	2.7 ± 0.4[a]	1.2 ± 0.2
Phospholipid	71 ± 13	76 ± 9	15.2 ± 0.9	14.2 ± 0.4
Protein	45 ± 7	48 ± 5	10.4 ± 1.2	9.3 ± 0.7

Donor livers were perfused in situ as described in Methods. At the end of the 2.5-h perfusion period, the perfusate was collected and the red blood cells were sedimented by low-speed centrifugation. VLDL was then harvested from the perfusate by ultracentrifugation and the lipid and protein content was analyzed as described in Methods. The values are the mean ± SEM of eight perfusions in each group.

[a]Different from control, $P < 0.01$.

plete interruption of the enterohepatic circulation and bile salt depletion for 72 h, the rate of bile acid secretion is equivalent to the rate of bile acid synthesis (34). No significant difference in bile salt synthesis was measured by this method between the rats administered the 25-OH cholesterol bolus and the vehicle (data not shown). These results in conjunction with the lack of change in 7α-hydroxylase activity (Table 4) suggest that the amount of free cholesterol contained in this pool regulates bile acid synthesis only to a minor extent, if at all.

Effect of orotic acid feeding on hepatic cholesterol metabolism, biliary cholesterol secretion, VLDL cholesterol secretion, and bile acid synthesis

An alternate explanation for the lack of change in biliary cholesterol secretion and bile acid synthesis with mevalonate administration is that the newly formed cholesterol is not immediately available for secretion from the liver by these routes. To test this possibility and to further demonstrate the relationship between biliary cholesterol and VLDL cholesterol secretion, we fed rats orotic acid 2 g % for 1 week. Previous work has demonstrated a sustained increase in cholesterol synthesis after 2 days of feeding orotic acid (35, 36). In addition, orotic acid feeding inhibits fatty acyl-CoA formation and therefore cholesteryl ester formation and triglyceride synthesis (35). In turn, these changes result in a marked decrease in VLDL secretion from the liver (37). An increased cholesterol synthesis coupled with the inhibition of cholesteryl ester formation and VLDL cholesterol secretion would be expected to increase the cholesterol available for both biliary cholesterol secretion and bile acid synthesis. This assumes that the cholesterol used as a substrate by ACAT, secreted in bile, converted to bile acids, and secreted in VLDL is derived from a common pool.

Consistent with the published effect of orotic acid on fatty acyl-CoA formation, we saw a substantial decrease in incorporation of [^{3}H]oleate into microsomal and VLDL triglyceride and cholesteryl esters and a significant decrease in microsomal cholesteryl ester content (**Table 7** and **Table 8**). Orotic acid feeding inhibited the secretion of all components of VLDL including free cholesterol and cholesteryl ester without changing the composition of the secreted particles (**Table 9**). The feeding of orotic acid stimulated both biliary cholesterol secretion independent of bile salt and phospholipid secretion (Table 8) and cholesterol synthesis as demonstrated by a striking in-

