Reduction of bovine plasma cholesterol concentration by partial interruption of enterohepatic circulation of bile salts: a novel hypocholesterolemic model

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Abstract Interruption of enterohepatic circulation (EHC) of bile salts in several species is known to cause a significant decrease in plasma concentrations of low density lipoprotein (LDL) cholesterol, but to have little effect on high density lipoprotein (HDL) cholesterol. The present study, for the first time, demonstrates that partial interruption of EHC dramatically reduces both plasma LDL and HDL cholesterol concentrations in cattle. Five adult Holstein cows were surgically altered to allow controlled portions of bile flow to be diverted from the body. The animals were fed a low-fat, cholesterol-free diet. In two experiments, bile was diverted at 50% and 22% of total flow rates. By day 8 of diversion, both rates reduced mean plasma cholesterol from baseline (85 mg/dl) to about 8 and 18 mg/dl, respectively. Cholesterol was reduced in equal proportions in all lipoprotein fractions. In addition, plasma concentrations of triglycerides and phospholipids were also dramatically reduced. All of these plasma lipids returned to baseline within 1 week after restoration of bile flow. To determine the hepatic response to bile diversion, liver cholesterol concentrations, cholesterol synthesis rates, and LDL receptor-binding activities were determined in biopsy samples. In response to bile diversion, hepatic cholesteryl esters were markedly depleted while hepatic cholesterol synthesis rates were increased by more than 10-fold. Nevertheless, because the basal cholesterol synthesis rate was so low, it was estimated that the increase in synthesis would have supplied no more than 5% of the sterols depleted during bile diversion (1.2 vs. 25 mmol/day). LDL receptor-binding activity was significantly elevated, suggesting an increased uptake of plasma lipoprotein cholesterol by the liver. These results suggest that the unique sensitivity of bovine plasma cholesterol to enterohepatic circulation interruption might occur as a result of the inherently low rate of hepatic cholesterol synthesis in cattle. This hypocholesterolemic model might serve as an interesting tool for the study of factors regulating plasma HDL cholesterol.—**Chen, Z., T. H. Herdt, J. S. Liesman, N. K. Ames, and R. S. Emery.** Reduction of bovine plasma cholesterol concentration by partial interruption of enterohepatic circulation of bile salts: a novel hypocholesterolemic model. *J. Lipid Res.* 1995. **36:** 1544–1556.

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Hepatobiliary excretion of cholesterol and its metabolites, the bile acids, is the major route for the elimination of cholesterol from the body. Such excretion thereby plays an important role in regulating body cholesterol metabolism. Under normal conditions, more than 95% of the bile acids secreted by the liver are reabsorbed in the ileum by an active transport mechanism and return to the liver via the hepatic portal vein (1, 2). Therefore, to maintain a steady state, the liver is normally required to synthesize only small amounts of bile acids to replace the fecal loss (3). However, when the enterohepatic circulation (EHC) of bile acids is interrupted, either by direct biliary drainage (4, 5) or by increasing fecal loss of bile acids (6–8), the rate of bile acid synthesis is augmented (4, 6, 9), leading to an increased requirement for cholesterol by the liver. The increase in cholesterol demand, in turn, leads to an increase in hepatic cholesterol synthesis rate (9–11). The

Abbreviations: EHC, enterohepatic circulation; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; apo, apolipoprotein; DPS, digitonin-precipitable sterols; MEM, minimum essential media.

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uptake and catabolism of plasma lipoprotein cholesterol by the liver is also enhanced (6, 9, 11, 12). As a result of this, plasma cholesterol concentration is usually reduced (6–12). The impact of interruption of EHC on cholesterol metabolism has been investigated in a number of species (6–12), yet similar information has not been available for ruminant animals such as cattle.

As in most mammals, the majority of the circulating cholesterol in cattle is carried by high density lipoprotein (HDL) (13, 14). However, there appear to be many differences in plasma cholesterol metabolism between cattle and other species in which the effects of interruption of EHC on plasma cholesterol metabolism have been studied. These differences include the lack of an apoE-like protein in bovine HDL particles (14, 15), very low level of cholesteryl ester transfer activity in bovine plasma (16), and a very low activity of hepatic triglyceride lipase (17, 18). Because of these differences, hepatic clearance of plasma cholesterol might be different in cattle than in other species. Thus, it would be interesting to examine the effect of interruption of EHC of bile acids on plasma cholesterol metabolism in cattle. In combination with the unique properties of bovine cholesterol metabolism, such studies might result in new information related to the general understanding of mechanisms regulating plasma cholesterol. As the first step to explore this possibility, the present study was carried out to determine the effects of controlled biliary diversion on plasma concentrations of cholesterol and other lipids and on hepatic cholesterol metabolism in cattle. Interestingly, we found that partial diversion of bile in this species caused a reduction of more than 90% in plasma concentrations of cholesterol and other lipids. Simultaneously, hepatic cholesterol synthesis rate and low density lipoprotein (LDL) receptor-binding activity were elevated. This study presents a unique animal model with which to investigate the regulation and the metabolic role of plasma lipoprotein cholesterol, especially HDL cholesterol.

MATERIALS AND METHODS

Materials

[^{3}H]water (100 mCi/mmol) and sodium [^{125}I]iodide (carrier free, pH 7–11) were purchased from New England Nuclear Corp. (Boston, MA). Minimum essential media (MEM 12000) was purchased from GIBCO (Grand Island, NY). Durapore filters (0.45 μm, 25 mm in diameter, catalogue number HVLP 02500) were obtained from Millipore Corp. (Bedford, MA). Bovine serum albumin (fraction V), newborn calf serum, and heparin (grade I; from porcine intestinal mucosa) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade obtained from commercial sources.

Animals and diet

Five healthy adult, nonpregnant, nonlactating Holstein cows (600–700 kg) were used. They were kept under environmentally controlled conditions (temperature maintained at 20°C) in individual pens. Throughout the entire study, the animals were fed ad libitum an alfalfa hay-based diet and had free access to water and trace-mineralized salt. The diet was supplemented daily with 4 pounds of concentrate containing ground corn, oats, and vitamins. Based on tables of nutrient composition of animal feeds (19), this diet contained less than 3% fat. In addition, there was essentially no dietary cholesterol since no animal ingredient was used. The animals were weighed weekly.

