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EFFECTS OF INTERRUPTION OF ENTEROHEPATIC CIRCULATION OF BILE SALTS ON CHOLESTEROL METABOLISM IN CATTLE: A NOVEL HYPOCHOLESTEROLEMIC MODEL

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ABSTRACT

EFFECTS OF INTERRUPTION OF ENTEROHEPATIC CIRCULATION OF BILE SALTS ON CHOLESTEROL METABOLISM IN CATTLE: A NOVEL HYPOCHOLESTEROLEMIC MODEL

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Enterohepatic circulation (EHC) of bile salts has a pivotal role in cholesterol homeostasis in the body. Its interruption is known to cause wide-ranging effects on cholesterol metabolism in an number of non-ruminant species, leading to a reduction in plasma cholesterol concentration. Similar information has not been available for ruminant animals. The objective of the present study was to determine the effects of interruption of EHC on plasma concentrations of cholesterol and other lipids and on hepatic cholesterol metabolism in cattle. Five adult, nonlactating, Holstein cows were fitted with reentrant intestinal cannula so that the portion of bile flow reaching the intestine could be controlled. Two 15-day bile diversion periods, i.e., 50% and 22% bile diversion, were studied to examine the effects of partial bile diversion on plasma lipids. Both bile diversion procedures caused a dramatic reduction in plasma cholesterol concentration. After 8 days of bile diversion, plasma cholesterol concentrations reached a low, stabilized level of about 10 mg/dl. Plasma concentrations of phospholipids and triglycerides were also decreased dramatically in response to bile diversion. After the termination of bile diversion, plasma concentrations of cholesterol and other lipids returned to initial values within a week. Subsequent experiments were conducted to determine if changes in hepatic cholesterol synthesis or uptake, during bile diversion, could account for the response in plasma cholesterol. Cholesterol

synthesis rates, hepatic cholesterol concentrations, and hepatic low density lipoprotein (LDL) receptor-binding activity were determined in liver biopsies taken in precannulation control and postcannulation control periods and taken at the end of a second 50% bile diversion period. Cholesterol synthesis rate was barely detectable in liver tissues during the two control periods. After 14 days of bile diversion, however, hepatic cholesterol synthesis rate was increased by more than 10-fold. Nonetheless, the estimated total amount of hepatic newly-synthesized cholesterol would provide no more than 5% of the sterols depleted from the body during bile diversion (1.2 vs. 25 mmoles/day). Moreover, bile diversion almost completely depleted the liver of cholesterol ester. This indicates that bovine liver must rely upon extrahepatic sources of cholesterol for biliary sterol formation during this period. In response to bile diversion, hepatic LDL receptor-binding activity was significantly elevated, suggesting an increased uptake of plasma lipoproteins by the liver. In conclusion, the present study, for the first time, shows that bovine plasma concentrations of cholesterol and other lipids respond to partial bile diversion with a dramatic reduction. The substantial hypocholesterolemia is suggested to occur as a result of the inherently low rate of cholesterol synthesis in the bovine liver. This study has defined a novel animal model with which to investigate the metabolic role of plasma cholesterol and regulation of plasma lipoprotein metabolism.

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CHAPTER I

REVIEW OF LITERATURE

I. INTRODUCTION

Cholesterol is an essential constituent of living tissues. It plays a critical role in the body as an indispensable component of biomembranes and as an obligatory precursor for the biosynthesis of steroid hormones and bile acids. Cholesterol is also required by the liver and intestine for the

synthesis and secretion of lipoproteins. In order to fulfill the cholesterol demands of various cells, a continuous supply of cholesterol is required. All mammalian cells can synthesize cholesterol to meet their requirement, but this does not usually occur. In fact, a significant amount of cholesterol used by the peripheral tissues is usually derived from plasma lipoproteins (1, 2). This is especially true for the steroidogenic tissues such as adrenals and corpora lutea of the ovary, which are dependent upon the uptake of circulating lipoprotein-carried cholesterol to maintain physiological levels of steroid hormone production (3-5). Therefore, an adequate supply of plasma cholesterol to the tissues is important for maintaining cellular and endocrine functions in the body.

Plasma cholesterol concentration in most animal species is maintained within a narrow range throughout different physiological stages, but this does not appear to be true for dairy cattle. For example, large fluctuations in blood cholesterol concentrations through the gestation-lactation cycle have been well documented in dairy cows (6). The mechanisms regulating these large changes in plasma cholesterol are not known. Nonetheless, the degree of peripartum reduction in plasma cholesterol concentration has been shown to be associated with metabolic diseases of the cow, including fatty liver and infertility (7-10). This sheds light on the necessity for understanding the physiological role and the regulation of plasma cholesterol concentrations in dairy cattle. To date, however, studies of the cholesterol transport system in cattle have been primarily focused on qualitative and quantitative aspects of the plasma lipoprotein profile. A major emphasis has been on the physical and chemical characteristics of the major lipoprotein particles and their apolipoproteins. Very little information is available regarding the pathways

of lipoprotein metabolism and their regulation in the system. In contrast to this, numerous investigations have been directed towards elucidation of the mechanism(s) of control of plasma cholesterol metabolism in other species, especially humans and experimental animals such as rats. The purpose of this chapter is to review current knowledge on the major pathways of plasma cholesterol metabolism and their regulation in these species, with emphasis on the role of liver. Extrapolation of this knowledge to cattle will then be made based on available information on physicochemical characteristics of bovine plasma lipoproteins.

II. THE LIPOPROTEIN CHOLESTEROL TRANSPORT SYSTEM

Nearly all cholesterol in plasma is carried by plasma lipoproteins, which in turn act as vehicles for transport of cholesterol through the aqueous medium of plasma. An abnormal plasma cholesterol concentration usually reflects an underlying change in the concentration of a specific lipoprotein. In order to understand plasma cholesterol metabolism, therefore, it is necessary to consider the nature and origin of plasma lipoproteins, their interconversions within the plasma and their extravascular metabolism.

A. Physicochemical properties of plasma lipoproteins

Lipoproteins are macromolecular complexes of protein, phospholipid, cholesterol, cholesterol ester and triglyceride. The hydrophobic components, i.e., triglycerides and cholesterol esters, form the core of the lipoprotein particles while polar phospolipids and unesterified cholesterol, together with apoproteins, form the surface monolayer coat(11).

Traditionally, lipoproteins are classified according to their hydrated densities into four major classes: very low density lipoproteins (VLDL) and chylomicrons, <1.006 g/ml; low density lipoproteins (LDL), 1.006 to 1.062 g/ml; and high density lipoproteins (HDL), 1.063 to 1.21 g/ml. In addition, based on the difference in their surface charge, lipoproteins can also be separated into four electrophoretic classes on media such as paper or agarose (12). Due to their large size, the chylomicrons remain at the origin while the three other classes migrate with different mobilities in an electric field. HDL and LDL are referred to as α- and β-lipoproteins because their mobilities are identical to α- and β-globulins, respectively. VLDL is pre-β-lipoprotein, so-called because its mobility is slightly greater than β-lipoproteins. The physicochemical characteristics of lipoproteins vary according to the lipoprotein density classifications. Table 1 is a summary of some major physicochemical properties of various classes of lipoproteins of human plasma. The density of lipoprotein particles is inversely related to their size, reflecting the relative amounts of low density, non-polar core lipid and high density, surface protein present. Chylomicrons and VLDL are large particles and are highly enriched in triglyceride, but they are low in cholesterol content. In contrast, LDL and HDL are small, relatively dense particles which are rich in cholesterol esters, but low in triglyceride content. It should be noted that HDL are polymorphic and consist of several more or less discrete subspecies. For example, there are two major subclasses of HDL, i.e., HDL_2 and HDL_3, in human plasma. The core diameter of HDL_2 is about 50% larger than that of HDL_3, resulting in a core volume which is 3.5 larger. Hence, HDL_2 contains 3-4 fold more cholesterol ester and triglyceride molecules than HDL_3 (14). Similar subpopulations of HDL particles can also be found in other species (15, 16).

Table 1. Physicochemical Properties of Human Plasma Lipoprotein Classes[a]

Lipoprotein	Density (g/ml)	Diameter (nm)	Electrophoretic mobility	Protein (% weight)	Major lipid
Chylomicrons	<0.93	75-1200	Remains at origin	1.5-2.5	TG[c]
VLDL[b]	0.93-1.006	30-80	Pre-β-lipoproteins	5-10	TG
IDL	1.006-1.019	25-35	Slow pre-β-lipoproteins	15-20	TG/CE
LDL	1.019-1.063	18-25	β-Lipoproteins	20-25	CE
HDL					
HDL_2	1.063-1.120	9-12	α-Lipoproteins	40-45	CE/PL
HDL_3	1.120-1.210	5-9	α-Lipoproteins	50-55	CE/PL

[a] Compiled from Myant (13) and Gotto et al (11).

[b,c] Abbreviations: very low density lipoprotein, VLDL; intermediate density lipoprotein, IDL; low density lipoprotein, LDL; high density lipoprotein, HDL; triglyceride, TG; cholesterol ester, CE; and phospholipid, PL.

The fate of lipoproteins is largely determined by the amount and the type of protein present on the surface. The protein composition of various lipoprotein classes is shown in Table 2. In human lipoproteins, apoA-I and apoA-II are the two major proteins of HDL, whereas apoB is the major protein of LDL and also comprises about half the protein of VLDL. The apoCs (i.e., C-I, C-II and C-III) are present in chylomicrons and VLDL and, to a minor extent, in HDL (13, 17). Likewise, apoE is a constituent of triglyceride-rich lipoproteins and is also detectable in HDL (17), especially the large HDL particles such as HDL_1, which is present in low concentrations in normal human plasma.

Table 2. Protein composition of human plasma lipoproteins*

Lipoprotein	Major	Minor	Trace
Chylomicrons	ApoB and apoC	apoE	apoA-I and apoA-II
VLDL	ApoB and apoC	apoE	apoA-I and apoA-II
IDL	ApoB and apoB	apoC	--
LDL	ApoB	--	apoC
HDL_2	ApoA-I and apoA-II	apoC	apoE and apoD
HDL_3	ApoA-I and apoA-II	apoC	apoE and apoD

* Adapted from Myant (13).

Bovine plasma lipoproteins are also commonly separated into VLDL, LDL and HDL fractions according to their hydrated densities, although this might not be an appropriate procedure because there is an overlap in the density of the various functional types of lipoproteins in this species (6). As with most other mammals, HDL is the predominant lipoprotein class in cattle, accounting for up to 90% of the total plasma lipoproteins (6, 16). Bovine HDL is characterized by its high concentration of cholesterol esters and by the presence of large HDL particles (18-20). These large HDL particles normally account for half of total bovine plasma HDL in lactating cows (18-20). They are distributed over the density interval of 1.06 to 1.12 g/ml and range in particle size from 12 to 16 nm in diameter (6, 18, 21). Such HDL particles are larger than human HDL_2 and are normally absent from human plasma. However, the large bovine HDL appear to posses physicochemical characteristics similar to those of HDL_1 from normal rat plasma (14, 16) or HDL_C isolated from animals such as pigs when they are fed a high-cholesterol diet (22). Unlike HDL_1 or HDL_C, however, the bovine

particles do not appear to contain apoE (6, 10, 23). While the majority of bovine α-lipoproteins can be isolated in HDL fraction, some have a density beyond the lower density limit of the HDL and are usually partitioned into LDL fraction (6, 19). Thus, unlike LDL from other species, bovine LDL contains α-lipoproteins as well as β-lipoprotein fraction (6, 19). These physicochemical properties of bovine HDL/α-lipoproteins reflect the unique features of plasma cholesterol transport in the cattle.

B. Lipoprotein metabolism and cholesterol transport

The liver and small intestine are the primary organs for lipoprotein synthesis and secretion. In plasma, the lipoproteins secreted from these organs undergo extensive metabolic transformation. Through interactions with enzymes and cell surface receptors, the apoproteins direct each lipoprotein to its site of metabolism. Many of the functions associated with apoproteins are summarized in Table 3 while the major pathways for the transfer of cholesterol through lipoprotein conversion are depicted in Figure 1.

Input of cholesterol into the plasma can come either from endogenous synthesis or from exogenous sources, i.e., dietary cholesterol. However, due to the low content of cholesterol in ruminant diets, the exogenous supply of cholesterol to ruminants must be very limited. Dietary cholesterol supply to this species may be further limited by the action of ruminal microflora which hydrogenate cholesterol in the rumen (24). Thus, ruminant animals must rely on endogenous cholesterol synthesis for cholesterol input. Cholesterol synthesized by the small intestine is exported to the plasma pool mainly as a component of chylomicrons. Upon entering the general circulation, the triglyceride core of chylomicrons is rapidly

hydrolyzed by lipoprotein lipase (LPL) on the endothelium of peripheral capillaries, with apoC-II acting as activator for the enzyme (11). During this process, chylomicrons are reduced in mass and the core becomes enriched with cholesterol esters. In addition, the chylomicrons acquire additional apoproteins, including apoE, leading to the formation of chylomicron remnant particles. As the size of the chylomicrons diminishes, the surface components, including unesterified cholesterol, are transferred to HDL (11, 14). The chylomicron remnants are removed from the plasma by the liver via hepatic receptors specific for apoE (25).

Table 3. Metabolic Roles of Plasma Apolipoproteins in Lipid Transport*

Function	Apolipoprotein
Lipoprotein biosynthesis/secretion	B-48 (intestine)
	B-100 (liver)
Enzyme activation	
Lipoprotein lipase	C-II
Lecithin-cholesterol acyltransferase	A-I
	C-I
	A-IV
Interaction of lipoproteins with cellular receptors	
LDL receptor recognition (B/E receptor)	B-100
VLDL/remnant receptor (E receptor)	E
HDL receptor	A-I; A-II
Inhibition of interaction with hepatic receptors	C-I
	C-II
	C-III

* Adapted from Gotto et al (11).

Ruminant animals also possess the capability to produce chylomicrons from the intestine (6, 26) and the resulting particles also appear to be removed by the liver (27). However, due to the low lipid content of ruminant diets, production of chylomicrons by ruminant intestine normally might occur at a very low rate. Moreover, the ingesta in the ruminant digestive tract are released from the rumen-reticulum in a continuous manner (28). The low intake of dietary fat plus this digestion and absorption pattern means a small continuous supply of dietary lipids to the small intestine of the cattle. This has been suggested to result in production of VLDL-like particles by the ruminant intestine, which have lower triglyceride content and a higher hydrated-density than typical chylomicrons (6, 29). Thus, plasma concentrations of typical chylomicrons are usually low in ruminants (6, 29). Nevertheless, cholesterol molecules required for intestinal triglyceride-rich lipoprotein production in ruminants are mainly synthesized within individual enterocytes, where the rate of cholesterol synthesis appears to be determined by the amount of triglycerides synthesized and secreted by these cells (30).

The liver exports cholesterol to the circulation as a component of VLDL (Figure 1). The VLDL particles are, like chylomicrons, also rich in triglyceride but they contain a larger amount of cholesterol ester. The major apoproteins in VLDL are apoB-100 and apoE (11, 17). After secretion from the liver, much of the core triglyceride of VLDL is removed in the capillary beds in a manner analogous to chylomicron metabolism (11). Concomitantly, some surface constituents, especially free cholesterol, are transferred to HDL. The remaining surface components and core lipids form IDL (11, 17). A significant fraction of IDL is taken up by liver, while the remainder undergoes further lipolysis of triglyceride and phospholipid

to give rise to LDL. The precise mechanism for this lipolysis process is unclear, but it appears to involve the action of hepatic lipase (31). During the conversion of VLDL to LDL, apoE is lost from the particle and only apoB-100 is retained (31). LDL is greatly enriched in cholesterol ester, with apoB-100 being the only significant protein component. LDL delivers cholesterol to peripheral tissues for utilization as well as to the liver for catabolism. Its uptake is mediated by the well-characterized LDL (or apoB/E) receptor, which is present on most cells (32). The apoprotein profile of ruminant VLDL appears to be similar to that of nonruminant species (6, 10, 23), suggesting that the above pathways for plasma VLDL metabolism might be applicable to ruminant animals. Nonetheless, ruminant liver is known to have very low capacity for VLDL secretion (10, 26). Thus, the hepatic contribution to the plasma cholesterol pool through VLDL secretion might be very small in this species.