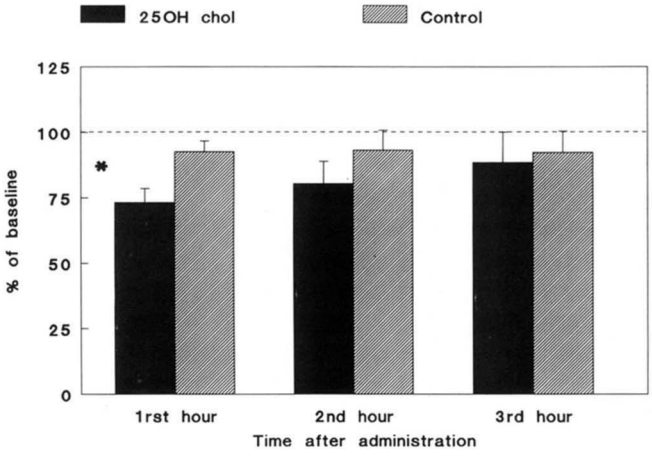

Fig. 2. Effect of 25-OH cholesterol on bile acid synthesis. Rats were pretreated with [26-^{14}C]cholesterol and bile acid synthesis was measured by the release of $^{14}CO_2$ as described in Methods. Two baseline values of bile acid synthesis were obtained after which the animals were treated with 25-OH cholesterol (solid bars) or the ethanol vehicle (hatched bars). After agent administration, the mean of four 10-min timed collections of expired $^{14}CO_2$ over a 1-h period was used to determine the rate of bile acid synthesis in each individual animal for that hour. In this figure, bile acid synthesis is expressed as percent of baseline, calculated by dividing an animal's mean hourly bile acid synthesis rate by its average baseline value. A small but statistically significant decrease in bile acid synthesis compared to the baseline was measured within the first hour after the administration of 25-OH cholesterol. By the second hour the effect was no longer present. No change in bile acid synthesis occurred over the 3-h period with the ethanol alone. The graphed values are the means ± SEM of five animals in each group. *Different from baseline, $P < 0.05$.

TABLE 7. Effect of orotic acid on HMG-CoA reductase, ACAT, and 7α-hydroxylase activities and microsomal cholesterol content

Variable	Orotic Acid	Control
HMG-CoA reductase activity (pmol mevalonate/mg/min)	507 ± 39[a]	112 ± 18
ACAT activity (pmol cholesteryl ester/mg/min)	144 ± 10	188 ± 31
7α-Hydroxylase activity (pmol 7α-hydroxycholesterol/mg/min)	26 ± 4	24 ± 4
Bile acid synthesis (nmol/min)	34 ± 3	29 ± 2
Microsomal free cholesterol (μg/mg protein)	15.4 ± 0.6[a]	20.1 ± 1.8
Microsomal cholesteryl ester (μg/mg protein)	0.8 ± 0.1[b]	1.3 ± 0.2

Rats were administered normal lab chow or chow supplemented with 2 g % orotic acid for a period of 7 days. One group of five orotic acid- and control-fed rats was administered [26-^{14}C]cholesterol (5 μCi) in order to measure bile acid synthesis by the timed release of $^{14}CO_2$ from [26-^{14}C]cholesterol as described in Methods. A second group of eight control- and orotic acid-fed animals were killed and microsomes were prepared for the measurement of HMG-CoA reductase, ACAT, and 7α-hydroxylase activities, and for microsomal cholesterol content. The values are expressed as the mean ± SEM.

[a]Different from control, $P < 0.001$.

[b]Different from control, $P < 0.05$.

crease in microsomal HMG-CoA reductase (Table 7). A somewhat surprising finding was the accompanying decrease in microsomal free cholesterol content (Table 7). Finally, orotic acid had no effect on bile acid synthesis as measured both by $^{14}CO_2$ release from [26-^{14}C]cholesterol and microsomal 7α-hydroxylase activity (Table 7).

DISCUSSION

The concept of metabolic compartmentalization of hepatic cholesterol used for biliary secretion, bile acid synthesis, and lipoprotein secretion has been suggested by studies both in the rat and humans (18, 30, 31, 38–40). However, the processes that regulate the size of these putative precursor pools of cholesterol and the interrelationship of these compartments have not been fully elucidated. Therefore, in this series of experiments we acutely manipulated the availability of hepatic free cholesterol and measured the effect on biliary cholesterol secretion, VLDL cholesterol secretion, and bile acid synthesis. The results are summarized in **Table 10.**

In the initial experiments we administered mevalonate to increase free cholesterol availability in the liver. In agreement with Spady, Turley, and Dietschy (5), these results demonstrate that an increase in free cholesterol is counteracted by inhibition of HMG-CoA reductase, thus blocking further cholesterol synthesis from acetate. The accompanying stimulation of intrahepatic cholesteryl ester formation demonstrates the primary role of ACAT in maintaining cellular free cholesterol homeostasis. In turn, these changes maintained microsomal free cholesterol content and biliary secretion constant at the expense of an increased cholesteryl ester content in microsomes and secreted VLDL.