Surgical procedures and post-surgical maintenance

The cows were surgically prepared and fitted with reentrant intestinal cannula using a modification of the procedure of Symonds, Matter, and Hall (20). Surgical anesthesia was induced by intravenous administration of guaifenesin and sodium thioamylal and maintained by inhalation of halothane. Under general anesthesia and using aseptic surgical technique, a 20-cm section of duodenum that included the insertion of the common bile duct was transected at each end. The ends were then closed to form an isolated segment. The remaining portions of the duodenum were joined to reestablish the patency of the intestine. A reentrant intestinal cannula (**Fig. 1**) was placed with one arm in the isolated intestinal

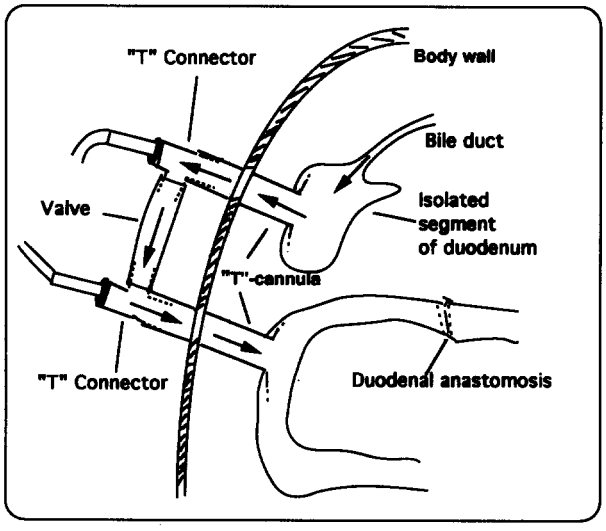

Fig. 1. Diagram of surgical modification to the duodenum. The direction of bile flow through the reentrant cannula is indicated by the arrows.

segment and the other in the intact duodenum. During the surgery, a liver biopsy (pre-cannulation control) was obtained for the experiments described in the following sections.

During the first 5 days after surgery, to minimize the internal pressure in the isolated segment of intestine, the reentrant cannula was interrupted and all bile flow was diverted from the isolated segment into a reservoir flask. Bile was infused via a peristaltic pump back to the cow, as it was collected, through the cannula arm connected to the intact duodenum. Infusion rates were adjusted occasionally so that only a small quantity of bile accumulated in the flask. Cows were given oxytetracycline (3 gm) through the intestinal cannula twice a day during this period. Thereafter, the reentrant intestinal cannula was reestablished and biliary secretions flowed directly from the isolated segment into the intact duodenum via the cannula. A one-way valve (Fig. 1) was used to prevent back-flow of ingesta into the cannula and isolated intestinal segment. Patency of the cannula was checked at least four times a day and the cannula was flushed once a day with physiological saline. The general health of the animals was monitored by physical examination and routine clinical chemistry determinations.

Experiment I

After surgery, cows were allowed at least a 4-week recovery period before any experiments were started. After the recovery period, to obtain baseline values of bile flow and total bile acid concentration and to examine the diurnal variations of these values, rates of bile flow were measured and bile samples were collected. For this purpose, the reentrant cannula was closed and all bile was collected in a flask. At 30-min intervals, bile volume was measured and 3-ml aliquots were taken. Collected bile was then infused into the duodenum at approximately the same rate as it was collected. Bile flow measurements were carried out for a period of 24 h. Twenty-four hours after the completion of bile flow rate measurement, bile diversion experiments commenced. Two bile diversion periods were studied. Initially, 50% of bile flow was diverted for 15 days. This was followed by a 21-day period of no bile diversion and completed by a 15-day period of 22% bile diversion.

During the periods of bile diversion, the reentrant cannula was interrupted and all bile was collected into a flask. Bile was aspirated from the collection flask into two separate, flexible plastic tubes, each connected to the same peristaltic pump (Model 1210; Harvard Apparatus Inc., South Natick, MA). Thus, the same rate of peristaltic motion was applied to each tube. One tube was connected to the duodenal arm of the cannula and the other to a discard flask. During periods of 50% bile diversion, tubes of equal diameter were used, while during periods of 22% bile diversion, a discard tube with one-third the cross-sectional area of the reinfusion tube was selected. The pumping rate was adjusted so that there was always a small amount of bile in the collection flask.

The volume of diverted bile was measured twice a day. A fresh bile sample (2–3 ml) was collected daily and stored at −20°C until analyzed. A coccygeal-vessel blood sample was collected into an EDTA-treated tube between 10–12:00 AM daily throughout the experiment. Plasma was immediately collected by centrifugation and stored at −20°C. When required, a large volume (30 ml) of plasma was collected and stored at 4°C for lipoprotein isolation.

Experiment II

After completion of Experiment I, the animals were allowed a 24-day period without bile diversion. At the end of this period, a second liver biopsy (post-cannulation control) was taken under local anesthesia. The cows were then allowed a further 10-day period without bile diversion. Following this baseline period, another 50% bile diversion was carried out for 14 days using the procedure as described for Experiment I. At the end of this bile diversion period, under regional anesthesia, final liver biopsies were taken. In addition, a 20-cm segment of jejunum was removed, immediately flushed with ice-cold phosphate-buffered saline and the mucosa was scraped for measurement of cholesterol synthesis rate. Immediately after collection of the terminal samples, the animals were euthanized by administration of an overdose of sodium pentobarbital. The liver and whole small intestine were dissected from the animals to determine the liver mass and total mass of small intestinal mucosa.

Using the procedures described above, control small intestine biopsies were obtained from four adult, nonpregnant, non-lactating, noncannulated Holstein cows that were fed a diet similar to that of the principal animals.

Determination of cholesterol synthesis rate

Cholesterol synthesis rate was determined in liver tissues and intestinal mucosal samples by measuring the incorporation rate of tritium from $[^3H]H_2O$ into digitonin-precipitable sterols (DPS) as described by Andersen and Dietschy (21). Immediately after collection, intestinal mucosal scrapings or thin liver slices (approximate 150 mg) were placed in screw-capped 20-ml glass vials containing 5 mCi of $[^3H]H_2O$ in 1 ml of MEM under an atmosphere of 95% O_2–5% CO_2. The vials were then incubated for 2 h at 37°C in a metabolic shaker or at 0°C in an ice bath. After incubation, 3 ml of 1.4 N KOH in 80% ethanol was added to the incuba-

tion mixture and incubated at 85°C for 3–4 h to saponify the lipids. Non-saponifiable lipids of the reactants were extracted into hexane and treated with digitonin to precipitate DPS (22). After precipitation, the resultant digitonides were washed with ethanol and diethyl ether and then split with pyridine. Thereafter, the free sterols were extracted into diethyl ether (22), dried, and assayed for radioactivity. According to thin-layer chromatographic analysis, about 90% of the radioactivity in DPS was associated with cholesterol. Rat liver slices were used as a positive control for the assays. Rat liver was obtained from adult Sprague-Dawley rats fed a commercial diet. All assays were performed in triplicate incubations.

The incorporation rate of tritium into DPS was corrected for bound $[^3H]H_2O$ (incorporated at 0°C) and expressed as nmole of ^3H incorporated per gram tissue per h (nmol/g h). To account for dilution with tissue water, the specific activity of the media water was determined after incubation by direct assay of the media. Absolute rates of cholesterol synthesis were estimated from the incorporation of ^3H assuming that 23 ^3H atoms are incorporated into each molecule of cholesterol (21, 23). Thus, the nmole cholesterol synthesized/gram wet tissue per day = (nmol ^3H incorporated/g h/23) × 24 h.