HDL is formed in the circulation from precursors secreted by the liver and intestine (Figure 1). The major apolipoproteins (i.e., apoA-I and apoA-II) of HDL are synthesized in both the liver and small intestine. A significant portion of HDL apolipoproteins are transferred from triglyceride-rich lipoproteins during their lipolysis. The initial particle secreted into the circulation is a discoidal lipoprotein composed of phospholipids, apolipoproteins, and small amounts of free cholesterol (14, 33). Because of the low lipid content of nascent HDL particles, input of cholesterol to the circulation as these HDL particles must be very limited. It has also been proposed that some apoA-I are secreted as the pure protein from the liver and that even the discoidal particles can be formed in the circulation (34, 35). The discoidal HDL is transformed into the mature, spherical HDL through the action of lecithin-cholesterol acyltransferase

(LCAT) (33), which hydrolyzes C18:2 fatty acid from surface lecithin and reesterifies it to free cholesterol (36). HDL can be viewed as the site of plasma cholesterol esterification. This is especially true for animal species such as ruminants, which have very low hepatic acyl-CoA:cholesterol acyltransferase activity (37). This enzyme is responsible for the cholesterol esterification inside the cell. In fact, almost all of the cholesterol esters in ruminant plasma are generated through the LCAT reaction (37, 38). The free cholesterol substrate for LCAT may come from other lipoproteins or from cell membranes, whereas the HDL phospholipid consumed by the this reaction appears to be mainly derived from the surface coat of triglyceride-rich lipoproteins during remnant formation (14). It is apparent that the majority of substrates for HDL formation come from the redundant surface lipids and proteins generated during the lipolysis of the triglyceride-transporting lipoproteins, i.e., VLDL and chylomicrons. Thus, HDL might be an essential product of triglyceride transport.

As with the cholesterol associated with other lipoprotein classes, HDL-carried cholesterol is also delivered to the liver and other tissues for metabolism. A portion of HDL cholesterol is transported directly with HDL particles, whereas other portions may be transferred to other lipoprotein classes and then delivered to the cells along with these lipoproteins. A detailed discussion of the pathways for HDL cholesterol uptake will be presented in section III B. The major apoproteins of HDL, apoA-I and apoA-II, are catabolized slowly *in vivo* . The circulating half-time of HDL protein in humans is 3-6 days (39). Liver and kidney are the major sites of catabolism of apoA-I (14, 39).

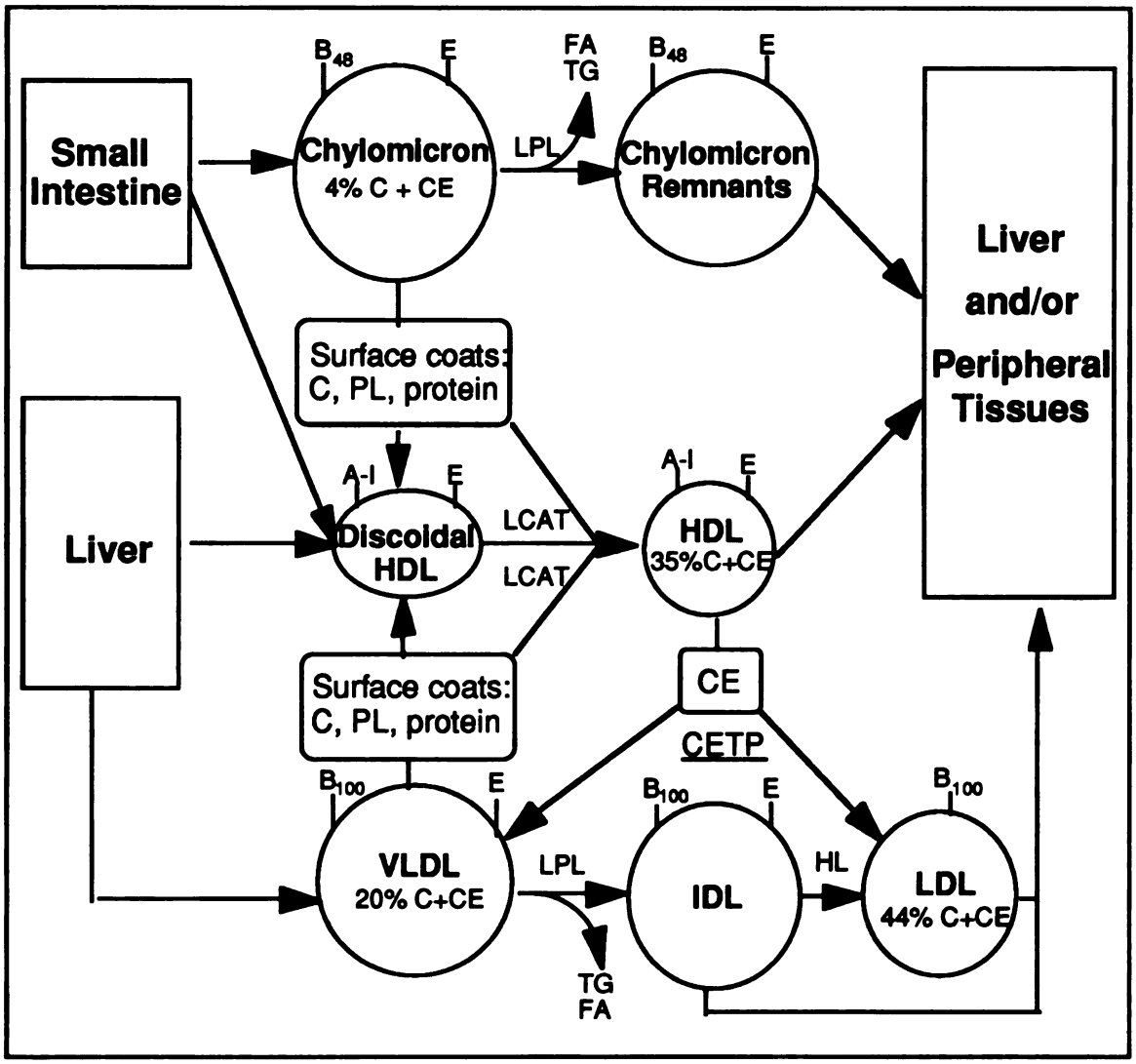

Figure 1: Overall summary of pathways of plasma lipoprotein metabolism and cholesterol transport. Lipoprotein components include unesterified cholesterol (C), cholesterol esters (CE), phospholipids (PL), triglyceride (TG) and fatty acids (FA). The letters projecting from each particle refer to the apolipoproteins: A-I, B and E, which mediate lipoprotein cholesterol metabolism. The important regulators of lipoprotein metabolism include lipoprotein lipase (LPL), lecithin-cholesterol acyltransferase (LCAT), hepatic lipase (HL), and cholesterol ester transfer protein (CETP).

C. Regulation of HDL subpopulation distribution

As mentioned earlier, there are several subpopulations of HDL particles in the plasma. These HDL subspecies undergo dynamic interconversions in the circulation and many of them are remodeled by acquisition of surface components of triglyceride-rich lipoproteins. It appears that the large HDL particles are formed during active lipolysis of triglyceride-rich lipoproteins when large amounts of surface lipids are transferred to small HDL particles such as HDL_3 (14). Thus, the concentration of HDL_2 in human plasma appears to be proportional to the rate of triglyceride transport in the circulation (40). Lipoprotein lipase is the key enzyme required for the hydrolysis of triglyceride-rich particles. The activity of this enzyme has been shown to be positively correlated with plasma HDL cholesterol concentrations in human populations (41). The elevated concentration in HDL cholesterol observed in human subjects with higher levels of lipoprotein lipase activity is primarily due to an increase in HDL_2 associated cholesterol (41, 42). Likewise, transgenic mice expressing human lipoprotein lipase have higher plasma concentrations of HDL cholesterol than their non-transgenic litter mates (43, 44). Conversely, a genetic defect in lipoprotein lipase in humans is known to result in reduced HDL_2 cholesterol concentration with a concomitant increase in VLDL- and IDL-cholesterol content (45).

The large HDL particles can be converted back into smaller particles in the plasma. The underlying mechanism for this conversion is still unclear. However, in species such as rabbits and humans, which have high levels of plasma cholesterol ester transfer activity (46), considerable quantities of cholesterol esters carried by the large HDL particles are transferred to other lipoprotein classes such as VLDL and LDL in an

exchange of triglycerides (47). The triglyceride which is transferred into the HDL particles can be rapidly hydrolyzed by hepatic lipase (14, 18). Moreover, since hepatic lipase also possesses potent phospholipid hydrolysis activity, it could partially deplete the HDL particles of surface phospholipid molecules (48). The hydrolysis of phospholipid has been suggested to be a critical step in the remodeling of HDL particles (48, 49). Genetic absence of either cholesterol ester transfer protein (47, 50) or hepatic lipase (51) has been shown to cause a remarkable increase in HDL_2 cholesterol concentration in humans. Furthermore, comparison of the plasma cholesterol ester transfer activity and lipoprotein profile among different animal species reveals a clear association between low levels of this activity in the plasma and the prominence of a subclass of large HDL, i.e., HDL_1. For example, dogs, rats, and mice, with low levels of plasma cholesterol ester transfer activity (46), have high plasma concentration of HDL_1 (16). Conversely, injection of cholesterol ester transfer protein into the rat results in reduction of the large HDL_1 species and formation of smaller HDL (52). This provides direct evidence for the dependence of the presence of prominent HDL_1 on low plasma cholesterol ester transfer activity. Taken together, there is convincing evidence that cholesterol ester transfer protein and hepatic lipase play a pivotal role in the conversion and metabolism of the large HDL particles. Interestingly, neither of these two proteins appears to be expressed at a discernible level in ruminants (46, 53-55). Concomitantly, bovine lipoprotein contains in high proportion a subclass of HDL_1-like large HDL particles (6). Thus, the accumulation of such HDL particles in bovine plasma might be due to the apparent absence of plasma cholesterol ester transfer activity and hepatic lipase in this species.

III. ROLE OF LIVER IN MAINTENANCE OF CHOLESTEROL HOMEOSTASIS

As the major site of both the synthesis and catabolism of plasma lipoproteins, liver has a central role in cholesterol metabolism and in the regulation of lipoprotein cholesterol concentration in the circulation. The major pathways concerning cholesterol metabolism in the liver are depicted in Figure 2. The control of the hepatic pathways is complex and interrelated. Presumably, perturbation of this system produces wide-ranging effects on lipoprotein cholesterol metabolism.

A. The role of liver in whole body cholesterol synthesis

Like all other mammalian cells, liver cells are capable of synthesizing cholesterol from acetyl CoA. Cholesterol biosynthesis occurs chiefly in the liver cell microsomes and the primary rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) into mevalonic acid, which is catalyzed by HMG CoA reductase (56). The activity of this enzyme is under tight control by feedback inhibition from cholesterol, or more specifically, oxysterol (56, 57). Liver is commonly considered to be the most important site for whole body cholesterol synthesis. This concept arose from early studies (58-60) where assays of rates of cholesterol synthesis were performed *in vitro* using various ^{14}C-labeled substrates such as ^{14}C-acetate. However, it became clear that the ability of different tissues to take up and utilize these ^{14}C-labeled substrates varies considerably and most of these substrates were poorly utilized in extra hepatic tissues (61). Moreover, the specific activity of the ^{14}C-acetyl CoA pool that is the immediate precursor for cholesterol biosynthesis appears to be

disproportionately (relative to the liver) diluted in many tissues by the unlabeled, endogenous acetyl-CoA (61). These observations raise the possibility that the role of the liver in whole body cholesterol synthesis might have been overestimated in some species. More recently, Dietschy and his colleagues have developed a more reliable system for measurement of *in vivo* or *in vitro* cholesterol synthesis rates based on measurements of the incorporation of ^{3}H from $^{3}H_2O$ into digitonin-precipitable sterols in tissues (61, 62). Using this method, these investigators have built up a detailed picture of cholesterol synthesis in the tissues of a number of animal species (2, 63). These recent studies have indeed demonstrated that the contribution of liver cholesterol synthesis to whole body synthesis has been overestimated in some species. Although in species such as the rat and monkey, the liver contributes more than 50% of the whole body cholesterol synthesis, the liver of some other species, including rabbits and guinea pigs, has relatively low activity for cholesterol synthesis. In these latter species, the contribution of the liver to whole body synthesis is less than 20% (1, 2, 63). Thus, it is probable that the quantitative importance of liver in whole body cholesterol synthesis varies considerably among animal species.

In contrast to the experimental animals, information on cholesterol synthesis rates by ruminant tissues is very limited. Only two studies have been reported on the measurement of cholesterol synthesis rates by several tissues from small ruminants, including goats (64) and sheep (30). These studies showed that the liver of these animals has a very low capacity for cholesterol synthesis, whereas the intestine and adipose tissues have high cholesterol synthesis rates (30, 64). Thus, it has been suggested that the liver is a minor site of body cholesterol synthesis in ruminants. However, these early studies used ^{14}C-labeled substrates, including ^{14}C-acetate and

^{14}C glucose, to determine tissue cholesterol synthesis rates. It is known that these substrates are poorly utilized by ruminant liver (65). Thus, the role of ruminant liver in cholesterol synthesis remains to be clarified. Nonetheless, because the ruminant liver does not normally produce significant amounts of VLDL (26), the hepatic contribution to cholesterol in the ruminant plasma pool might be very limited. The intestine of ruminants might be the major contributor of blood cholesterol. This is not only because this organ appears to have high cholesterol-synthesis activity (30, 64), but also because it is the predominant site of triglyceride-rich lipoprotein production (6, 26). Thus, feed intake, or any other dietary factors which potentially affect intestinal lipoprotein production might have a large influence on plasma cholesterol concentrations in ruminants.

B. Hepatic lipoprotein receptors and hepatic clearance of plasma cholesterol

While the liver may play only a minor role in whole body cholesterol synthesis in some species, it nevertheless remains the key organ for the regulation of cholesterol balance within the intact animal. The terminal catabolism of most lipoproteins occurs mainly in the liver by means of receptor-mediated pathways (Figure 2). The best characterized pathway for cellular lipoprotein uptake is LDL receptor-mediated endocytosis. The LDL receptor, discovered by Brown and Goldstein in cultured human fibroblasts (66), is now recognized to be mainly operative *in vivo* on the surface of hepatocytes (32). This receptor is a cell surface glycoprotein, and it binds specifically to apoB-100, the major apoprotein in LDL. It also binds to apoE which is found in several lipoproteins including VLDL remnants and a subspecies of HDL in most of the mammals (67). Thus, this receptor

mediates the removal from the blood of most of these apoB/apoE containing lipoprotein particles. After binding to the receptor, the lipoprotein particles are internalized and then digested in lysosomes (32). The cholesterol esters are hydrolyzed to yield free cholesterol, which may be catabolized into bile acids by the hepatocytes and then secreted into the intestine for their removal from the body (Figure 2). The importance of the LDL receptor in mammalian cholesterol metabolism is best manifested by the examples of genetic defect in this receptor occurring either naturally or experimentally. The genetic deficiency of LDL receptors in humans (32), rabbits (68), and rhesus monkeys (69) causes severe hypercholesterolemia. Likewise, experimental disruption of the LDL receptor gene in mice results in a several-fold increase in plasma cholesterol concentration and the hypercholesterolemia is reversed when expression of the receptor protein is restored in these mice (70). Expression of the LDL receptor is regulated by the cellular cholesterol status (32). The LDL receptor gene is always partially suppressed. This suppression is part of a feedback regulatory mechanism designed to maintain constant intracellular levels of cholesterol in liver and other cells (32). When the intracellular cholesterol level falls, the LDL receptor gene is activated as the cell attempts to obtain more cholesterol. Regulation of the hepatic LDL receptor is a major factor controlling the plasma concentration of atherogenic lipoprotein classes in human. Hence, much interest has been centered on this receptor in hepatocytes and on its dietary, hormonal and pharmacological regulation in physiological and pathological states. The LDL receptor has also been identified in bovine tissues including liver (71). However, the physiological significance of this receptor in regulation of bovine plasma cholesterol metabolism is yet to be determined. In view of the low concentration of LDL

in bovine plasma and the lack of apoE in bovine HDL particles (6, 10, 23), it appears that this receptor might have a less significant role in cattle than other species such as humans or rats, which have either high plasma LDL concentration or large proportion of apoE-rich HDL particles.

The liver also takes up cholesterol of intestinal origin, including dietary cholesterol, which is contained in chylomicron remnants (Figure 2). This uptake occurs by way of a high-velocity, saturable transport system that probably depends on the presence of receptors on the hepatocytes that interact with the apoE of the remnants (72). Since the Km for this transport is very low, the plasma is cleared completely of the particle within minutes (72, 73). The identity of the chylomicron remnant receptor has not been fully established, but a vast body of evidence suggests that the recently cloned LDL-related protein/α-2-macroglobulin receptor is a candidate for this receptor (72-74). Several recent studies (75, 76) have also suggested that hepatic lipase plays an important role in the receptor-mediated endocytosis of the remnant particles by the liver cell. Unlike the LDL receptor, the chylomicron remnant receptor does not seem to be tightly regulated by cellular cholesterol balance (73). However, endocytosis of the remnant particles, like that mediated by the LDL receptor, leads to the same sequence of processing within hepatocytes. It is apoE of the surface of remnant particles that directs these particles towards the liver for clearance. Thus, ablation of the apoE gene in mice causes massive accumulation of remnant particle-associated cholesterol in the blood, especially after the ingestion of a high-fat or high-cholesterol meal (77, 78). Although the expression of the remnant receptor in the ruminant liver has not been examined, liver has been shown to be the major site of removal of chylomicron cholesterol in sheep (27). Moreover, apoE has been identified in

the bovine triglyceride rich-lipoprotein particles (6, 23). Thus, it is likely that the cholesterol carried in chylomicrons in bovine plasma is also cleared by the liver.