Using a variety of acute pharmacologic manipulation, we have previously demonstrated that the precursor pool of free cholesterol destined for biliary secretion is coordinately regulated by the rates of cholesterol synthesis and esterification (18). Our current data demonstrate a comparable effect on biliary cholesterol secretion in the perfused liver, suggesting that this is a direct effect of the oxysterol 25-OH cholesterol in the biliary precursor pool. This is just one more example of the potential physiologic importance of oxysterols in regulating cholesterol homeostasis in the cell (41–43). The stimulatory effect of 25-OH cholesterol on ACAT resulted in the secretion of VLDL enriched with these newly formed cholesteryl esters, thus reaffirming the important role of intrahepatic cholesterol esterification by ACAT in determining VLDL cholesteryl ester content (13). Furthermore, these studies extend this concept by demonstrating that ACAT coordinately regulates biliary free cholesterol secretion and VLDL cholesteryl ester secretion and suggest a reciprocal relationship between these two routes of cholesterol egress from the liver.

The studies of orotic acid feeding also suggest a common biliary/VLDL pool of cholesterol. Orotic acid feeding resulted in an increased cholesterol synthesis, an inhibition of cholesteryl ester formation, and a decreased VLDL cholesterol secretion; changes diametrically opposed to the effect of 25-OH cholesterol administration (see Table 10). If our concept of the regulation of this common precursor pool of cholesterol is correct, this should

TABLE 8. Effect of orotic acid feeding on [^{3}H]oleate incorporation into lipids and biliary lipid secretion in the isolated perfused liver

Content	Orotic Acid	Control
Microsomes (dpm/mg/h)		
Cholesteryl esters	18 ± 2[a]	53 ± 5
Triglycerides	349 ± 82[b]	2768 ± 205
VLDL (× 10^3 dpm/g liver/h)		
Cholesteryl esters	0.4 ± 0.1[b]	3.7 ± 0.6
Triglycerides	17 ± 6[b]	316 ± 3
Biliary lipids (μmol/kg body wt/h)		
Bile salt	124 ± 8	120 ± 6
Cholesterol	2.8 ± 0.6[a]	1.48 ± 0.06
Phospholipid	11.1 ± 0.3	8.7 ± 0.4

Donor rats were fed standard lab chow or chow supplemented with 2 g% orotic acid. Livers were perfused for 2 h with a recirculating perfusate containing [^{3}H]oleate to measure incorporation of the label into VLDL and microsomal lipids. Bile flow was maintained by constant infusion of taurocholate into the portal vein as described in Methods. The results are expressed as the mean ± SEM of seven orotic acid- and control-fed liver perfusions.

[a]Different from control, $P < 0.05$.

[b]Different from control, $P < 0.001$.

TABLE 9. Effect of orotic acid feeding on VLDL secretion and composition in the isolated perfused liver

Content	Output		Composition	
	Orotic Acid	Control	Orotic Acid	Control
	μg/g liver/h		*% by wt*	
Triglycerides	140 ± 28[a]	439 ± 38	71.7 ± 1.0	70.1 ± 1.0
Free cholesterol	7 ± 2[a]	22 ± 3	3.0 ± 0.3	3.6 ± 0.4
Cholesteryl ester	4 ± 1[b]	9 ± 1	1.7 ± 0.2	1.4 ± 0.2
Phospholipid	24 ± 6[a]	85 ± 8	11.4 ± 1.1	13.4 ± 0.7
Protein	22 ± 3[a]	71 ± 6	12.3 ± 1.4	11.3 ± 0.5

Livers from orotic acid-fed and control-fed donors were perfused for a 2-h period after which the red blood cells were separated from the perfusate by low speed centrifugation. The red cell free perfusate was then ultracentrifuged and the VLDL was harvested and analyzed as described in Methods. The results are expressed as the mean ± SEM of seven orotic acid and control perfusions.