Isolation and labeling of LDL

LDL were isolated from the serum of a healthy lactating cow by ultracentrifugation (24) and affinity chromatography on heparin-Sepharose as described (25). The LDL preparation was further purified by gel filtration chromatography using Bio-Gel A5-M (26) to remove very low density lipoprotein (VLDL) particles. The purified LDL was radiolabeled with ^{125}I according to a modified McFarlane procedure (27) as described (28). The specific activities of ^{125}I-labeled LDL ranged from 350 to 520 cpm/ng protein. More than 95% of the ^{125}I radioactivity in ^{125}I-labeled LDL was precipitable by tricholoroacetic acid. The labeled LDL were sterilized through a 0.45-μm Durapore filter, stored at 4°C, and used within 2 weeks.

Hepatic LDL receptor binding assay

Within 1 h after liver biopsy, a portion (about 2.5 g) of the liver sample was homogenized in 10 ml of buffer A (NaCl, 50 mM; $CaCl_2$, 1.0 mM; Tris-HCl, 20 mM, pH 7.5). The homogenates were flushed through a 19-gauge needle 10 times and thereafter stored at −70°C until assayed.

LDL receptor-binding activities of the tissues were determined by measuring heparin-sensitive binding of ^{125}I-labeled LDL to the liver homogenate as described (29). Fifty milligrams of homogenate protein was incubated on ice for 2 h in 150 μl of buffer B (NaCl, 100 mM; $CaCl_2$, 1 mM; BSA 20 mg/ml; Tris-HCl, 50 mM, pH 7.5) containing a fixed amount of ^{125}I-labeled LDL (50 μg protein/ml). After incubation, aliquots (60 μl) of the binding reaction mixture were applied onto Durapore filters which were mounted on a sampling manifold (catalogue number: XX 2702550; Millipore Corp., Bedford, MA). The filters were washed eight times with 3 ml of ice-cold buffer C (100 mM NaCl, 0.5 mM $CaCl_2$, BSA 0.1 mg/ml, 50 mM Tris-HCl, pH 7.5) by applying suction to the filters. After the final wash, the filters were incubated at 4°C for 20 min with 2 ml of buffer D (NaCl, 50 mM; HEPES, 10 mM, pH 7.5) alone (for total binding) or with 10 mg/ml of heparin (for heparin-resistant binding). After incubation, suction was applied and the filters were washed twice with 3 ml of buffer C. ^{125}I activity in the filters was determined in a gamma-counter. Heparin-sensitive binding of ^{125}I-labeled LDL was calculated by subtracting the heparin-resistant binding from total binding. All binding assays were performed in triplicate.

Miscellaneous procedures

Total cholesterol was measured enzymatically using a reagent kit (Sigma, St. Louis, MO). Plasma concentrations of phospholipids were determined by an enzymatic method (WAKO Chemical Ltd., Dallas, TX). Plasma triglycerides were measured by a colorimetric procedure using a triglyceride reagent kit (Sigma, St. Louis, MO). Lipoprotein fractionation was performed by discontinuous density-gradient ultracentrifugation in a vertical-tube rotor as described previously (24). Total bile acids in bile were enzymatically determined with a reagent kit (Sigma, St. Louis, MO). Cholesterol in bile was measured enzymatically after treatment of bile with alcoholic KOH to remove the bile pigments and extraction of the biliary cholesterol with hexane/isopropanol.

Protein contents of liver homogenates and LDL were determined using a modified Lowry procedure (30). The concentrations of cholesterol and cholesteryl esters in the liver were determined enzymatically after lipid extraction (31) and isolation of unesterified cholesterol and cholesteryl esters by thin-layer chromatography using silica gel HL Uniplates (Analtech Inc., Newark, DE) and a solvent system of hexane–diethyl ether–acetic acid 85:15:1 (v/v/v).

Calculations and statistics

Values are presented as means ± SEM. The data were analyzed by analysis of variance and differences accepted as significant when $P < 0.05$.

RESULTS

Validity of the experimental model

The animals were healthy and maintained stable body weights throughout the entire study. Plasma albumin and bilirubin concentrations were normal, indicating that hepatic protein synthesis and excretion functions were not impaired. During the first 4 days after the surgery, blood cholesterol concentrations were decreased by 20%, compared to samples collected before surgery, but they returned to near the pre-surgical values by the end of the first post-surgical week. When no bile was diverted, the averaged daily rates of bile flow and total bile acid secretion were 132 ± 11.5 ml and 5.3 ± 0.82 mmol per h per 100 kg body weight, respectively. These values are comparable to those previously reported for adult cattle by other investigators (20). Based on the measurements obtained at 30-min intervals throughout a 24-h period, no significant diurnal changes in bile flow and bile acid secretion rates were observed (data not shown). This is consistent with the pattern of continuous bile secretion that is typical of ruminant animals (20, 32), probably due to the continuous passage of ingesta from the rumen to the lower gut. Taken together, these data suggested that the surgical modification per se had no effects on plasma cholesterol concentrations and EHC of bile acids of these animals.

In addition, partial bile diversion did not appear to have a negative effect on feed intake and general health of the animals. No side effects from the liver biopsy procedure were observed.

Effects of controlled bile diversion on bile flow and bile acid secretion

To examine the effects of interruption of EHC on plasma lipids, we initially diverted 50% of bile flow from the animals for a period of 15 days. In response to this bile diversion treatment, both bile flow and total bile acid secretion dropped sharply during the first 24 h and then stabilized at 30% and 8%, respectively, of their initial values. The values normalized after the termination of bile diversion (**Fig. 2**). Total bile acid concentrations in bile were also reduced by several fold during bile diversion (data not shown), thus the dramatic decline in bile acid output rate occurred as a result of the combined effects of a reduced bile flow rate and the lowered concentration of bile acids.