The liver is an important site for the catabolism of HDL. Because HDL can serve as an acceptor for excessive cellular cholesterol from the extrahepatic tissues (39), the HDL particles deliver to the liver for catabolism not only the cholesterol originating from liver and intestine, but also cholesterol removed from a variety of peripheral tissues (Figure 2). This is the so-called reverse cholesterol transport system and is believed to play an important role in maintenance of cholesterol homeostasis in the body. The mechanisms that are responsible for the removal of HDL, or of the cholesterol carried in HDL, from the plasma are complex and still poorly understood. Four pathways have been proposed to play a role in this process (Figure 2). The first pathway depends on plasma cholesterol ester transfer activity. As noted earlier, this activity mediates the net transfer of HDL cholesterol ester to VLDL or LDL, apparently in exchange for triglyceride (47). The transferred cholesterol esters are then cleared from the circulation via the LDL receptor-mediated pathway. The cholesterol ester transfer activity in plasma is high in some species such as humans and rabbits (46). This pathway has been suggested to play the most important role in the disposition of HDL-cholesterol in humans (47, 73). A genetic defect in this protein causes a marked increase in HDL and in plasma total cholesterol concentrations (50, 79). However, plasma cholesterol ester transfer activity is very low in most mammals including ruminants (46), hence, this pathway might not be operative in cattle.

Another pathway for HDL cholesterol removal involves the interaction of LDL receptors with apoE contained in some HDL particles,

leading to the LDL receptor-mediated endocytosis of these particles by hepatocytes. ApoE-rich HDL particles can be found in the plasma of most mammals. In species such as rats, up to 60% of total HDL cholesterol is carried in apoE-rich HDL particles (15, 67). Presumably, this pathway might play an important role in the hepatic HDL cholesterol uptake in this species. In contrast, no apoE nor apoE-like protein has been found in bovine HDL or α-lipoproteins (6, 23). This suggests that the LDL receptor-mediated pathway plays a minimal role in hepatic uptake of bovine HDL particles.

A third pathway for HDL cholesterol removal is dependent on the action of hepatic lipase. Owing to its phospholipase A activity (39, 80), it appears that this enzyme is able to hydrolyze HDL phosphatidylcholine at the surface of hepatocytes or endothelial cells, leading to the production of lysophosphatidyl choline (80). This in turn causes fusion of the surface of HDL to the external leaflet of the plasma membrane, thereby facilitating transfer of cholesterol esters into the cell (39, 80). In this case, the enzyme would exert a "receptor" function. This process leads to the selective uptake of cholesterol esters from HDL without the latter being internalized. The role of hepatic lipase in promoting HDL cholesterol ester transfer into cells has been well documented in cell culture (39) and rat liver (81, 82), though the quantitative significance of this pathway is poorly understood. Nonetheless, because the ruminant liver is almost devoid of hepatic lipase activity (53-55), it is obvious that this pathway might not be operating in ruminant animals.

The final pathway for hepatic HDL cholesterol uptake is mediated by a specific, saturable process in which the hepatocytes remove cholesterol esters from the apoE-free HDL particles and release the apoproteins without the latter necessarily being degraded (39, 83, 84). This process leads

to a greater fractional rate of the uptake of cholesterol esters than that of HDL apolipoproteins. When HDL containing ^{125}I-tyramine cellobiose-labeled apo A-I and [^{3}H]-cholesterol ethers (non-hydrolyzable tracers for apoprotein and cholesterol ester, respectively) was injected into rats, it was observed that hepatic uptake of the cholesterol ester tracer was at least two-fold more rapid than that of the apoprotein tracer (85). The underlying mechanism(s) of this pathway is still poorly defined. The binding of HDL particles to the surface of the cell appears to be the initial event in this pathway and thereafter, the cholesterol esters could be delivered to the cell through either of the following two separate mechanisms. One is by the internalization and lysosomal degradation of the whole HDL particle (14, 39, 84), while the other is by selective uptake of cholesterol esters without the internalization of the HDL particles (83, 86) after which the cholesterol esters appear to be hydrolyzed extralysosomally (87) by a neutral cholesterol ester hydrolase (88). The former mechanism might be mediated by a specific receptor. In fact, a high-affinity HDL-binding site has been identified on hepatocytes (14, 39, 89) and on liver membranes from various species including cattle (90, 91). Furthermore, several membrane-bound HDL binding proteins have also been purified from hepatic tissues (92, 93). These observations support the existence of a putative HDL receptor on the cell surface. The role of such a receptor in selective uptake is yet to be clarified. Regardless of the underlying mechanism, this pathway has been shown to play a quantitatively important role in the clearance of HDL cholesterol esters by the liver. It is the dominant pathway for the hepatic uptake of HDL cholesterol esters in the rat (94). Even in species such as rabbits, which have about four times the plasma cholesterol ester transfer activity of humans (46), a significant amount of HDL cholesterol ester is

taken up by the liver through this pathway (95, 96). Because the other three pathways, as discussed above, do not appear to be operative in cattle, it could be readily imagined that this pathway plays a particularly important role in the catabolism of HDL cholesterol in the bovine species. It is intriguing to speculate that this species might serve as a unique animal model for the study of hepatic HDL cholesterol uptake through this pathway.

C. Biliary sterol formation and enterohepatic circulation of bile salts

The lipoprotein cholesterol reaching the liver can be reutilized either for biogenesis of biomembranes or for synthesis and secretion of lipoproteins. It can also be re-esterified and stored as cholesterol esters in the cell. Alternatively, cholesterol can be secreted into the biliary system either as intact cholesterol or, after conversion, as bile acids (Figure 2). The hepatic conversion of cholesterol to bile acids is a major mechanism whereby cholesterol is eliminated from the body (13, 63).

The first and rate-limiting step of this process is hydroxylation at the 7α position of the sterol nucleus, catalyzed by a microsomal P_{450}-dependent enzyme, cholesterol 7α-hydroxylase (97). Bile acid synthesis appears to be under feedback regulation by bile acids returning to the liver (63, 97, 98). There is also some evidence suggesting that bile salt synthesis is regulated by free cholesterol availability in some specific precursor pool (99, 100). The cholesterol substrate for bile acid synthesis can come either from cholesterol synthesized *de novo* in the liver, or from the uptake of lipoprotein cholesterol. However, under normal physiological conditions, the majority of cholesterol utilized for bile acid synthesis is derived from the uptake of plasma lipoproteins (63, 101-103). Furthermore, in rats, it has

been shown that the uptake of esterified cholesterol from HDL rather than from LDL is more efficiently coupled to the formation of bile acids (101, 104, 105). It appears that free cholesterol from HDL_2 is preferentially utilized for bile acid synthesis (63, 101, 102, 104). Free cholesterol from chylomicron remnants is also a preferred substrate for bile acid synthesis (63, 101).

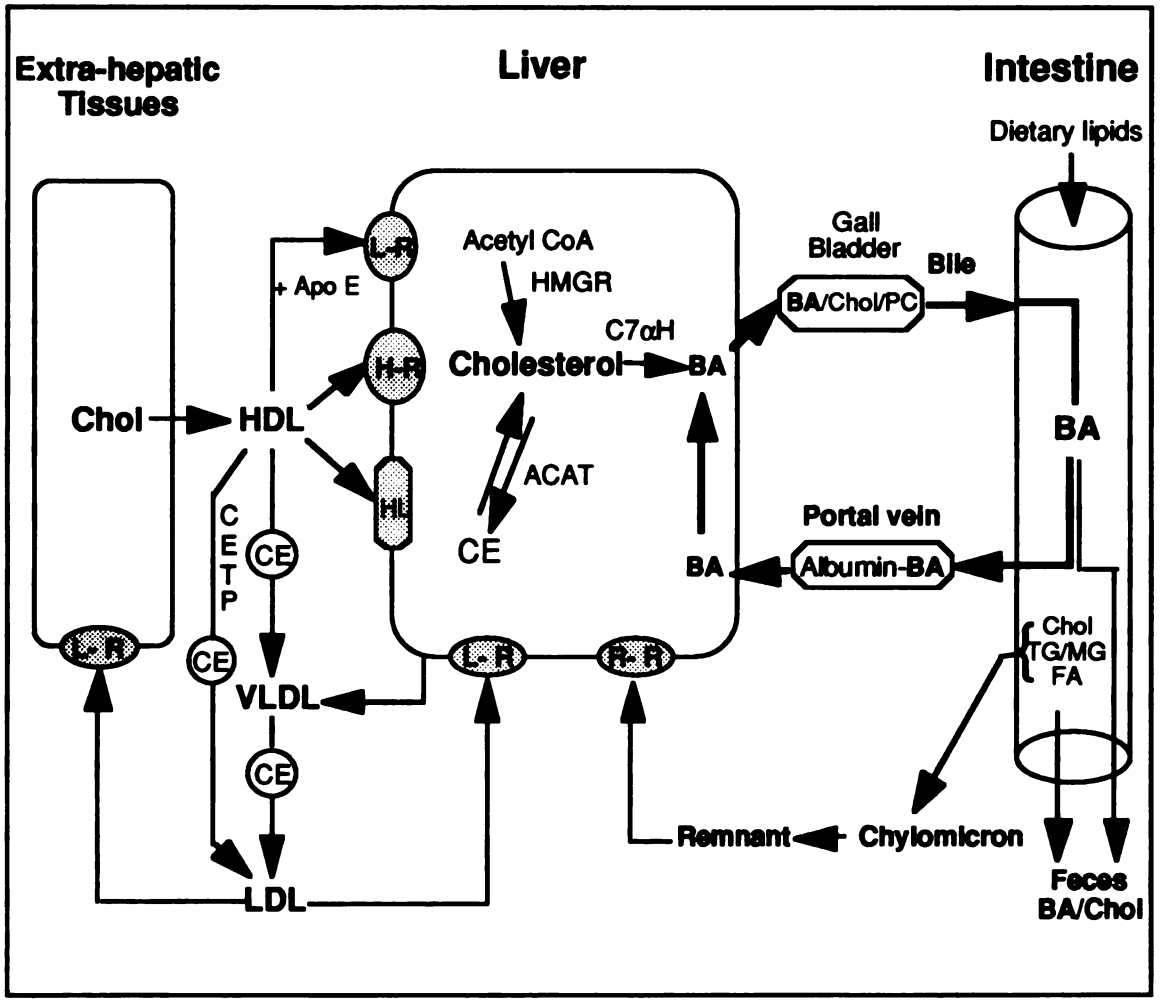

Figure 2: Lipoprotein receptor-mediated pathways for hepatic clearance of plasma cholesterol and their interactions with enterohepatic circulation of bile salts. The meanings of the abbreviations used in this schematic are as follows: HMGR, HMG-CoA reductase; C7aH, cholesterol 7 α-hydroxylase; ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesterol ester; R-R, remnant receptor; L-R, LDL receptor; H-R, HDL receptor; HL, hepatic lipase; CETP, cholesterol ester transfer protein; Chol, cholesterol; BA, bile acids; PC, phosphatidylcholine; TG, triglyceride; MG, monoglyceride; and FA, fatty acids.

Like the cholesterol pool utilized for bile acid synthesis, preformed cholesterol is the preferred source of cholesterol secreted into the bile (63, 102, 103). Free cholesterol carried in the HDL fraction, especially HDL_3, is preferentially used for the formation of biliary cholesterol (102, 106). The rate of cholesterol secretion, however, is determined by the species and rate of bile acid secretion, as well as the rate of biliary phospholipid secretion (63, 107). The lipoproteins, presumably HDL, are also the major source of biliary phospholipids (108).

In human bile, bile salts comprise more than 60% of total solute weight, with phospholipids accounting for about 20% and free cholesterol, about 5% (109). Bile also contains bile pigments and a mixture of proteins and electrolytes. After secretion from the liver, the bile is stored in the gallbladder until this organ, under the influence of gastrointestinal hormones, discharges it into the intestine (109). Bile salts in the duodenum and ileum aid in the digestion and absorption of dietary fats. Small quantities of bile salts can be absorbed by passive diffusion along the length of the intestine, whereas the major portion is absorbed by an active transport system in the terminal ileum (109, 110). As shown in Figure 2, after entering the portal vein, the absorbed bile salts are bound to albumin and then transported rapidly to the liver, cleared efficiently, and resecreted (109, 111). This enterohepatic circulation (EHC) of bile salts is a highly efficient process and more than 95% of the bile salts are reabsorbed into the circulation (109-111). Thus, to maintain EHC, the liver normally only has to synthesize a small amount of bile salts to replace the fecal loss. The details of EHC have been worked out in a number of animal species (111-115). For example, the quantitative aspect of this circulation in humans is depicted in Figure 3. In contrast to other species, very little information on EHC is

available for ruminants. Based on some early studies, it appears that one of the characteristics of EHC in ruminants is the pattern of continuous secretion and the lack of a clear diurnal change in bile salt secretion (116, 117). This might occur as a result of the continuous flow of digest from the rumen into the lower digestive tract (28).

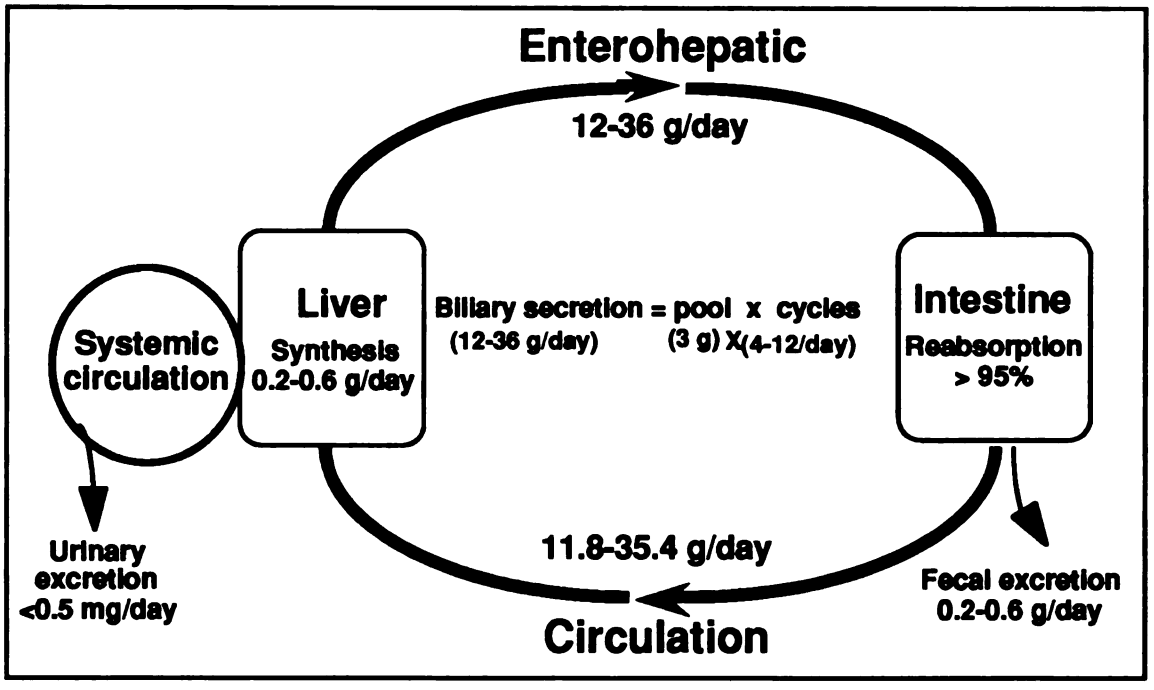

Figure 3: Mass balance of bile acids around the enterohepatic circulation showing typical kinetic values from healthy humans. The pool size of bile acids in human EHC is about 3 g. This small pool of bile acids recirculates through the intestinal tract 4-12 times per day, resulting in a large daily flux of bile acids. Due to the high efficiency of reabsorption of bile salts by the intestine and uptake of bile acids by the liver, however, only a very small proportion of bile acids is lost from the EHC. Thus, the liver is only required to synthesize a small amount of bile acids to maintain a normal EHC. The data presented in this diagram were taken from Carey et al. (109).