[a]Different from control, $P < 0.001$.

[b]Different from control, $P < 0.01$.

have resulted in an increased secretion of free cholesterol in bile. The experimental data agree with this prediction. However, the decreased microsomal free cholesterol with orotic acid feeding was a somewhat surprising and novel observation suggesting two possible explanations. It is possible that this common pool is not contained in the microsomes, or is a small subpool of the microsomes; several studies have provided evidence suggesting this (40, 44). Alternatively, as Tokmakjian and Haines have proposed (35), orotic acid acts to directly enhance biliary cholesterol secretion which then results in a stimulation of cholesterol synthesis by decreasing negative feedback on the enzyme. Although our orotic acid feeding data do not differentiate between these two possibilities, they do nevertheless suggest the existence of a common precursor pool of cholesterol available for secretion in bile or VLDL. Chronic feeding studies by Nervi et al. (38) have also suggested the existence of this common precursor pool.

In distinction, our data do not support the concept that bile acid synthesis is regulated by cholesterol availability in this common bile/VLDL precursor pool. The regulation of bile acid synthesis by cholesterol availability has recently been the subject of much research. To date several groups using both mevalonate to stimulate newly formed cholesterol and an HMG-CoA reductase inhibitor to decrease newly formed cholesterol have presented data suggesting that the availability of newly synthesized cholesterol regulates both 7α-hydroxylase activity and the rate of bile acid synthesis (21, 23, 24, 45, 46). In distinction, several different papers have presented convincing evidence that suggests that neither the amount of microsomal cholesterol nor the degree of saturation of the enzyme with cholesterol nor the level of HMG-CoA reductase is a major direct regulator of 7α-hydroxylase in the rat (22, 47, 48). Other authors have also suggested that hepatic cholesterol used for bile acid synthesis is contained in a compartment that is functionally distinct from that utilized for biliary cholesterol secretion (38, 39, 49–51). Despite the administration of mevalonate and the feeding of orotic acid, two processes that increase sterol synthesis, and 25-OH cholesterol, which decreases sterol synthesis, we saw no substantive change in bile acid synthesis as measured by 7α-hydroxylase activity or by $^{14}CO_2$ release from [26-^{14}C]cholesterol. We suggest that this occurs because either the pool of cholesterol used for bile acid synthesis is not common with the pool used for biliary cholesterol secretion and VLDL cholesterol secretion or the enzyme 7α-hydroxylase is saturated with cholesterol and therefore not subject to regulation by the amount of free cholesterol contained in the bile/VLDL precursor pool. However, most of our experiments were of a short term nature and an effect of changing free cholesterol availability on bile acid synthesis over a longer experimental period cannot be ruled out.

In conclusion, our results suggest that in the rat the cholesterol secreted in VLDL and biliary cholesterol are derived from a common pool that is regulated by cholesterol synthesis and esterification. It is not known whether a similar relationship exists in humans. Studies demonstrating an increase in hepatic HMG-CoA reductase activity and a decreased ACAT activity in patients with cholesterol gallstones suggest that the biliary precursor

TABLE 10. Summary of the effects of altering cholesterol metabolism on hepatic cholesterol secretion into plasma and bile

	Mevalonate	25-OH Cholesterol	Orotic Acid
Cholesterol metabolism			
Synthesis	↑	↓	↑
Esterification	↑	↑	↓
Biliary cholesterol	–	↓	↑
VLDL cholesterol	↑	↑	↓
Bile acid synthesis	–	–	–

pool of free cholesterol in humans is also regulated by these two processes (52, 53). Furthermore, in humans the reported negative correlation between serum cholesterol and cholesterol gallstones might be explained by a common pool of cholesterol available for either excretion into the plasma or bile (54).

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