It has been previously reported that the rhesus monkey can increase its bile acid synthesis rate to compensate for the loss of biliary sterols when up to 24% of the bile is diverted (4). To compare cattle to this monogastric species, we have also examined the effects of 22% bile diversion on bile flow and bile acid secretion in these cows. As shown by Fig. 2, diversion of 22% of bile

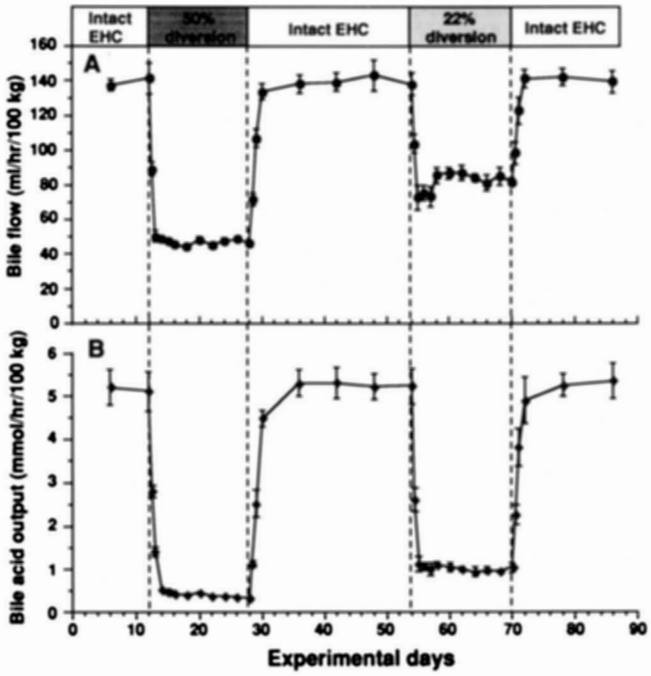

Fig. 2. Effects of controlled biliary diversion on rates of bile flow (A) and bile acid output (B). Bile flow rates were measured as described in Materials and Methods. Total bile acid concentration was determined enzymatically. Values represent means ± SEM (n = 5).

flow caused a marked decrease in the rates of bile flow and bile acid output in a manner comparable to 50% bile diversion. The reduction in bile flow and bile acid secretion rates was only slightly greater when 50% of bile flow was diverted, as compared to 22% diversion. These data suggest that bovine liver has a smaller capacity than the rhesus monkey to increase its bile acid synthesis in response to the induced loss of biliary sterols.

Effects of controlled bile diversion on plasma lipids

As a result of bile diversion, plasma total cholesterol concentration decreased dramatically, reaching a stable level 8 days after bile diversion (**Fig. 3**). Based on the stabilized levels, diversion of 50% of bile caused a significantly greater reduction than 22% bile diversion (91% vs. 78%; $P < 0.001$, n = 5). When the bile diversion procedures were terminated, however, plasma cholesterol concentrations returned towards the baseline values.

In addition to the decrease in blood cholesterol, partial bile diversion also resulted in a dramatic decline in plasma phospholipids and triglycerides. The changes in plasma phospholipid concentration followed a pattern similar to that of cholesterol (Fig. 3). However, the differences in plasma phospholipid concentrations between the 50% and 22% bile diversion periods were not significant ($P > 0.05$). Plasma triglyceride concentrations

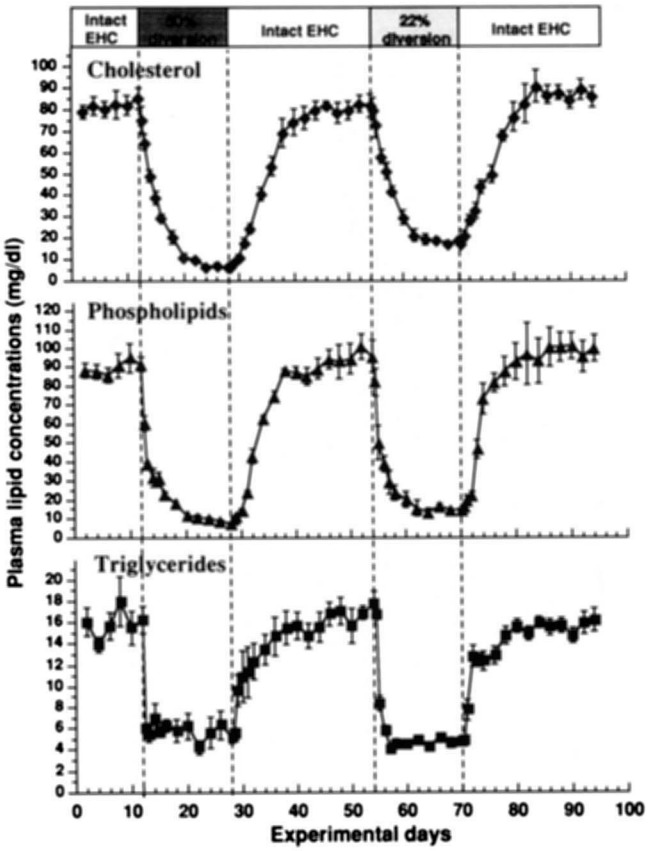

Fig. 3. Plasma concentrations of cholesterol, phospholipids, and triglycerides before, during, and after partial bile diversion. Plasma concentrations of total cholesterol, triglycerides, and phospholipids were determined by enzymatic methods. Values are means ± SEM (n = 5).

dropped sharply after the initiation of bile diversion and stabilized within 24 h thereafter (Fig. 3). This is in contrast to plasma cholesterol and phospholipid concentrations which did not decline so rapidly. When steady state was reached, both bile diversion procedures caused a 70% reduction in triglycerides compared to pre-diversion values. The rapid declines in plasma triglycerides and in bile acid secretion appeared to occur in a coordinated manner. This suggests that the dramatic drop in plasma triglycerides observed in this study might be due to the reduced availability of bile acids for lipid absorption by the small intestine.

To characterize the changes in cholesterol concentrations of different lipoprotein fractions in response to bile diversion, lipoproteins were isolated from plasma samples collected at 0 h, 12 h, and 14 days of bile diversion and cholesterol concentrations in each fraction were determined. As shown in **Table 1,** at 12 h after bile diversion, VLDL cholesterol and LDL cholesterol were significantly ($P < 0.01$) reduced by about 50% for both bile diversion procedures, whereas there were no significant changes in HDL cholesterol. By day 14 of bile diversion, however, like VLDL and LDL cholesterol, HDL cholesterol was reduced by 90% and 85% for 50% and 22% diversions, respectively (Table 1). Thus, distribution of cholesterol among different lipoproteins on day 14 of bile diversion was not different from initial values (Table 1).