D. Effects of interruption of EHC

The maintenance of a normal EHC relies on the efficient reabsorption of bile salts by the intestine. Interruption of EHC can be achieved either by surgical procedures such as ileal resection and biliary diversion, or by pharmacological means such as bile acid sequestrant (e.g., cholestyramine) treatment. These procedures exaggerate the fecal loss of bile sterols and diminish the amount of bile salts returning to the liver. This results in the release of feedback inhibition of bile acid formation in the liver and hence, the rate of bile acid synthesis increases (63, 107, 112, 118). Due to the large daily flux of biliary sterols through the intestinal tract, however, a slight increase in the percentage of loss requires a large proportional increase in synthesis to maintain the EHC. If the interruption is only partial, the increase in bile acid synthesis may be sufficient to maintain a normal rate of secretion of bile salts into the intestine and the size of bile acid pool remains unchanged. For example, it has been shown that rhesus monkeys can compensate for up to a 20% biliary loss and maintain a normal bile acid pool size by increasing their production rate of the acids (112). If, however, the rate of fecal loss of bile salts exceeds the maximal capacity of the liver for synthesizing bile acids, the bile salt pool decreases and a new steady state is achieved in which the rate of secretion of bile salts is subnormal or greatly reduced. Total diversion of bile produces a rapid and dramatic drop in bile secretion rate while the rate of bile acid synthesis is maintained at the maximal level (109, 112, 113, 115).

The augmented bile acid synthesis rate resulting from the interruption of EHC in turn largely enhances the demand for cholesterol in the liver cell. In response to this, the liver increases its capacity for *de novo* cholesterol synthesis. In rats, a five to ten-fold increase in the rate of

hepatic cholesterol synthesis can be observed after bile diversion or cholestyramine treatment (101, 107, 118). Similar results have also been reported in a number of other species (63, 101, 119). The increased demand for cholesterol can also cause the mobilization of hepatic cholesterol stores, i.e., the esterified cholesterol in the liver (101, 119). Under most circumstances, however, the local cholesterol supply is not sufficient to meet the increased cholesterol requirement. Hence, the liver has to increase its recruitment of lipoprotein cholesterol from the circulation. In response to the interruption of EHC, increased expression of LDL receptor on the hepatocytes is usually induced (32, 63, 120-122). This may serve as an important mechanism for the enhanced uptake of lipoprotein cholesterol by the liver. Yet, the effect of interruption of EHC on other hepatic pathways of lipoprotein uptake is unclear.

As a result of the enhanced hepatic uptake of lipoprotein cholesterol, however, plasma cholesterol concentration may be reduced. The hypocholesterolemic effects of ileal bypass surgery or bile resin therapy have been well documented in humans (101, 120, 123) as well as in laboratory animals such as rabbits (121, 124) and hamsters (119, 125). These treatments cause a reduction of 25-35% in plasma total cholesterol concentrations in these species and the reduction occurs almost exclusively in LDL cholesterol. Furthermore, because these treatments fail to reduce plasma cholesterol in patients with homozygous hypercholesterolemia (126), who lack functional LDL receptors (32), their hypocholesterolemic effects appear to be entirely dependent on the ability of the liver to increase its uptake of plasma cholesterol through the LDL receptor-mediated pathway. In contrast to LDL, plasma VLDL concentration may increase in response to these treatments in humans (101, 123, 127, 128) and rhesus

monkeys (129). This has been shown to occur as a result of an increased VLDL production rate (127-129), although the mechanism is still poorly understood. Likewise, HDL cholesterol concentration is not affected or is slightly increased during bile resin therapy (63, 101) or following ileal bypass surgery in humans (123). Nonetheless, there appears to be a significant alteration in distribution of cholesterol among HDL subfractions. These treatments have been shown to increase the plasma concentration of HDL_2 cholesterol with minimal effects on HDL_3 (101, 123). While the effect of treatment on HDL fractional rate of clearance is not known, the production rate of HDL might be expected to increase as the turnover rate of VLDL is known to be increased (127, 128). In addition, it has been shown that intestinal production of nascent HDL particles is enhanced during biliary diversion in the rat (130). Furthermore, the esterification of cholesterol through the LCAT system also appears to be induced by cholestyramine treatment in humans (101), leading to the accumulation of cholesterol esters in HDL_2.

Most of the previous studies on the effects of interruption of EHC were focused on its effectiveness in lowering LDL cholesterol, the atherogenic cholesterol in humans. Because of this focus, these studies have been on species such as humans, rabbits and hamsters, in which LDL serves as the major carrier of plasma cholesterol (16). Consequently, the understanding of such effects on species with HDL as the predominant plasma lipoprotein is very limited. However, it is known that in one of these species, i.e., the rat, induction of fecal loss of biliary sterols does not result in appreciable reductions in plasma cholesterol concentration (113, 118, 131). The reason for this difference between the rat and the other species is not known. However, the explanation might be found in the higher hepatic cholesterol

synthesis rate of the rat, compared to most other mammals (1, 2). This would allow the rat to alter its rate of cholesterol synthesis over a very wide range to adapt to changes in fecal loss of sterols (118). It is not known whether interruption of EHC could reduce plasma cholesterol concentration in other "HDL" mammalian species such as cattle. As noted earlier, however, unlike the rat, cattle appear to have low rates of hepatic cholesterol synthesis. Thus, it would be interesting to examine the effects of interrupting EHC on hepatic cholesterol metabolism and on plasma cholesterol concentration in bovine species.

IV. IMPLICATIONS OF UNIQUE FEATURES OF PLASMA CHOLESTEROL METABOLISM IN DAIRY COWS

Most comparative lipoprotein studies have been undertaken with the objective of increasing our understanding of human hyperlipoproteinemia and atherosclerotic disease processes. Research on bovine lipoprotein metabolism, on the other hand, has been conducted primarily with the objective of developing ways to improve productivity of ruminant animals. With respect to dairy cattle, the major goals of lipoprotein investigations have been related to either animal health or milk fat synthesis and secretion. Although the understanding of bovine lipoprotein metabolism is still incomplete, these previous studies have revealed some interesting phenomena in bovine plasma lipoprotein cholesterol metabolism. Knowledge of the underlying mechanisms for these phenomena would provide insight into the role and regulation of plasma cholesterol in dairy cows.

A. Gestation-lactation cycle related changes in plasma cholesterol concentration

The large fluctuation in plasma cholesterol concentrations that occurs through the gestation-lactation cycle is a well documented phenomenon in dairy cattle. As early as in 1931, Maynard and co-workers (132) demonstrated that blood lipids decrease prior to parturition and then begin to increase again during lactation. Of all the lipid classes, the greatest changes and the highest concentrations were in serum total cholesterol. Since then, this finding has been corroborated frequently by other investigators (8-10, 133-137). Typically, the maximum cholesterol concentration occurs about midway during lactation and the minimum concentration at or near parturition, with a two-three fold difference between these two values. The decline in plasma cholesterol concentration during the peripartum period is not peculiar to dairy cows since some other species, including guinea pigs (138), non-human primates (139) and rabbits (140) also undergo a marked reduction in serum cholesterol concentration in the later stages of pregnancy. However, the large increase in plasma cholesterol during lactation appears to be unique to cattle.

B. Possible mechanisms for the lactational hyperalphalipoproteinemia

The lactational hypercholesterolemia of cattle is due to a marked increase in HDL, occurring mainly in the cholesterol ester-enriched, large HDL-particle fraction (6, 19, 134). Thus, it can be generally considered as hyperalphalipoproteinemia. The underlying mechanism(s) has not been examined. However, as discussed earlier, the formation of large HDL particles in other animal species is known to be related to triglyceride transport activity. Plasma triglyceride-rich lipoproteins are important

sources of triglyceride for milk fat synthesis (29, 141). During lactation, the mammary lipoprotein lipase activity is elevated (142), leading to extensive lipolysis of triglyceride-rich lipoproteins by the mammary tissue. Moreover, the production rate of triglyceride-rich lipoproteins by the intestine might also be largely increased during midlactation when feed intake has peaked. When combined, the increased rates of lipolysis and triglyceride-rich lipoprotein secretion would result in an increased rate of lipoprotein turnover. Surface remnants of these lipoproteins would provide cholesterol substrate for the LCAT system, as well as other components for HDL formation. In addition, as the adipose tissue is depleted during early lactation, efflux of cholesterol from depot stores into the blood provides additional cholesterol for HDL cholesterol ester synthesis through the LCAT reaction.

While the rate of formation of the cholesterol ester-enriched large HDL particles is increased during lactation, cattle appear to lack the ability to efficiently metabolize these particles. This is because plasma cholesterol ester transfer activity and hepatic lipase are almost absent in the bovine species (46, 53, 54). As noted earlier, both of these two factors play a critical role in the conversion of large HDL particles into smaller ones for metabolism. Moreover, it has been shown in humans (143) and other species (14) that the fractional catabolic rate of HDL-apoproteins is inversely correlated with particle size and the ratio of cholesterol esters/(apo AI + apo AII), implicating that the removal rate of the large bovine HDL particles might be very slow. Taken together, the marked increase in HDL-cholesterol during lactation appears to be the result of an active lipolysis process combined with the relative low capacity of the ruminant liver to dispose of remnant particles and their metabolites. Nonetheless, the

accumulation of these large HDL particles might serve as a reservoir of apoCII for activating lipoprotein lipase and also of essential fatty acids. In ruminants, essential fatty acids are stored in plasma mainly in form of phospholipids and cholesterol esters (65).

C. Peripartum hypocholesterolemia and its association with cow diseases

The mechanism(s) responsible for the fall of plasma cholesterol during the peripartum period is still poorly understood. Feed intake is usually reduced shortly before parturition and during early lactation. Presumably, the intestinal production of triglyceride-rich lipoproteins and thus, cholesterol synthesis is reduced in association with the depressed feed intake. This in turn results in a diminished entry rate of cholesterol into the plasma pool as the intestine is the major contributor of plasma triglyceride-rich lipoproteins in cattle (26). On the other hand, the removal of blood cholesterol might be increased as a result of the formation of colostrum. Plasma cholesterol has been shown to be an important source of milk cholesterol in several species (144, 145) including ruminants (146). Bovine colostrum is enriched with cholesterol, phospholipids and β-carotene but not with triglycerides (137, 147), as compared to mature milk, suggesting the possible mammary uptake of LDL or/and HDL particles. Although this does not appear to have been experimentally proven, it is unlikely that mammary tissues lack the lipoprotein receptors as both LDL receptor and HDL receptor appear to be ubiquitously distributed among mammalian cells.

Concomitant with the peripartum fall in plasma cholesterol concentration, there is a sharp increase in the plasma concentration of

non-esterified fatty acids. A number of studies (148-150) have demonstrated that plasma cholesterol ester transfer activity can be activated *in vitro* by free fatty acid. Although this activity is low in normal bovine plasma (46), it has yet to be determined whether the activity is increased by non-esterified fatty acids during the peripartum period. If this is the case, this activity might mediate the transfer of cholesterol esters from HDL to LDL, leading to an enhanced catabolic rate of HDL cholesterol via the LDL receptor-mediated pathway.

While the mechanism for the regulation of bovine plasma cholesterol concentration has yet to be clarified, previous studies have indicated that the degree of reduction in plasma cholesterol concentration during the peripartum period is associated with morbidity and mortality in dairy cattle (7, 151, 152). The nature of the association between blood cholesterol and disease incidence is not clear, but blood cholesterol concentration and hepatic fat concentration are inversely related in peripartum cows (7-10, 152). It is generally assumed that fatty liver has a direct adverse effect on health and that hypocholesterolemia occurs secondary to reduced hepatic lipoprotein secretion (10). It is possible, however, that there may be additional, direct effects of hypocholesterolemia on cow health. For example, it has been shown that lipoproteins can exert a modulating effect on immune system components (153-155). While any direct effect of hypocholesterolemia on immunity in dairy cows is speculative, it is known in cows that both the phagocytic and lymphocytic arms of the immune system are suppressed during the peripartum period, the same period during which blood cholesterol concentrations are at their lowest. Moreover, it is very clear that life-threatening disease in dairy cows is also most prevalent in the peripartum, hypocholesterolemic period (156). Thus,

it appears that a clearer understanding of cholesterol metabolism in dairy cows may lead to a better appreciation of the mechanisms of some peripartum diseases.

In addition to possible effects on disease incidence, it has also been suggested that reduced plasma cholesterol concentration might impair the reproductive performance of the cow (7-9). This may be due to a reduction in the synthesis of sex steroid hormones such as progesterone from the corpus luteum of the ovary. Plasma lipoprotein-bound cholesterol is an important source of cholesterol for cells in general and specifically for steroidogenic tissues. Cholesterol is the precursor for all steroid hormones, including progesterone. Although steroidogenic cells are capable of *de novo* cholesterol synthesis, they rely on the uptake of lipoprotein cholesterol to maintain physiological levels of steroid hormone production. This has been demonstrated by numerous *in vitro* studies using luteal or granulosa cells from bovine (157-159) or other sources (3, 5, 160). Similarly, an impairment in the supply of plasma cholesterol to steroidogenic tissues *in vivo* has been shown to result in a lower production of steroid hormone in rats (5) and in humans (4). Thus, the reduced peripartum plasma cholesterol concentration in the cow might limit the supply of cholesterol for progesterone synthesis, leading to impaired reproductive performance. Moreover, recent studies have also suggested that plasma lipoprotein supply is involved in embryo development (161-163). A reduced concentration of serum cholesterol appears to be associated with low embryo recovery from superovulated cows (162, 163). Thus, in addition to its role in steroidogenesis, plasma cholesterol might also play some other important roles in the reproductive system of the animal.

It is apparent that a more complete understanding of the role of plasma cholesterol in ruminant metabolism will help us develop strategies to improve productivity of the cow. An experimental technique of modulating blood cholesterol is critical to the design of *in vivo* experiments with which to study the role of plasma cholesterol in ruminant metabolism. However, such a technique is not available at the present time. Since interruption of EHC has been shown to produce reversible reduction in blood cholesterol concentration in a number of animal species, it was the first objective of the following studies to examine the possibility of using this method to modify blood cholesterol levels in dairy cows. The second objective was to determine the effects of interruption of EHC on bovine hepatic cholesterol metabolism. The elucidation of these effects might provide valuable information for understanding of the role of ruminant liver in regulating plasma lipoprotein cholesterol metabolism.

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CHAPTER II

PARTIAL DIVERSION OF BILE DRAMATICALLY REDUCES PLASMA CHOLESTEROL CONCENTRATION IN CATTLE

INTRODUCTION

Hepatobiliary excretion of cholesterol and its major metabolites, the bile acids, is the key 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 salts secreted by the liver are reabsorbed in the ileum by an active transport mechanism (1) and return to the liver via the hepatic portal vein (2, 3). To maintain a steady state, therefore, the liver is normally required to synthesize only small amounts of bile salts to replace the fecal loss (4). However, when the enterohepatic circulation (EHC) is interrupted, either by direct biliary drainage (5,6), or by inducing fecal loss of bile sterols by means of bile acid sequestrants (7, 8) or ileal bypass (7, 9), the rate of bile acid synthesis is augmented (5, 7, 10). The increase in bile acid synthesis in turn leads to an enhanced uptake and catabolism of plasma lipoprotein cholesterol by the liver (7, 10, 11). 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, including cattle.

Like most of the mammals, the majority of the circulating cholesterol in cattle is carried by high-density lipoprotein (HDL) (13, 14). However, unlike other species, in which circulating cholesterol levels are normally

maintained within a relative narrow range through different physiological stages, plasma cholesterol concentration in cattle undergoes considerable fluctuations during the gestation-lactation cycle (14-16). The mechanisms regulating these large changes in plasma cholesterol are not completely understood. Nonetheless, the degree of peripartum reduction in plasma cholesterol concentration has been shown to be associated with metabolic diseases of the cow (17-19). To understand the role of plasma cholesterol in ruminant metabolism, we attempted to use interruption of EHC as a tool to study the metabolic effect of reduced plasma cholesterol concentration in cattle.

The objective of this study was to determine and characterize the effects of controlled biliary diversion and subsequent restoration of EHC on plasma concentrations of cholesterol and other lipids in cows. Interestingly, we found that partial diversion of bile caused a reduction of more than 90% in plasma concentrations of cholesterol and other lipids. This study presents a unique animal model with which to investigate the metabolic role of plasma lipoprotein cholesterol.

MATERIALS AND METHODS

Animals and diet

Five healthy adult, nonpregnant, nonlactating Holstein cows were used. They were kept under environmentally controlled conditions (temperature maintained at 20 C) in individual pens. Throughout the entire experimental period, the animals had free access to alfalfa hay, water and trace-mineralized salt. The diet was supplemented daily with four pounds of concentrate containing ground corn, oats, and vitamins. The animals were weighed weekly.

Surgical procedures

The cows were surgically prepared and fitted with reentrant intestinal cannula using a modification of the procedure of Symonds et al (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 then joined to reestablish the patency of the intestine. A reentrant intestinal cannula (Figure 4) was placed with one arm in the isolated intestinal segment and the other in the intact duodenum. Through this cannula flowed all biliary secretions. When required, controlled portions of bile flow could be diverted from the body (Figure 4).

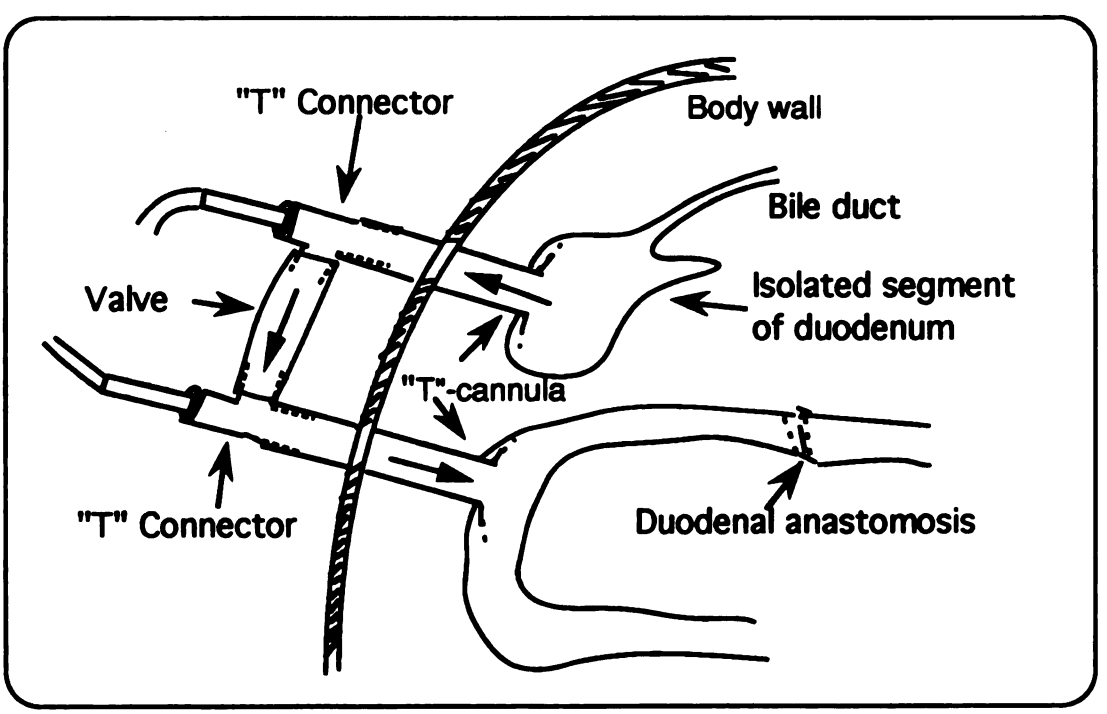

Figure 4: Diagram of surgical modification to the duodenum.

Animal maintenance and experimental procedures

During the first 5 days following the surgery, to minimize the internal pressure in the isolated segment of intestine, the reentrant cannula was interrupted and all of the bile flow was diverted from the isolated segment into a reservoir flask. Bile was infused to the cow, as it was collected, through the cannula arm connected to the intact duodenum using a peristaltic pump (Model 1210; Harvard Apparatus Inc., South Natick, MA). Infusion rates were adjusted occasionally so that only a small amount 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 into the intact duodenum via the cannula. A one-way valve (Figure 4) was used to prevent back-flow of ingesta into the cannula and isolated intestinal segment. Bile flow through the cannula was checked at least four times a day and the cannula flushed once a day with physiological saline. 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 collected. For this purpose, the reentrant cannula was closed and all bile 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 hours.

Twenty-four hours following 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 collected into a flask. Bile was aspirated from the collection flask into two tubings, 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 tubing. One tube was connected to the duodenal arm of the cannula and the other to a discard flask. During periods of 50% bile diversion, tubings of equal diameter were used, while during periods of 22% bile diversion, a discard tubing with one third the cross-sectional area of the reinfusion tubing 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.

Biochemical analyses

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). Lipoproteins were isolated

by discontinuous density-gradient ultracentrifugation in a vertical-tube rotor as described previously (21). Total bile acids in bile were enzymatically determined with a reagent kit (Sigma, St. Louis, MO).

Calculations and statistics

Bile flow rates were expressed in milliliters per 100 kg body weight per h. Output rates of total bile acids were determined by multiplying bile flow by bile acid concentration. Plasma lipid values were expressed in milligrams per deciliter. Values are presented as means ± S.E.M. The data were analyzed by analysis of variance and the significance of difference determined by appropriate *t* test, at a $P<0.05$ level of significance.

RESULTS

Validity of the Experimental Model

The animals were healthy and maintained constant 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 four 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 rates of bile flow and total bile acid secretion were nearly constant throughout a 24 h period (Figure 5). This is consistent with the pattern of continuous bile secretion which is typical of the ruminant animal (20, 22). The average bile flow rate was 132 ml/100 kg body weight/h, which is

comparable to the values previously reported on adult cattle (20). These results suggested that a normal EHC was maintained in these cows.

In addition, partial bile diversion did not appear to have any negative effect on feed intake and general health of the animals.

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 biliary 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 (Figure 6). Total bile acid concentrations in the 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 (5). 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 cows. As shown by Figure 6, diversion of 22% bile flow caused a remarkable decrease in the rates of bile flow and bile acid output in a manner comparable to 50% bile diversion. The reduction in bile and bile acid secretion rates was only slightly greater when 50% of bile 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.

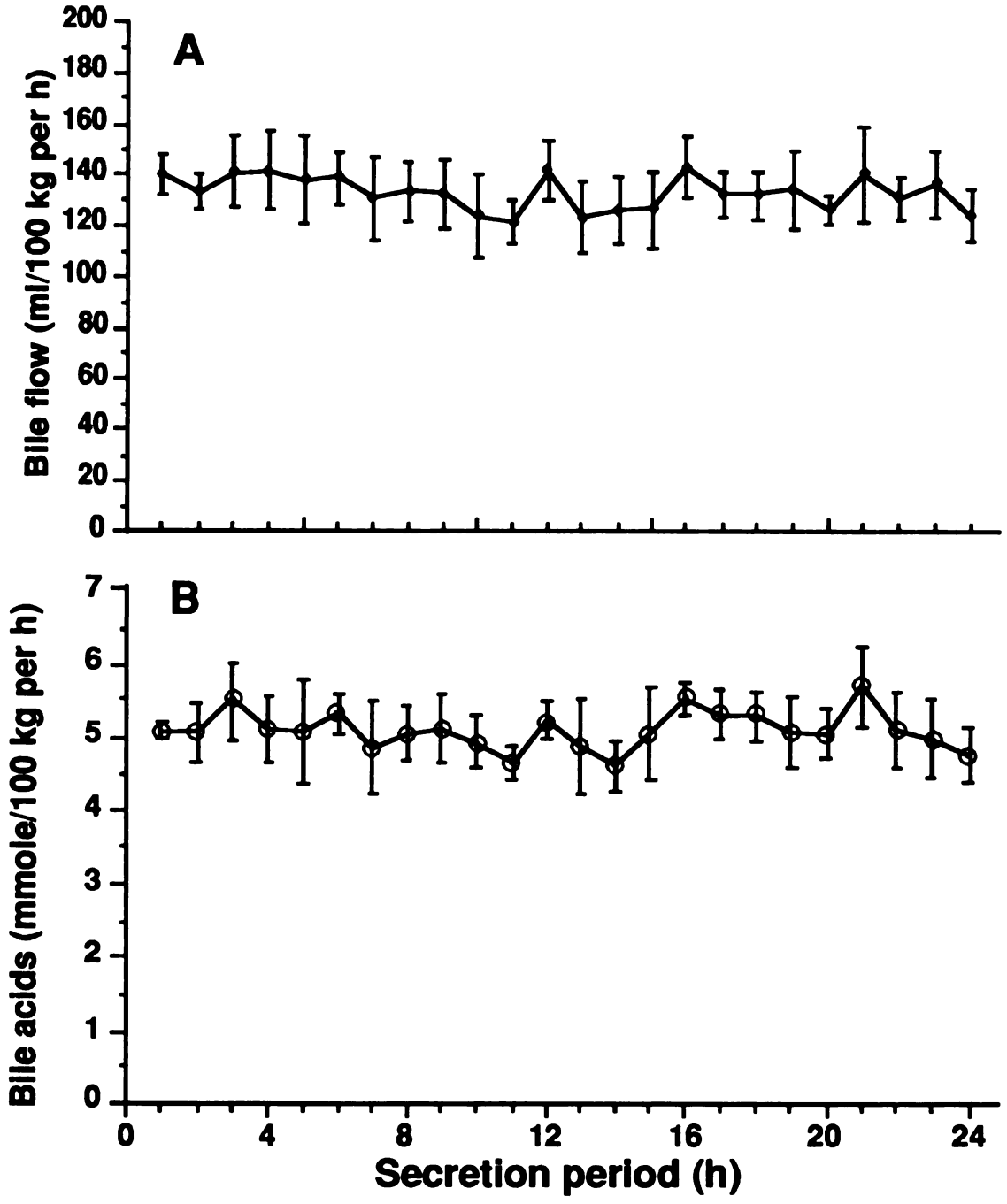

Figure 5: Diurnal changes in rates of bile flow (A) and bile acid secretion (B). Values represent means ± SEM (n=5).

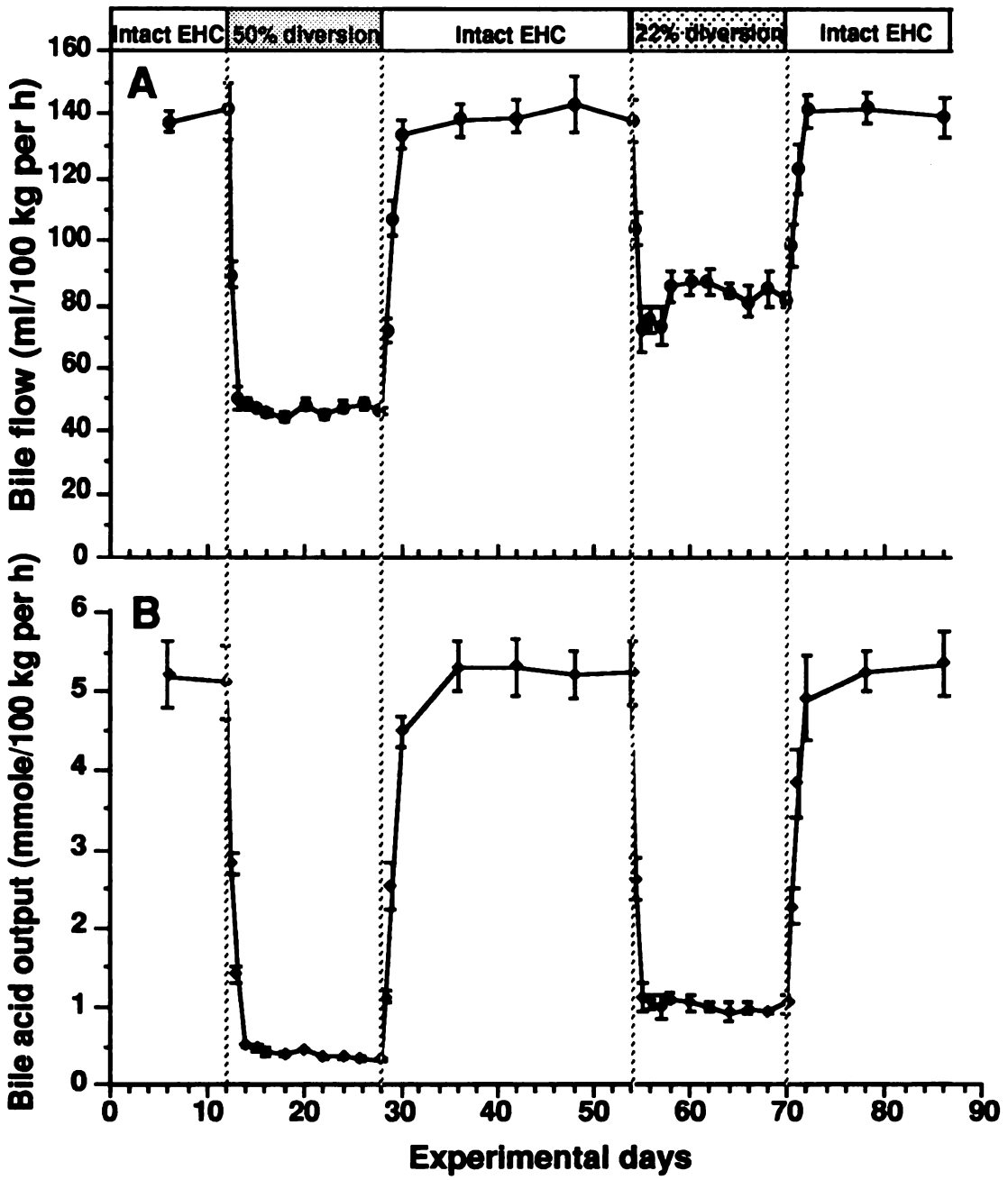

Figure 6: Effects of controlled biliary diversion on rates of bile flow (A) and bile acid output (B). Values represent means ± SEM (n=5).

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 (Figure 7). Based on the stabilized levels, diversion of 50% bile flow caused a significantly greater reduction than 22% bile diversion (91% vs. 78%; $P<0.001$, $n=5$). When the bile diversion protocols were terminated, however, plasma cholesterol concentrations returned towards the control values rapidly. In no other species has interruption of EHC been shown to result in such a dramatic reduction in plasma cholesterol.

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 (Figure 8). However, the differences in plasma phospholipid concentrations between the 50% and 22% bile diversion periods were not significant ($P>0.05$). In addition, it appeared that concentration of plasma phospholipids decreased more rapidly than that of plasma cholesterol.

Plasma triglyceride concentrations dropped sharply after the initiation of bile diversion and stabilized within 24 h thereafter (Figure 9). This is in contrast to plasma cholesterol and phospholipid concentrations which did not drop 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.

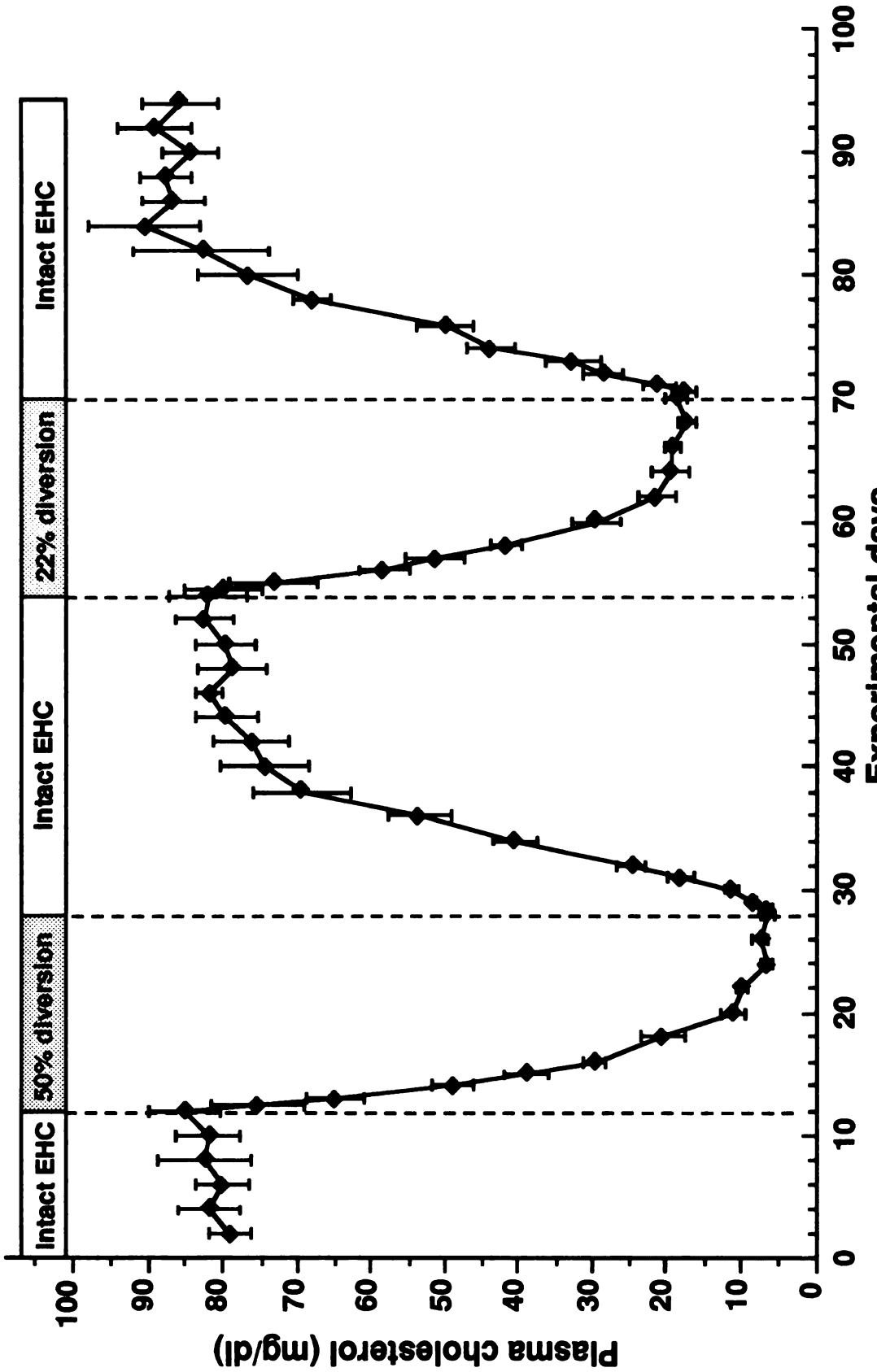

Figure 7: Plasma concentration of cholesterol before, during and after controlled biliary diversion. Values are means ± SEM (n=5).