Biliary sterol depletion and its effects on hepatic cholesterol profile

In no other species has interruption of EHC been shown to result in such a dramatic reduction in plasma cholesterol. To understand why this occurs in cattle, a second experiment was carried out to estimate the effects of bile diversion on the dynamics of cholesterol metabolism in cattle. This experiment used the same animals as in the first experiment, after a 25-day recovery period during which no bile was diverted. Plasma cholesterol concentrations were stable at about 85 mg/dl for 2 weeks before this experiment began. Fifty

TABLE 1. Effects of partial biliary diversion on plasma concentrations and distributions of lipoprotein cholesterol

	VLDL-Cholesterol		LDL-Cholesterol		HDL-Cholesterol	
Diversion Time	50% Diversion	22% Diversion	50% Diversion	22% Diversion	50% Diversion	22% Diversion
0 h						
mg/dl	3.04 ± 0.22	3.03 ± 0.33	11.68 ± 0.58	13.79 ± 1.43	66.77 ± 3.13	72.73 ± 1.48
%	3.76 ± 0.39	3.36 ± 0.27	14.90 ± 0.90	15.33 ± 1.24	81.84 ± 1.10	81.32 ± 1.44
12 h						
mg/dl	0.94 ± 0.12[a]	1.31 ± 0.16[a]	5.65 ± 0.49[a]	8.82 ± 1.03[a]	62.78 ± 4.92	66.52 ± 2.74
%	1.38 ± 0.23[a]	1.70 ± 0.19[a]	8.31 ± 1.01[a]	11.59 ± 1.51[a]	90.31 ± 1.15	86.72 ± 1.60
14 days						
mg/dl	0.43 ± 0.04[a]	0.63 ± 0.06[a]	1.83 ± 0.21[a]	2.12 ± 0.27	7.78 ± 0.78[a]	10.51 ± 1.07[a]
%	4.31 ± 0.40	4.84 ± 0.57	18.22 ± 1.19	16.25 ± 2.39	77.48 ± 0.90	78.91 ± 2.83

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. Lipoproteins were isolated by ultracentrifugation. Cholesterol concentrations in each lipoprotein fraction were determined enzymatically. Values are means ± SEM (n = 5).

[a]Significantly different from the corresponding control values (i.e., 0 h) at $P < 0.01$.

percent of bile flow was diverted and, as expected, plasma cholesterol concentrations again declined dramatically, reaching a stable level of about 8 mg/dl in about 8 days. The pattern of decline (data not shown) was similar to the previous period of 50% bile diversion. During this bile diversion period, biliary sterol loss through bile diversion was 180 mmol during the first 24 h (**Fig. 4**). Thereafter, the amount of diverted sterols sharply declined and stabilized at a steady-state level of about 25 mmol/day. The majority of diverted biliary sterols were bile acids, whereas biliary cholesterol accounted for less than 5% of the total biliary sterols (Fig. 4).

After 14 days of 50% bile diversion, liver content of esterified cholesterol was reduced by more than 90% ($P < 0.0001$), as compared to the pre-cannulation or post-cannulation controls, whereas the unesterified cholesterol content remained unchanged ($P > 0.05$) (**Fig. 5**). No significant difference in hepatic cholesterol profile between the two controls was observed. These data demonstrate that 50% bile diversion depleted the liver of cholesterol stores.

Hepatic and intestinal cholesterol synthesis and its response to biliary diversion

During the control periods, incorporation rate of tritium from [^{3}H]H_2O into DPS was barely detectable in the bovine liver tissues. It was only about 10% of that of the bovine intestinal mucosa, or 5% of the rat liver tissues (**Fig. 6**). Nevertheless, as with intestinal mucosa

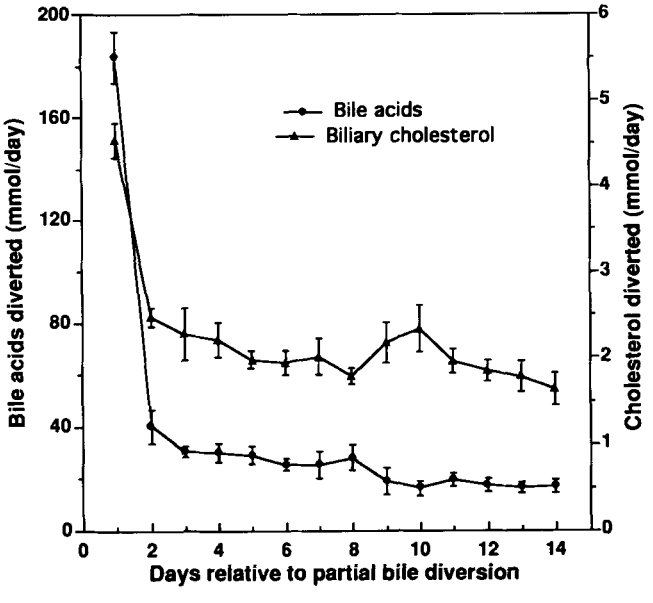

Fig. 4. Depletion of biliary sterols induced by chronic diversion of 50% of bile flow. Bile diversion was carried out as described in Materials and Methods. Total bile acid and biliary cholesterol concentrations were determined by enzymatic methods. Each data point represents the mean ± SEM (n = 5).

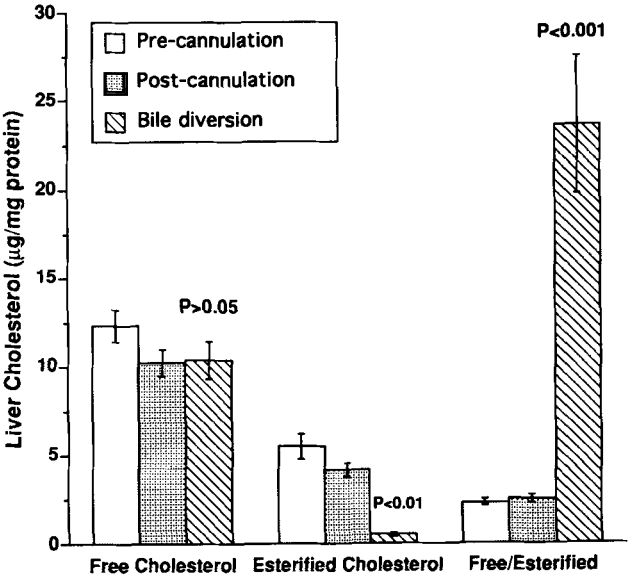

Fig. 5. Effects of bile diversion on hepatic cholesterol and cholesteryl ester concentrations. Total lipids were extracted from liver biopsy samples and free cholesterol and esterified cholesterol were separated by thin-layer chromatography, re-extracted with ether, and then quantified by enzymatic methods. Each value represents the means ± SEM (n = 5).

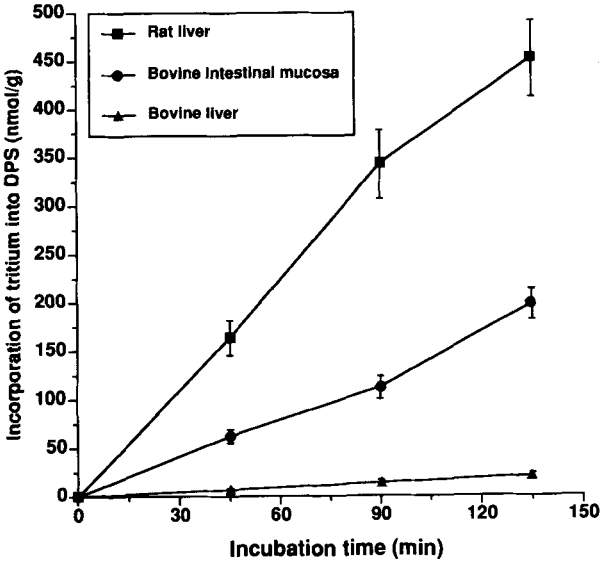

Fig. 6. Time-course of incorporation of tritium from tritiated water into bovine and rat liver tissues and bovine intestinal mucosa. Liver biopsies were taken from the principal animals at the time of intestinal cannulation surgery. Intestinal (jejunum) mucosal biopsies were obtained from control animals. The tissue explants (150 mg) were incubated in 1 ml MEM containing 5 mCi tritiated water at 37°C for the indicated time periods. After incubation, the digitonin-precipitable sterols (DPS) were isolated from the incubation mixture and the radioactivities of the DPS were determined. Incubations were performed in triplicate. Each data point represents the means ± SEM of three separate experiments.