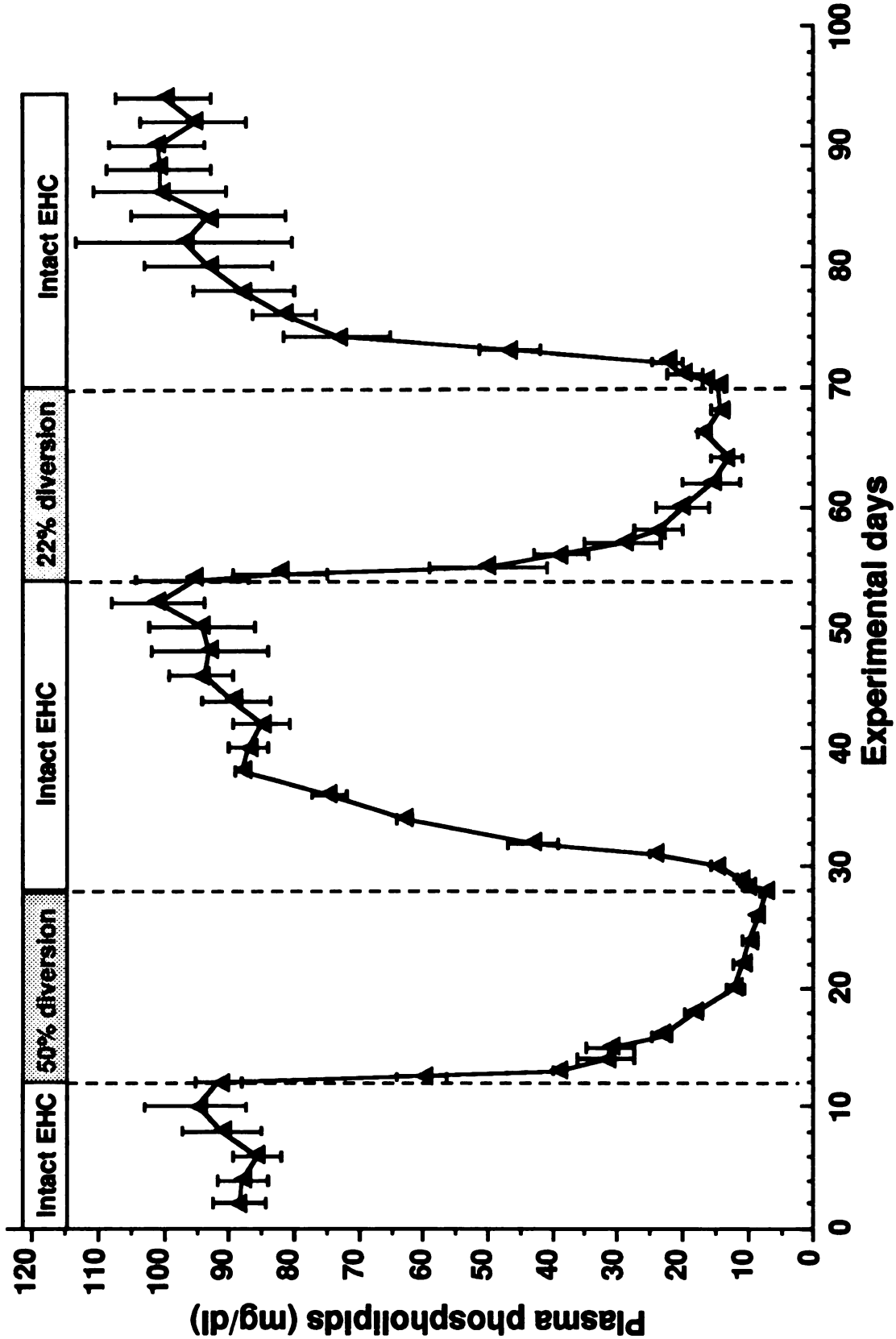

Figure 8: Plasma concentration of phospholipids before, during and after controlled biliary diversion. Values are means ± SEM (n=5).

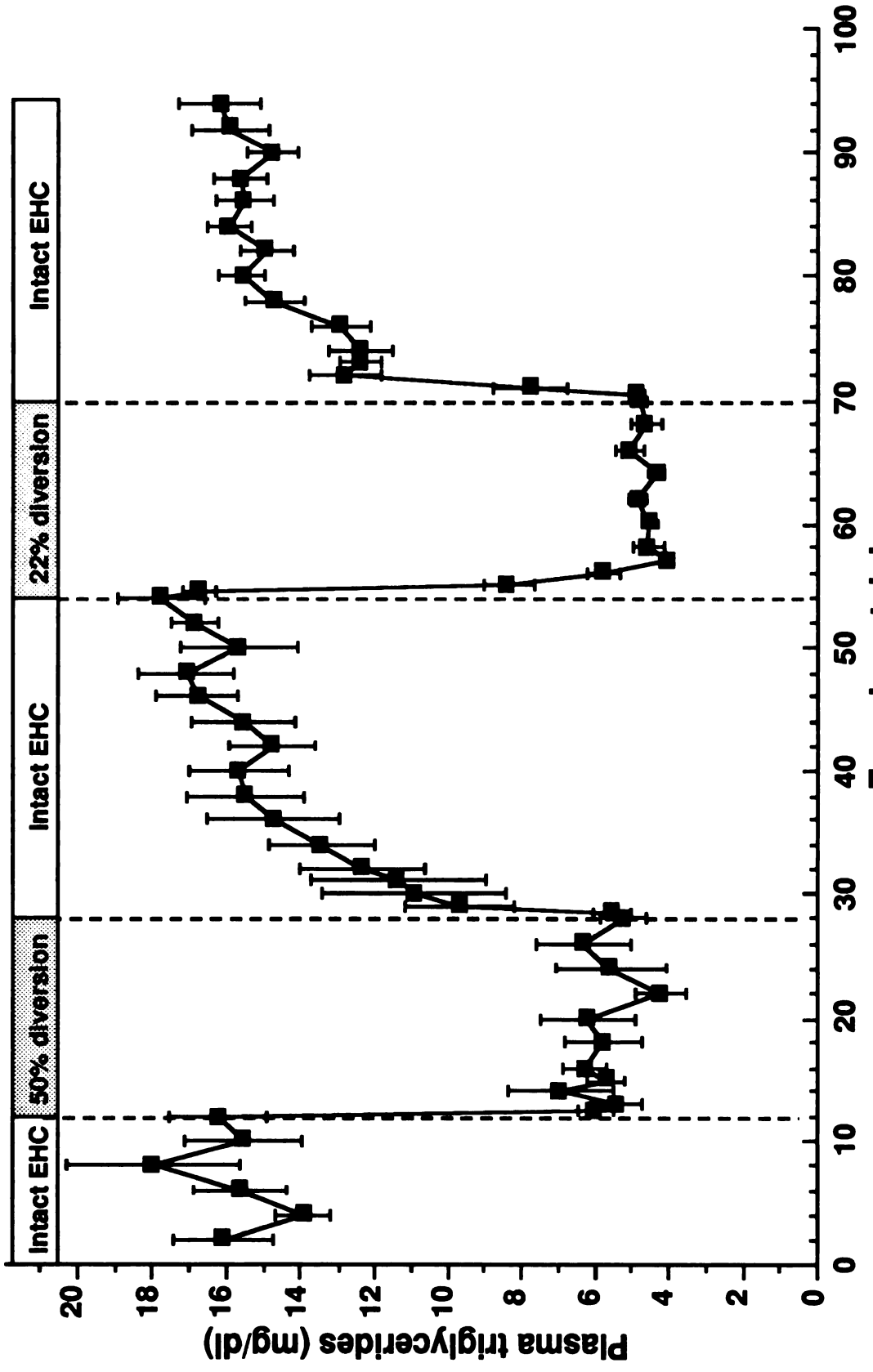

Figure 9: Plasma concentration of triglycerides before, during and after controlled biliary diversion. Values are means ± SEM (n=5).

Effects of controlled bile diversion on lipoprotein cholesterol concentrations

To further characterize the effects of partial bile diversion on plasma cholesterol metabolism, we determined the cholesterol concentrations in various plasma lipoprotein fractions. Plasma samples were collected at 0 h, 12 h, and 14 days of bile diversion, and lipoproteins were isolated and cholesterol concentrations in the lipoprotein fractions were determined. The results are shown in Table 4. At 12 hours 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 (Table 4). These data demonstrate that, in response to bile diversion, HDL cholesterol declined less rapidly than VLDL- and LDL-cholesterol, possibly due to the low turnover rate of HDL, compared to other lipoproteins. By day 14 of bile diversion, however, HDL cholesterol was reduced by 90% and 85% for 50% diversion and 22% diversion, respectively. The corresponding LDL cholesterol and VLDL cholesterol concentrations were reduced by an extent comparable to that of HDL. Thus, distribution of cholesterol among different lipoproteins on day 14 of bile diversion was not different from initial values (Table 4).

DISCUSSION

The role of EHC in determining cholesterol homeostasis and the effects of its interruption on plasma cholesterol metabolism have been well documented in humans and a number of other animal species (6-12). No similar study has been reported on ruminant animals. The present study has characterized the changes in plasma cholesterol and other lipids during chronic diversion of bile in cattle. This study demonstrates that

Table 4. Effects of partial diversion of bile flow on cholesterol concentration and distribution among the plasma lipoprotein fractions

	Cholesterol concentration (mg/dl)					
	VLDL		LDL		HDL	
Diversion period	50% diversion	25% diversion	50% diversion	25% diversion	50% diversion	25% diversion
0 h	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	0.94 ± 0.12*	1.31 ± 0.16*	5.65 ± 0.49*	8.82 ± 1.03*	62.78 ± 4.92	66.52 ± 2.74
	(1.38 ± 0.23*)	(1.70 ± 0.19*)	(8.31 ± 1.01*)	(11.59 ± 1.51*)	(90.31 ± 1.15)	(86.72 ± 1.60)
14 days	0.43 ± 0.04*	0.63 ± 0.06*	1.83 ± 0.21*	2.12 ± 0.27*	7.78 ± 0.78*	10.51 ± 1.07*
	(4.31 ± 0.40)	(4.84 ± 0.57)	(18.22 ± 1.19)	(16.25 ± 2.39)	(77.48 ± 0.90)	(78.91 ± 2.83)

Figures in parentheses indicate the percentage of cholesterol in the lipoprotein fractions compared to total plasma cholesterol.
Values are means ± S.E.M. (n=5): * significantly different from the corresponding control values (i.e., 0 h) at $P < 0.01$.

interruption of EHC dramatically influences plasma cholesterol concentration in this species.

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 in our animal model by partial bile diversion, whereas only moderate reductions have been reported in other species (5, 6, 8, 12), including human (7, 9), in response to either complete or partial interruption of EHC. The dramatic change in blood lipids was not due to the surgical modification of the animals *per se* since the blood cholesterol levels and other lipid concentrations normalized when the animals recovered from the surgery. Furthermore, these animals maintained a normal bile secretion pattern and bile acid secretion rate during the control periods after surgery, implicating that EHC was functional. While both 22% and 50% bile diversion caused a dramatic drop in plasma cholesterol, diversion of 50% bile produced a significantly greater reduction in blood cholesterol than 22% bile diversion. This indicates that the response in plasma cholesterol concentration is dependent on the amount of bile diverted. In addition to plasma cholesterol concentration, plasma concentrations of triglycerides and phospholipids were also reduced greatly after bile diversion. This also appears to be unique for the ruminant animal since no significant reduction in these two plasma lipids has been observed in other species. In contrast, it has been shown in a number of monogastric species that interruption of EHC causes an increase in plasma triglyceride concentration (8,11). All of these changes in blood cholesterol, phospholipids, and triglycerides were reversible since plasma concentrations of these lipids returned to normal values after the termination of bile diversion.

Because HDL-cholesterol accounts for more than 80% of plasma cholesterol in cattle, the dramatic decrease in total cholesterol concentration mainly depends on the reduction in HDL cholesterol. Thus, although VLDL- and LDL-cholesterol were reduced by more than 50% during the first 12 h of bile diversion, only a minor decline was observed in plasma total cholesterol because HDL-cholesterol did not change significantly during this period. Plasma concentration of total cholesterol did not reach a new steady-state level until a week after the initiation of bile diversion, suggesting a low clearance rate of HDL. This might be due to at least in part the low level of plasma cholesterol ester transfer protein in this species (23), which plays an important role in the transport of HDL cholesterol to the liver (24). A recent study has shown that a genetic defect in this transport protein in human causes remarkably delayed catabolism of HDL without affecting its production rate (25). Furthermore, apoprotein E, which is present in more than 50% of the HDL particles in other species including rats (26), does not appear to exist in bovine HDL (14). As HDL-associated apoE functions to mediate the rapid clearance of HDL cholesterol by the liver (26), the lack of this apolipoprotein in HDL would be expected to further delay hepatic clearance of HDL-cholesterol in bovine plasma.

While partial bile diversion remarkably reduced HDL-cholesterol in our animal model, a similar observation has not been reported for other species. 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 (9, 11). On the other hand, in the high HDL-cholesterol species such as rats (13), bile diversion only produces a small drop in plasma total cholesterol (6, 10), suggesting that HDL-cholesterol was not affected

significantly. It is not known whether the dramatic decline in HDL-cholesterol in our model is due to an increase in HDL clearance rate or a decrease in HDL synthesis or both. Nonetheless, it has been shown in the rat that biliary diversion does not affect intestinal HDL secretion (27). However, a full understanding of HDL-cholesterol metabolism in our animal model requires detailed kinetic studies.

In addition to the changes in plasma lipids, both 22% and 50% bile diversion sharply decreased bile flow rate and bile acid secretion. The decrease in the availability of bile acids for the absorption of dietary lipids by the small intestine might be the primary mechanism for the rapid decline in plasma triglyceride concentration in response to bile diversion. This is because triglycerides which are absorbed by the small intestine and secreted into the blood as chylomicrons are the major source of circulating triglycerides in ruminant animals (28, 29). In fact, previous studies have shown that bile diversion greatly impaired the secretion of chylomicrons in rats (27, 30). The reduced intestinal secretion of triglyceride-rich lipoprotein may lead to decreased intestinal cholesterol synthesis and secretion. This would also contribute to the hypocholesterolemic effect of bile diversion.

The exact mechanism responsible for the unprecedented hypocholesterolemic effects of interruption of EHC in cattle observed in this study remains to be elucidated. Nonetheless, because the dramatic reduction in plasma cholesterol is reversible and because the degree of reduction appears to be proportional to the amount of bile diverted from the animal, this animal model might be of important value for the study of the role of plasma cholesterol in ruminant metabolism. In addition, no special drug treatment was required for this experimental technique, further signifying the usefulness of this animal model. Although bile acid

sequestrants can be used in a number of monogastric animals to provide an alternative means for the surgical approach to interrupt EHC by inducing fecal loss of bile sterols (7, 8, 11), these agents do not seem to be effective in ruminant animals. A previous study (31) has shown that feeding of a bile acid sequestrant to cows failed to produce any influences on fecal excretion of lipid and on blood cholesterol and other blood lipid concentrations. The intestinal concentration of the sequestrant in cattle might be too low for effective action, due to the large mass of ingesta in the ruminant digestive tract compared to monogastric animals. Thus, it appears that surgical intervention is a necessary approach to interrupt the EHC in ruminants.

In summary, the present study, for the first time, shows that plasma concentrations of cholesterol and other lipids in cattle responded to partial bile diversion with a dramatic reduction. Since no pharmacological treatments were involved and the animals maintained a healthy condition during bile diversion, this animal model provides excellent opportunities to further understanding of the regulation of plasma lipoprotein concentrations, as well as the metabolic role of circulating cholesterol in animals.

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CHAPTER III

INHERENTLY LOW RATE OF CHOLESTEROL SYNTHESIS IN THE LIVER EXAGGERATES THE HYPOCHOLESTEROLEMIC EFFECTS OF PARTIAL BILE DIVERSION IN CATTLE

INTRODUCTION

The liver plays a central role in the regulation of plasma cholesterol concentration. Input of cholesterol into the plasma pool can come from the secretion of hepatically derived plasma lipoproteins, especially very-low-density lipoprotein (VLDL) (1). Cholesterol for lipoproteins can be derived from its *de novo* synthesis by the liver (1, 2), and, in most species, the liver is the major site of cholesterol production (3, 4). On the other hand, the liver is also the organ primarily responsible for the clearance of plasma lipoproteins and the catabolism of cholesterol to bile acids (5, 6). Biliary excretion of cholesterol and bile acids provides the major pathway determining cholesterol homeostasis in the body.

The role of ruminant liver in regulating cholesterol metabolism is poorly understood and might be expected to differ from many monogastric animals. Unlike many other species, ruminant liver appears to be a minor site for body cholesterol synthesis (7, 8). The ruminant liver also has a very limited capacity to synthesize and secrete VLDL (9, 10). Therefore, the contribution of ruminant liver to plasma cholesterol might be minimal. With respect to cholesterol removal, however, the ruminant liver possesses mechanisms for the clearance of plasma cholesterol similar to those of other species. For example, the liver is the primary organ for the uptake of

chylomicron cholesterol in sheep (11). Furthermore, low-density lipoprotein (LDL) receptor has been demonstrated in liver tissues from cattle and has been suggested to play a role in regulating bovine serum cholesterol concentration (12).

Recently, we have reported that partial diversion of bile produces a 90% reduction in plasma cholesterol concentration in cattle (13). In no other species has interruption of enterohepatic circulation (EHC) of bile been shown to cause such a dramatic reduction in plasma cholesterol. The objective of the present study was to determine if changes in hepatic cholesterol synthesis or uptake, during bile diversion, could account for the previously observed response in plasma cholesterol.

MATERIALS AND METHODS

Materials

[^{3}H]-water (100 mCi/mmole) 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 adult, non-pregnant, non-lactating, Holstein cows were used. The cows were kept in an environmentally controlled facility in individual pens and fed an alfalfa hay-based diet as described previously (13).

Experimental procedures

The cows were surgically altered as described previously (13) and fitted with cannula for the collection of bile. Following surgery, the animals were initially used to study the effects of bile diversion on plasma lipid concentrations (13). After completion of the initial study, the animals were allowed a 14-day adjustment period without bile diversion. At the end of this period, a liver biopsy was taken as described in the following section. The cows were then allowed a further 10-day adjustment period. Plasma cholesterol concentrations before and after the liver biopsy had restabilized at about 85 mg/dl, which is in the range of normal values for nonlactating cows. Following the adjustment period, 50% of the total bile flow was diverted from the animals according to the procedure described previously (13) for an experimental period of 14 days. The volume of the diverted bile was measured twice a day and 3-ml aliquots of fresh bile were taken daily and stored at -20 C until analyzed. Coccygeal-vessel blood samples were taken daily and plasma collected as described (13) for the measurement of plasma cholesterol concentration.

Three liver biopsies and one intestinal mucosal biopsy were taken during the course of the experiment. The first liver biopsy was taken during the initial surgery (pre-cannulation control). Subsequent liver biopsies were carried out under local anesthesia. The second liver biopsy was taken after the adjustment period and before the experimental period (post-cannulation

control) while the third was taken at the completion of the experimental period. Also at the end of the experiment, under regional anesthesia, a 20-cm segment of jejunum was removed, immediately flushed with ice-cold phosphate-buffered saline and the mucosa scraped for measurement of cholesterol synthesis rate. Immediately following collection of the terminal samples, the animals were euthanized by administration of an over-dose 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.

For comparison, small intestine biopsies were obtained from four adult, non-pregnant, non-lactating, noncannulated, Holstein cows using the procedure described above.

Determination of Cholesterol synthesis rate

Cholesterol synthesis rate was determined in liver tissues and intestinal mucosal samples by measuring the incorporation rate of [^{3}H]-H_2O into digitonin-precipitable sterols (DPS) as described by Andersen et al (14). 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 [^{3}H]-H_2O in 1 ml of MEM medium 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. Following incubation, 3 ml of 1.4 N KOH in 80% ethanol was added to the incubation mixture and incubated at 85 C for 3-4 hr to saponify the lipids. Non-saponifiable lipids of the reactants were extracted into hexane and treated with digitonin to precipitate DPS (15). Following 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 (15), 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, male 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 [^{3}H]-H_2O (incorporated at 0 C) and expressed as nmole of ^{3}H incorporated per gram tissue per hr (nmole/ g.hr). 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 ^{3}H assuming that 23 ^{3}H atoms are incorporated into each molecule of cholesterol (14, 16). Thus, the nmole cholesterol synthesized/gram tissue per day = (nmole ^{3}H incorporated/g.hr /23) x 24 hours.

Isolation and labeling of LDL

Low density lipoproteins were isolated from the serum of a healthy lactating cow by ultracentrifugation and affinity chromatography on heparin-Sepharose as described by Cordle et al. (17). The LDL preparation was further purified by gel filtration chromatography using Bio-Gel A5-M (18) to remove VLDL particles. LDL was radiolabeled with ^{125}I according to a modified McFarlane procedure (19) as described (20). The specific activities of ^{125}I-LDL ranged from 350 to 520 cpm/ng protein. More than 95% of the ^{125}I radioactivity 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 following liver biopsy, a portion (about 2.5 grams) of the liver sample was homogenized in 10 ml of buffer A (50 mM NaCl / 1.0 mM $CaCl_2$ / 20 mM Tris, 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-LDL to the liver homogenate as described by Rudling et al (21). Fifty milligrams of homogenate protein were incubated on ice for 2 h in 150 ul 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 Tis-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 (50 mM NaCl/10 mM Hepes, 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-LDL was calculated by subtracting the heparin-resistant binding from total binding. All binding assays were performed in triplicate.

Miscellaneous procedures

Protein contents of liver homogenate and LDL were determined as described by Lowry et al (22). Biliary bile acid concentration was assayed by an enzymatic method (Sigma, St. Louis, MO). Plasma cholesterol was determined enzymatically using a reagent kit from 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. The concentrations of cholesterol and cholesterol ester in the liver were determined enzymatically after lipid extraction (23) and isolation of unesterified cholesterol and cholesterol ester 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).

The data are presented as means ± S.E.M. and were analyzed using analysis of variance and appropriate *t* test.

RESULTS

Biliary sterol depletion and plasma and hepatic cholesterol

Diversion of 50% of bile flow led to the loss of approximately 180 mmole biliary sterols during the first 24 h (Figure 10). Thereafter, the amount of diverted sterols sharply declined and stabilized at a steady-state level of about 25 mmole per day. The majority of diverted biliary sterols were bile acids, with biliary cholesterol accounting for less than 5% of the total biliary sterols (Figure 10).

As shown in Figure 11, 50% bile diversion was followed by a dramatic decline in plasma cholesterol concentration. After 8 days, plasma cholesterol concentration reached a steady-state level of 8 mg/dl, less than

10% of the original values. The decrease of plasma cholesterol concentration was in a pattern similar to that observed in our previous study (13).

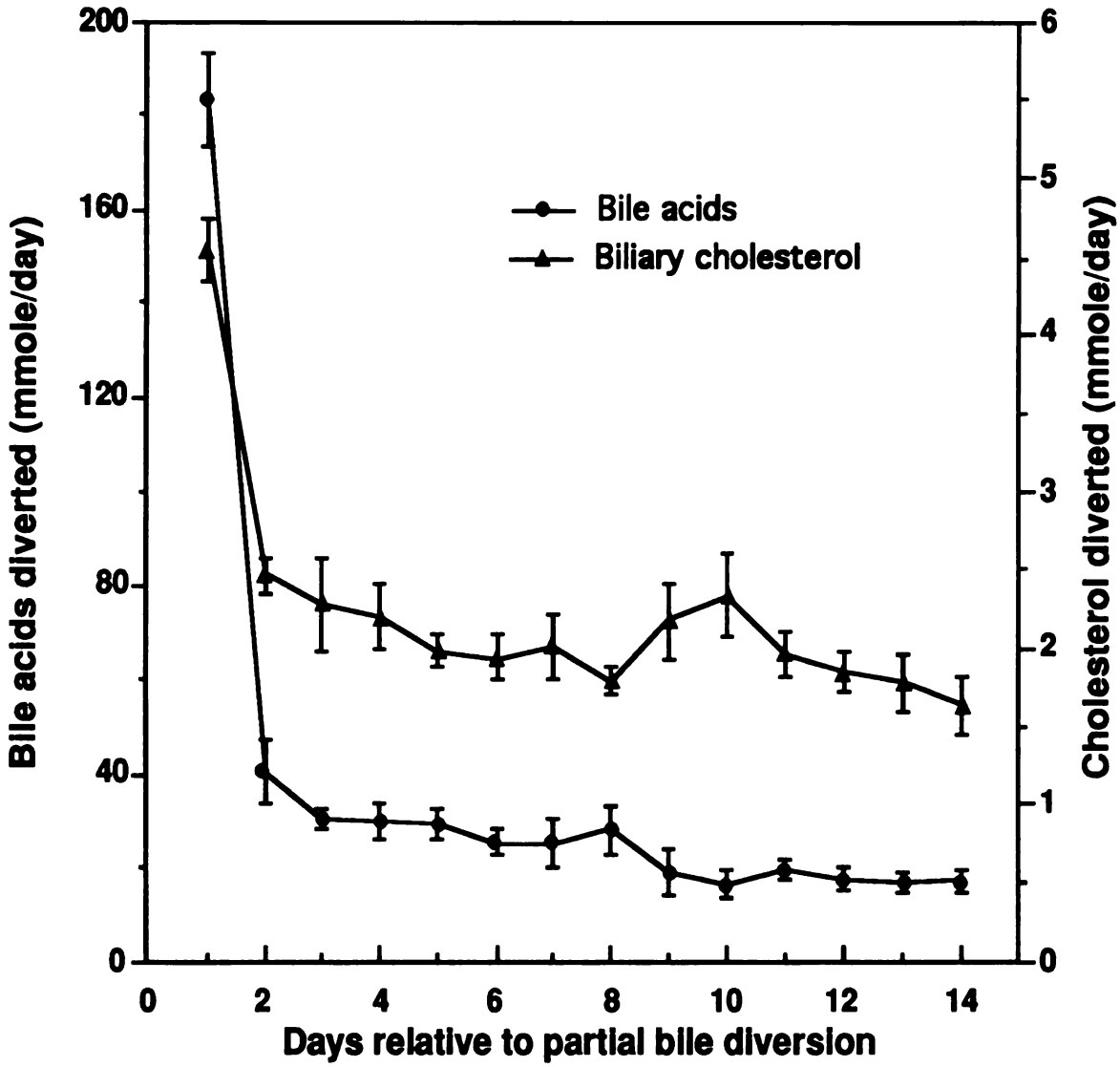

Figure 10: Depletion of biliary sterols induced by chronic diversion of 50% of bile flow. Bile diversion was carried out as described in Experimental Procedure. The amount of bile diverted was measured every 24 h and sampled. Total bile acids and biliary cholesterol concentrations were determined by enzymatic methods. Each data point represents the mean $\pm$ SEM; n=5.

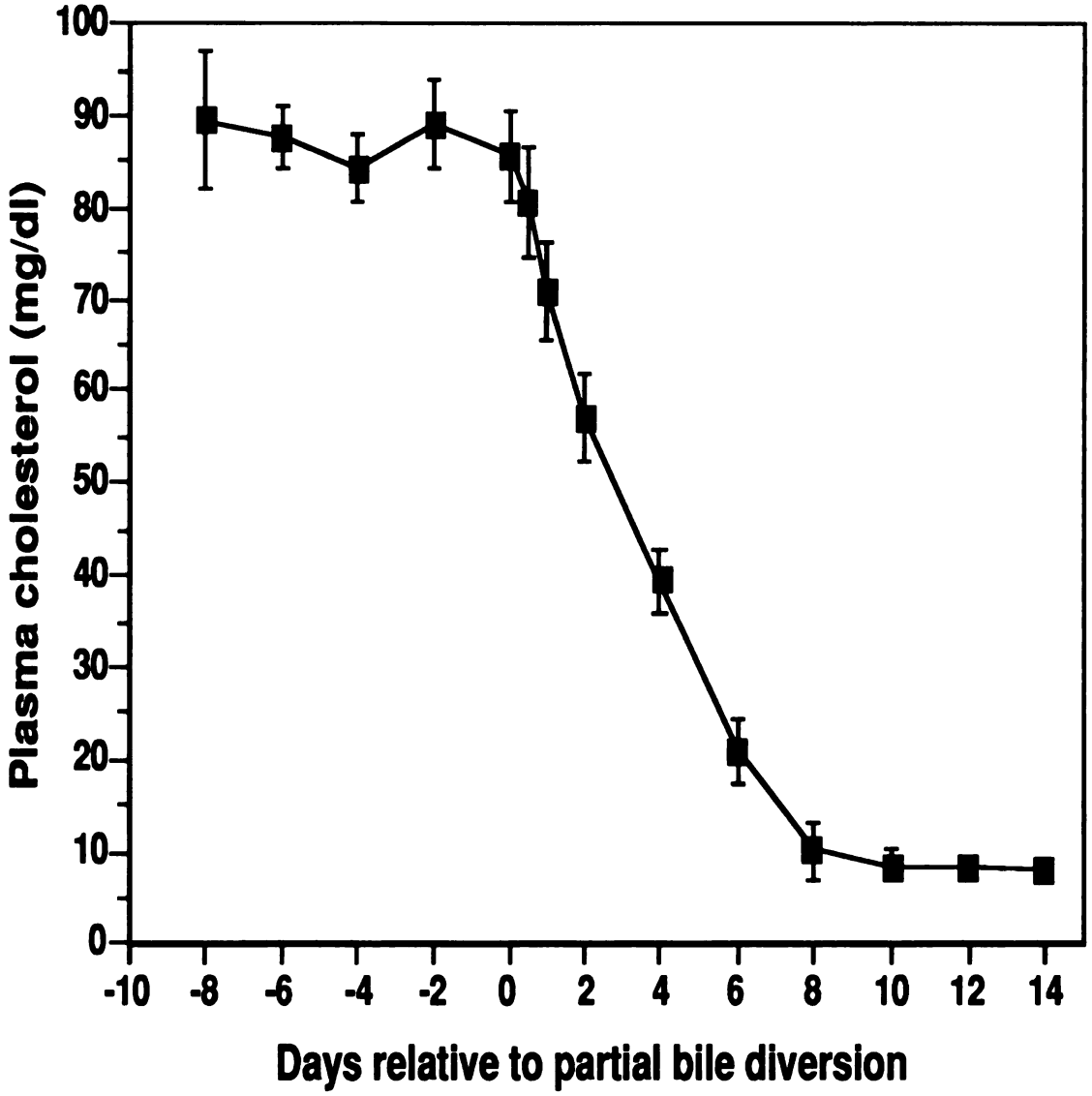

Figure 11: Effects of bile diversion on plasma cholesterol concentration. Plasma samples were obtained every other day and cholesterol concentrations were determined by enzymatic methods. Each value represents the means ± SEM (n=5).

In response to bile diversion, liver content of esterified cholesterol was reduced by more than 90% ($p<0.0001$) by day 14, as compared to the pre-cannulation or post-cannulation controls, whereas the unesterified cholesterol content remained unchanged ($p>0.05$) (Figure 12). These data demonstrate that 50% bile diversion depleted the liver of cholesterol stores.