and the rat liver tissues, incorporation of tritium into DPS by the bovine liver tissues was essentially linear with respect to time over a 2.3-h incubation period (Fig. 6). Furthermore, tritium incorporation into DPS was also proportional to the amount of the bovine liver tissues incubated per vial, at least from 50 to 200 mg (data not shown). To examine whether the low incorporation rate was due to a limitation of substrates in the MEM, we compared the incorporation rates of tritium into DPS by bovine liver slices incubated in 1 ml MEM alone or with 15 µmol acetate, propionate, lactate, octanoate, or glucose. The tritium incorporation rates ranged from 7.2 ± 1.2 to 8.3 ± 1.1 nmol/g h (n = 3 incubations) under these conditions and no significant difference among these values was observed. These data further confirm an inherently low rate of cholesterol synthesis in bovine liver.

After 14 days of bile diversion, however, the incorporation rate of tritium into DPS in the liver was increased by more than 10-fold ($P < 0.0001$) (Fig. 7), whereas there was no significant difference in the incorporation rates between the pre-cannulation and post-cannulation controls (**Fig. 7**). This indicates that the hepatic cholesterol synthesis rate was greatly increased in response to bile diversion. In contrast to this, the incorporation rate of tritium into DPS of the intestinal mucosa of these cows following bile diversion was only slightly greater than that of the intestinal mucosa from the control animals (35%; $P < 0.05$; Fig. 7).

Based on the incorporation rate of tritium from $[^3H]H_2O$ into tissue DPS and the weights of liver and small intestinal mucosa, the amounts of cholesterol synthesized by the liver and intestinal mucosa were estimated. As shown in **Fig. 8,** in the basal state, the estimated rate of small intestinal cholesterol synthesis was five times greater than that of liver. After bile diversion, as a result of the dramatic increase in the hepatic cholesterol synthesis rate, the apparent hepatic contribution to cholesterol synthesis was twice that of the small intestinal mucosa (Fig. 8). The estimated rate of hepatic cholesterol synthesis was approximately 1.2 mmol per day (Fig. 8). This amount of cholesterol, however, would provide no more than 5% of the sterols lost during bile diversion (1.2 vs. 25 mmol/day).

Responses in hepatic LDL receptor-binding activity

To further understand the possible changes in bovine hepatic cholesterol metabolism as a result of bile diversion, we have also determined the response in hepatic LDL receptor-binding activity. The binding of ^{125}I-labeled bovine LDL to liver homogenates was in a saturable manner (**Fig. 9**). Scatchard plot analysis (33) of the binding data revealed a linear plot with an equilibrium dissociation constant (K_d) of 16 µg LDL protein/ml (Fig. 9), reflecting a single class of LDL binding sites in this tissue. The LDL receptor-binding activity of the liver

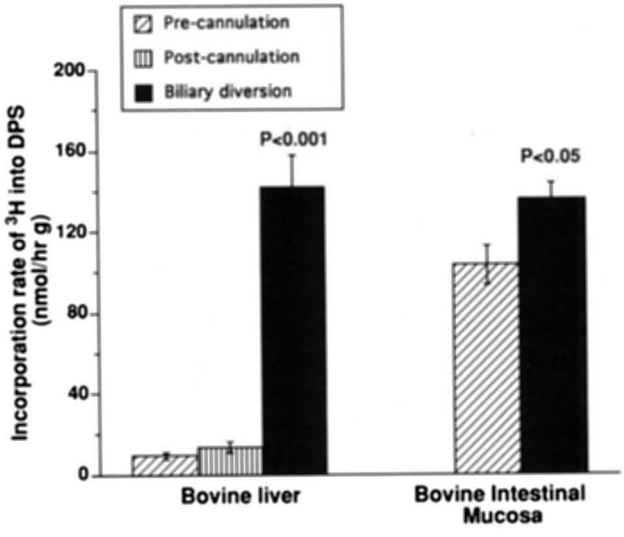

Fig. 7. Effects of bile diversion on the incorporation rate of tritium from tritiated water into digitonin-precipitable sterols (DPS) by bovine liver tissues and intestinal mucosa. Liver biopsies were obtained from the same principal animals at three different time periods. Control intestinal samples were obtained from control animals. Incorporation rates of tritium into DPS were determined as described in Materials and Methods. Each point represents the mean ± SEM; n = 5 except for control intestinal mucosa (n = 4).

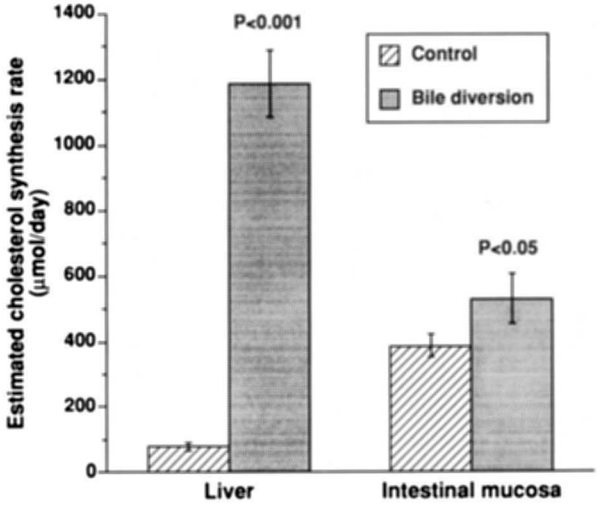

Fig. 8. Estimated quantities of cholesterol synthesized by the whole liver and intestinal mucosa before and after bile diversion. The absolute amount of newly synthesized cholesterol was estimated based on the incorporation rate of tritium into digitonin-precipitable sterols described in Fig. 7 and the total mass of the liver and intestinal mucosa, as described in Materials and Methods. Tritium incorporation rates of liver tissues from the principal animals during post-cannulation control period were used for estimating basal rate of liver cholesterol synthesis. Control intestinal tritium incorporation rates were obtained from four control animals as described in Fig. 7. Each point represents the mean ± SEM; n = 5.

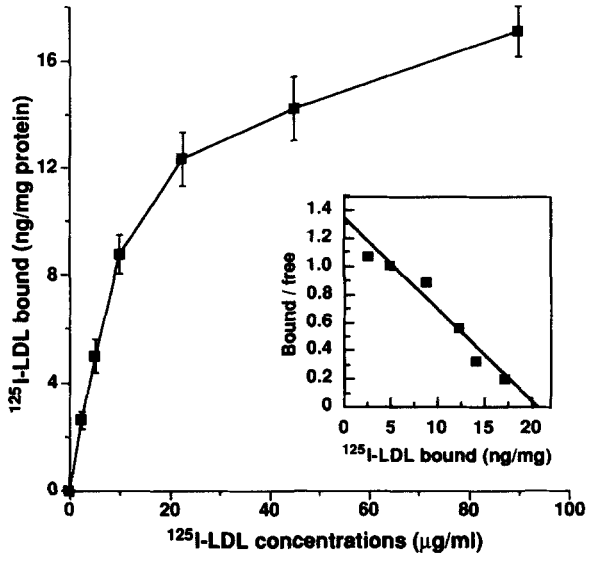

Fig. 9. Binding of radiolabeled bovine LDL to bovine liver homogenate as a function of ^{125}I-labeled LDL concentration. Liver homogenates (53 mg proteins) were incubated in 150 μl of binding assay buffer (buffer B) with the indicated concentrations of ^{125}I-labeled LDL for 2 h on ice. Specific bindings were determined by measuring the heparin-sensitive bindings. Each data point represents the means ± SEM of three incubations. Inset: Scatchard plot of the specific binding. Bound/free represents the amount of specifically bound LDL (ng/mg) divided by the concentration of unbound LDL in the reaction mixture (μg/ml).

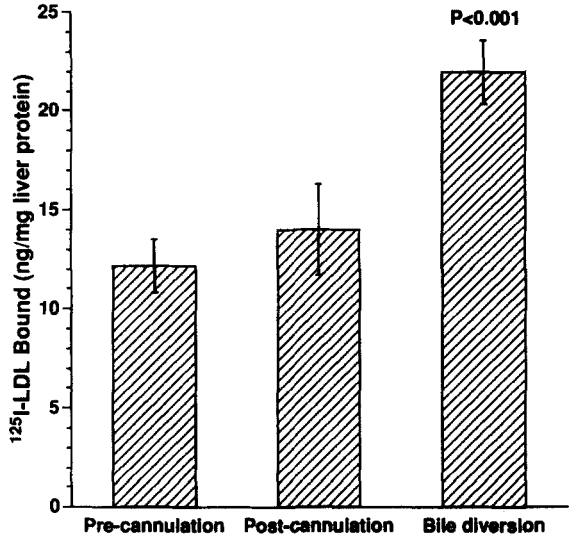

Fig. 10. Effects of bile diversion on hepatic LDL receptor-binding activity. LDL receptor-binding activities were determined on liver homogenates using a fixed concentration of ^{125}I-labeled LDL as described in Materials and Methods. Each value represents the mean ± SEM (n = 5).

tissue samples was then determined by measuring the heparin-sensitive binding of ^{125}I-labeled LDL to liver homogenates under a fixed concentration of ^{125}I-labeled LDL. The results (**Fig. 10**) show that, by day 14 of bile diversion, liver LDL receptor-binding activity was increased by 40% ($P < 0.001$) as compared to pre-cannulation and post-cannulation control samples, while there was no change in the binding activity between samples taken at the two control times (Fig. 10). These data implicate an enhanced uptake of LDL during bile diversion.

DISCUSSION

The role of EHC of bile acids in determining cholesterol homeostasis and the effects of its interruption on cholesterol metabolism have been studied in humans and in a number of other animal species (6–12). No similar study has been reported on ruminant animals, a species that uses HDL as the predominant form of circulating cholesterol carrier. The present study, for the first time, demonstrates that partial interruption of EHC dramatically reduces bovine plasma cholesterol concentration. Furthermore, we have also shown that bovine liver responds to partial interruption of EHC with a depletion of cholesterol stores, a marked increase in cholesterol synthesis rate, and an enhanced hepatic LDL receptor-binding activity.

The effects of EHC interruption on plasma cholesterol observed in this study are unprecedented. A greater than 90% reduction in plasma cholesterol concentration was achieved by 50% bile diversion. In contrast, no reductions of greater than 40% have been reported in other species (5–12), including humans (6, 8), in response to either complete or partial interruption of EHC. In further contrast to our results, interruption of EHC has not been shown to significantly affect plasma cholesterol concentrations in other HDL mammals such as rats (5, 9). In addition to plasma cholesterol, plasma concentrations of triglycerides and phospholipids were also markedly reduced after bile diversion. This also appears to be peculiar to the ruminant animal as no significant reduction in these two plasma lipids has been observed in other species in response to bile diversion. In fact, it has been shown in a number of monogastric species that interruption of EHC causes an increase in plasma triglyceride concentration (7, 10), probably due to an increase in hepatic VLDL production (34, 35).

While both 22% and 50% bile diversion caused a dramatic decrease in plasma cholesterol in this study, diversion of 50% of bile flow produced a significantly greater reduction in blood cholesterol than 22% bile diversion. This suggests that the response in plasma cholesterol concentration is dependent on the amount

of bile diverted. Like 50% bile diversion, 22% diversion also resulted in a dramatic reduction in plasma phospholipids and triglycerides. However, as the 22% interruption period was carried out after the 50% interruption, it may have exaggerated the effects of 22% diversion on plasma lipids. Nonetheless, plasma cholesterol and other lipids and the secretion rates of bile acids had returned to initial values for more than a week before the initiation of the subsequent experiments. The confounding effects between each diversion period, if any, might be minimal.

The underlying mechanism for the observed substantial hypocholesterolemia is not completely understood. However, our studies on the hepatic responses in cholesterol metabolism to bile diversion suggest that the inherently low rate of cholesterol synthesis in the bovine liver might be a possible explanation for this phenomenon. Cholesterol demand by the liver to replace sterol losses at steady state during 50% bile diversion should be at least equal to the amount of biliary sterols diverted, i.e., 25 mmol/day, because other losses of bile acids could be expected to be relatively small. The hepatic cholesteryl esters were almost completely depleted after bile diversion in our experiment, indicating that the reserve cholesterol supply was depleted. Thus, the liver must have relied on either newly synthesized cholesterol, an extrahepatic supply, or both to provide cholesterol for the increased bile acid synthesis during bile diversion. Our results suggest that both responses occurred.

In response to bile diversion, the bovine hepatic cholesterol synthesis rate was augmented by a factor of 10-fold. This is not unexpected because a decrease in cellular cholesterol level is known to up-regulate 3-hydroxy-3-methylglutaryl CoA reductase activity (36). In addition, similar effects of EHC interruption have been reported for a number of other species (9–11, 36). However, as the basal rate of bovine hepatic cholesterol synthesis is so low, the absolute magnitude of this increase appears to contribute negligibly to the replacement of biliary sterol losses. It was estimated that the increase in newly synthesized cholesterol by the liver could provide no more than 5% of the sterols lost through 50% bile diversion. In the absence of a sterol balance study, this estimate was based on in vitro hepatic cholesterol synthesis rate. It might be possible that the in vivo cholesterol synthesis rates have been underestimated by the in vitro measurements. However, based on extensive studies of a variety of tissues from several species, a previous study (37) has reported that the in vitro cholesterol synthesis rate obtained by using [^{3}H]water as labeled substrate represented 10–40% of the in vivo rate. Therefore, it is unlikely that the discrepancy between the estimated and the actual in vivo cholesterol synthesis rate could explain the large difference between the rates of sterol depletion and the hepatic cholesterol synthesis during bile diversion.

Information on the relative importance of bovine liver and intestine to total body cholesterol production has not been previously available, yet earlier studies (38, 39) on other ruminant animals have suggested that ruminant liver has a very low cholesterol synthesis rate. In the present study, we estimated that, at the organ level, the small intestinal mucosa synthesizes five times as much cholesterol as does the liver in the adult bovine female. Liver cholesterogenic activity appears to be much lower in cattle than in other mammalian species (37, 40). The reason for the inherently low rate of cholesterol synthesis in the ruminant liver is not known. However, it appears that this difference between ruminants and other species makes the bovine liver highly dependent on uptake of lipoprotein cholesterol for the increased biliary sterol formation during bile diversion. This increased dependence on plasma cholesterol could, thus, explain the dramatic decline in bovine plasma cholesterol, compared to other species in which bile diversion has been studied (6–12). As expected, bile diversion resulted in an increase in bovine hepatic LDL receptor-binding activity, providing evidence for the enhanced catabolism of lipoprotein cholesterol.

It is possible that a decrease in intestinal triglyceride-rich lipoprotein production also contributes to the hypocholesterolemic effect of partial bile diversion. Interruption of EHC has been shown to decrease the absorption of dietary lipids by the small intestine (10, 41–43). The coordinated pattern of the rapid declines in plasma triglyceride concentrations and in bile acid secretion rate observed in our study after bile diversion suggests that the production rate of intestinal triglyceride-rich lipoproteins might be impaired by bile diversion. However, as our animals were fed a low-fat diet throughout the entire study, the basal rate of intestinal lipoprotein production could be expected to be very low. Hence, it is unlikely that the changes in chylomicron production played an important role in the development of the observed hypocholesterolemia. In fact, a slight increase, rather than a decrease, in intestinal cholesterogenesis was observed after bile diversion, as compared to the control animals. Furthermore, as a cholesterol-free diet was used in our study, the possible impairment in dietary cholesterol absorption resulting from EHC interruption could also be excluded as a mechanism for the observed hypocholesterolemia.

It is important to reemphasize that the hypocholesterolemia observed in our study must have relied almost exclusively on the reduction in HDL-cholesterol. In species such as humans, in which LDL is the major carrier of plasma cholesterol (13), interruption of EHC

mainly reduces LDL-cholesterol while concomitantly causing a slight increase in HDL-cholesterol (8, 10). Moreover, in high HDL-cholesterol species such as rats (13), bile diversion only produces a small decrease in plasma total cholesterol (5, 9), suggesting that HDL-cholesterol was not affected significantly. It is not known whether the dramatic decline in HDL-cholesterol observed in our animals is due to an increase in HDL clearance rate or a decrease in HDL synthesis or both. Biliary diversion does not appear to affect intestinal HDL secretion in rats (42, 44). However, the maturation of HDL particles in the plasma requires a constant supply of surface coat components including free cholesterol, phospholipids, and certain apolipoproteins from triglyceride-rich lipoproteins during lipolysis (44). Thus, a decrease in chylomicron secretion resulting from bile diversion could potentially influence plasma HDL synthesis. Further studies on HDL kinetics are required to fully elucidate the mechanism for the hypocholesterolemia observed in this study.

Regardless of the exact mechanism(s) leading to the observed decrease in plasma HDL-cholesterol, it is clear that, due to the limited capacity of the bovine liver to increase its cholesterol synthesis, a large amount of HDL-cholesterol needs to be taken up by the bovine liver for biliary sterol formation during bile diversion. This suggests that HDL actively transports cholesterol from the extrahepatic tissues to the bovine liver for catabolism during bile diversion. The origins of this HDL-cholesterol are not clear. However, the majority of cholesteryl esters in ruminant plasma are generated through the lecithin:cholesterol acyltransferase system in the plasma (45, 46). It remains to be examined whether bile diversion stimulates the synthesis and efflux of cholesterol in the extrahepatic tissues such as adipose tissue, which is known to be a major site of cholesterol synthesis in ruminants (38).

While an increased hepatic LDL receptor-binding activity was observed in our study, this increase might not necessarily lead to an increase in HDL-cholesterol uptake by the bovine liver. This is due to the lack of apoE in bovine HDL particles (14, 15) and the low cholesteryl ester transfer activity in bovine plasma (16), although it is not known whether the latter can be induced during bile diversion. It appears that HDL-cholesterol uptake by the bovine hepatocytes mainly depends on the "HDL pathways." Such pathways could include the selective transfer of cholesteryl esters from HDL to the cells, without the uptake the whole HDL particles (47–49). This process has been shown to operate in several cell types including hepatocytes (48–50). The molecular basis of this process is yet to be elucidated, but it appears that it could be facilitated by hepatic lipase (51, 52). Although the role of hepatic lipase in HDL-cholesterol uptake during bile diversion remains to be determined, it is known that ruminant liver possesses very little hepatic lipase activity (17, 18). Alternatively, HDL can be taken up by the HDL-receptor pathway. This pathway involves binding of HDL to the putative HDL receptor on the cell surface, followed by the internalization of the whole HDL particle and the release of cholesterol without the HDL apoproteins necessarily being degraded (47, 50). Although a high affinity binding site for apoE-free HDL has been demonstrated in a variety of cell types and tissues (47, 53) including bovine liver plasma membranes (54), neither the identity nor the physiological significance of the putative HDL receptor has been established. However, this pathway might play an important role in the hepatic clearance of HDL-cholesterol in our animal model.

In summary, the present study has revealed that partial interruption of EHC of bile acids can dramatically reduce plasma cholesterol concentration in cattle, a species in which HDL is the predominant circulating lipoprotein. It appears that a possible reason for this striking hypocholesterolemia is the inherently low rate of cholesterol synthesis in the bovine liver, rendering these animals more dependent on plasma cholesterol for bile acid synthesis. Nonetheless, further studies are required to fully understand the exact mechanism. With the unique characteristics of plasma cholesterol metabolism in this species, such studies might enhance our basic understanding of the regulation of HDL-cholesterol metabolism.

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