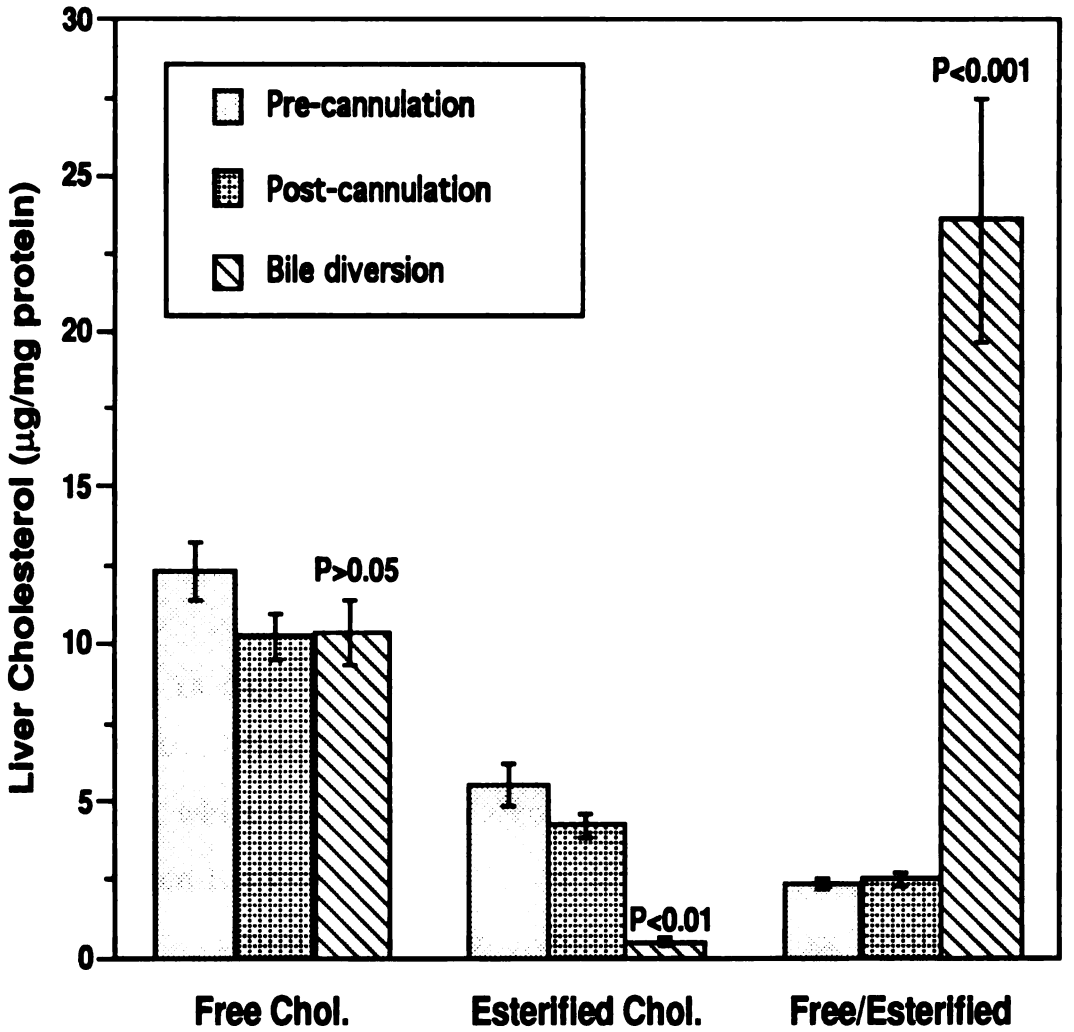

Figure 12: Effects of bile diversion on hepatic cholesterol and cholesterol ester concentrations. Lipids were extracted from liver tissue samples and free cholesterol and esterified cholesterol were separated by thin layer chromatography, reextracted with ether, and then quantified by enzymatic methods. Each value represents the means $\pm$ SEM (n=5).

Hepatic and intestinal cholesterol synthesis and its response to biliary diversion

Incorporation rate of [^{3}H]-H_2O into DPS was barely detectable in the bovine liver tissues, only about 10% of that of the bovine intestinal mucosa, or 5% of the rat liver tissues (Figure 13). Nevertheless, as with intestinal mucosa and the rat liver tissues, incorporation of [^{3}H]-H_2O into DPS by the bovine liver tissues was essentially linear with respect to time over a 2.3-h incubation period (Figure 13). Furthermore, incorporation 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 substrate in the MEM medium, incorporation rates of [^{3}H]-H_2O into DPS by bovine liver slices incubated in MEM media supplemented with various substrates were determined (Figure 14). The results showed that inclusion of additional substrate into MEM had no effects on the incorporation rates (Figure 14). This further confirms an inherently low rate of cholesterol synthesis in bovine liver.

After 14 days of bile diversion, however, the incorporation rate of [^{3}H]-H_2O into DPS in the liver was increased by more than 10 fold ($p<0.0001$) (Figure 15), while there was no significant difference in the incorporation rates between the two controls (Figure 15). This indicates that the hepatic cholesterol synthesis rate was greatly increased in response to bile diversion. In contrast to this, the [^{3}H]-H_2O incorporation rate 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% ; $p<0.05$; Figure 15).

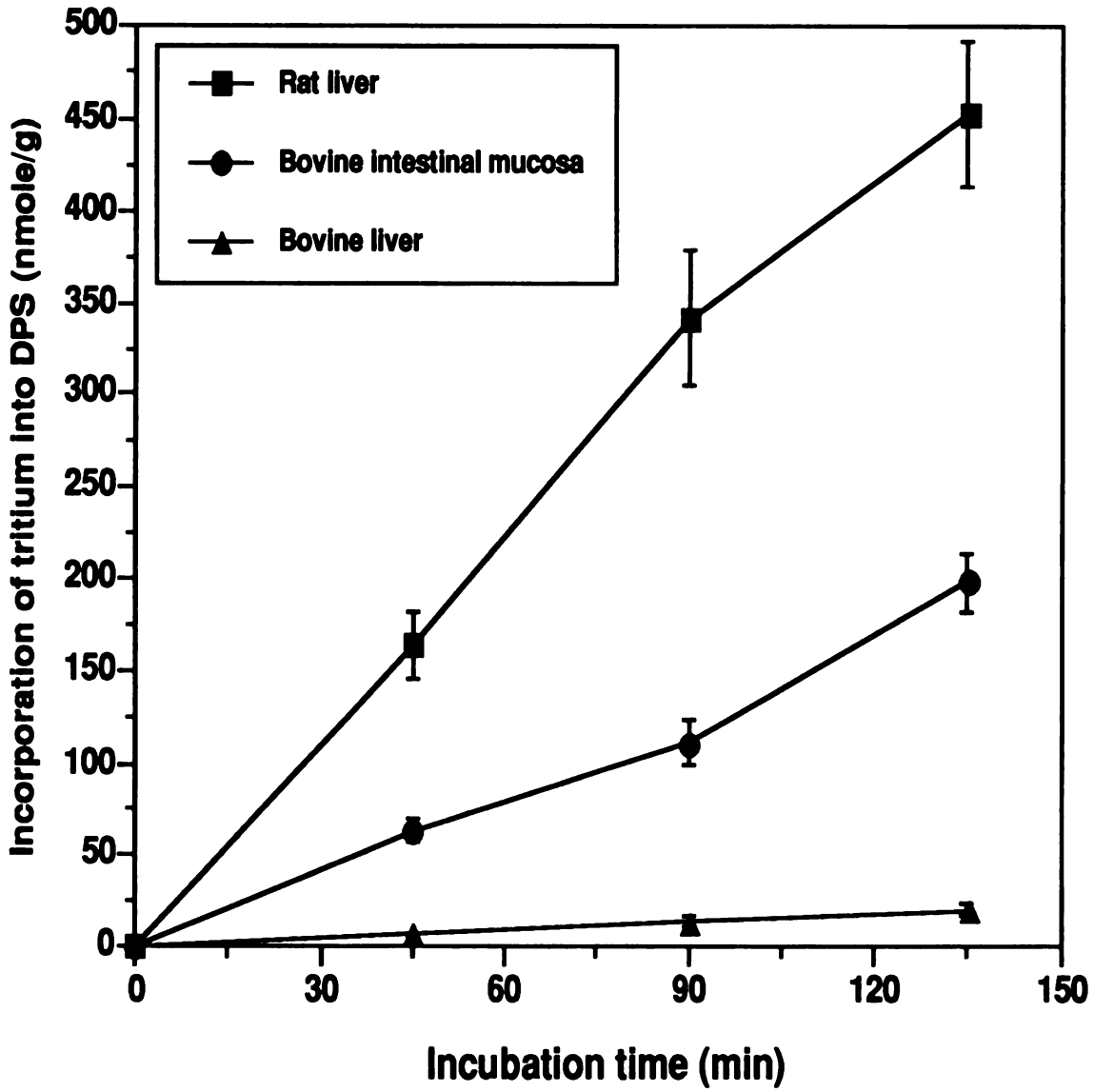

Figure 13: Time-course of incorporation of tritium from tritiated water into cow and rat liver tissues and cow intestinal mucosa,. The tissue explants (150 mg) were incubated in 1 ml MEM containing 5 mCi tritiated water at 37 C for the indicated time periods. After incubation, the digitonin precipitable 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 $\pm$ SEM of three separate experiments.

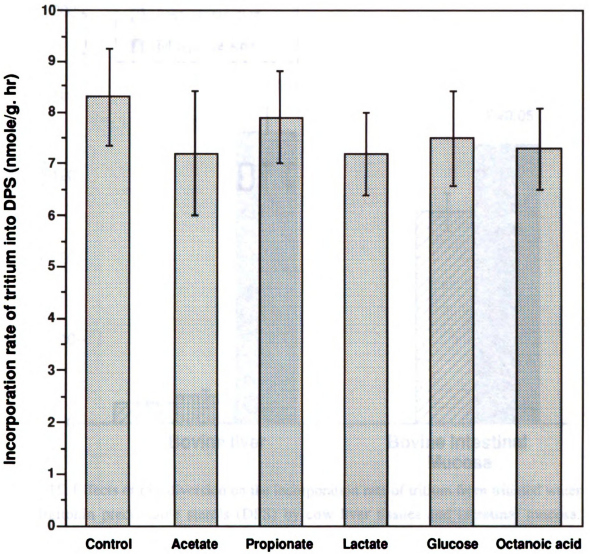

Figure 14: Effects of supplement of various substrates to MEM on incorporation rate of tritium from tritiated water into DPS by cow liver tissues. About 150 mg of cow liver explants were incubated in 1 ml of MEM containing 5 mCi of tritiated water alone or with 15 μmoles of acetate, propionic acid, lactate, glucose, or octanoic acid. After incubation, the digitonin precipitable sterols (DPS) were isolated from the incubation mixture and the radioactivities of the DPS were determined. Each data point represents the means ± SE of three incubations.

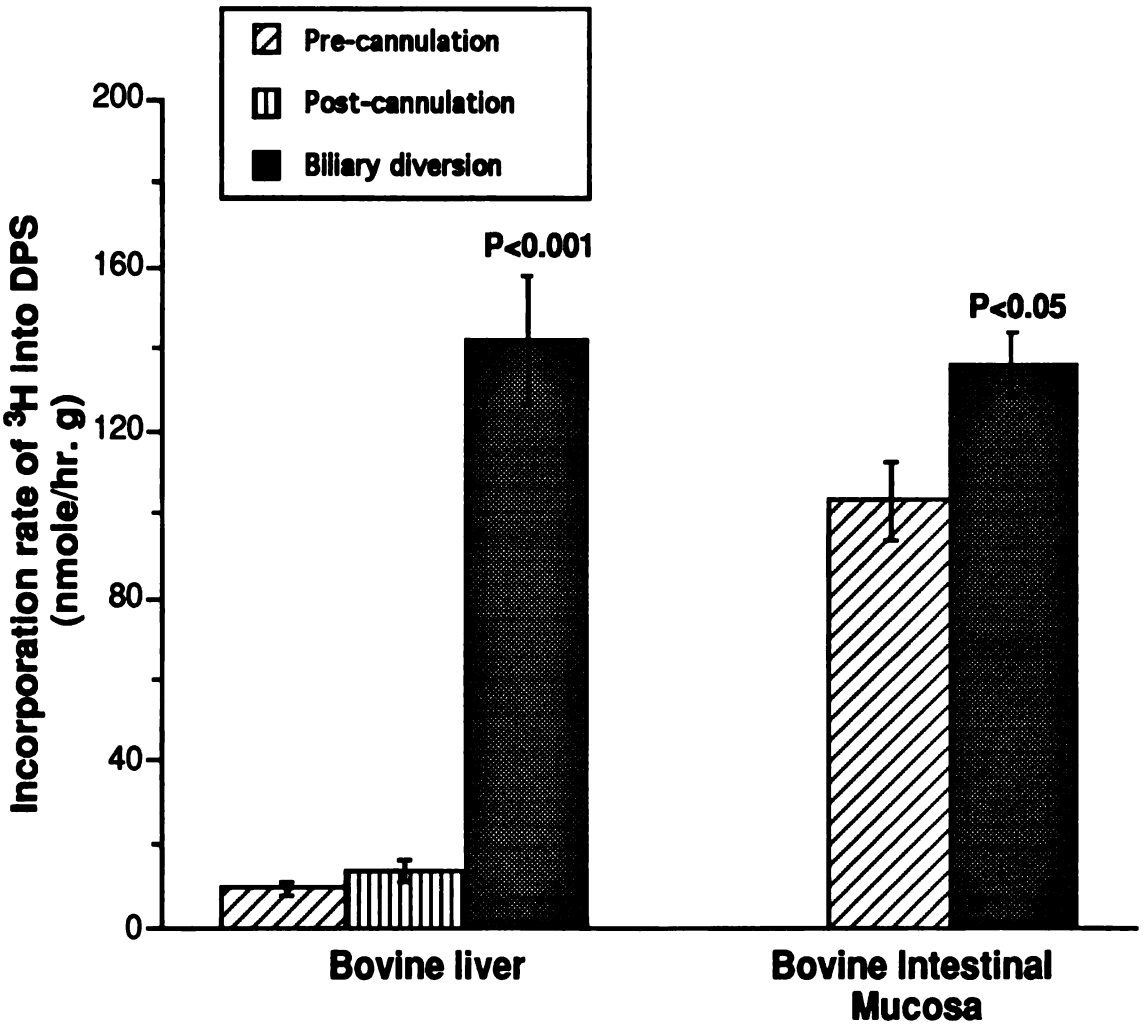

Figure 15: Effects of bile diversion on the incorporation rate of tritium from tritiated water into digitonin precipitable sterols (DPS) by cow liver tissues and intestinal mucosa. Incorporation rates of tritium into DPS were determined as described in Materials and Methods. Each point represents the mean ± SEM; n=5 except for control intestinal mucosa (n=4).

Based on the incorporation rate of [^{3}H]-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 Figure 16, in the normal 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 (Figure 16). The estimated rate of hepatic cholesterol synthesis was approximately 1.2 mmoles / day (Figure 16). This amount of cholesterol, however, would provide no more than 5% of the sterols lost during bile diversion (1.2 vs. 25 mmoles/day).

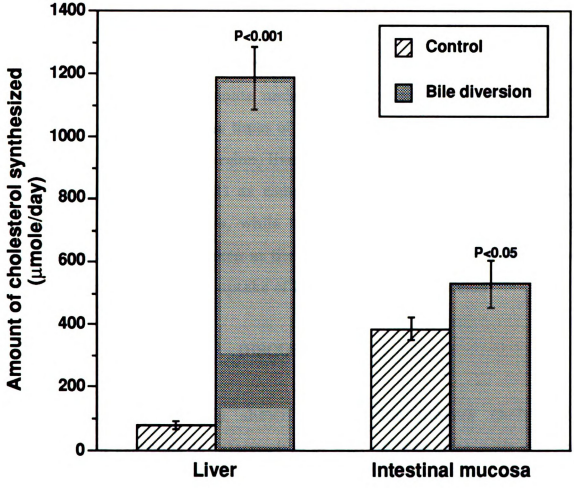

Figure 16: Estimated quantities of cholesterol synthesized by the whole liver and intestinal mucosa before and after bile diversion. The absolute amount of newly synthesized cholesterol was estimated based on the incorporation rate of tritium into DPS and the total mass of the liver and intestinal mucosa as described in Materials and Methods. Each point represents the mean $\pm$ SEM; n=5 except for control intestinal mucosa (n=4).

Hepatic LDL receptor binding activity

To further understand the possible changes in bovine hepatic cholesterol metabolism in response to bile diversion, we have also determined the response in liver LDL receptor-binding activity . The binding of ^{125}I-labeled bovine LDL to liver homogenate was in a saturable manner (Figure 17). Scatchard plot analysis (24) of the binding data revealed a linear plot with an equilibrium dissociation constant (Kd) of 16 μg LDL protein /ml (Figure 17), reflecting a single class of LDL binding sites in this tissue. The LDL-receptor binding activity of the liver tissue samples was then determined by measuring the heparin-sensitive binding of ^{125}I-LDL to liver homogenate under a fixed concentration of ^{125}I-LDL which is approximately three times of the Kd. The results (Figure 18) 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 (Figure 18). These data implicate an enhanced uptake of LDL during bile diversion.

DISCUSSION

We have previously shown that bovine plasma cholesterol concentration responds to interruption of EHC of bile salts with a much more dramatic decrease than that previously reported in other species (13). To understand the underlying mechanism for this observation, in the present study, we have determined the responses in hepatic and intestinal cholesterol metabolism induced by bile diversion.

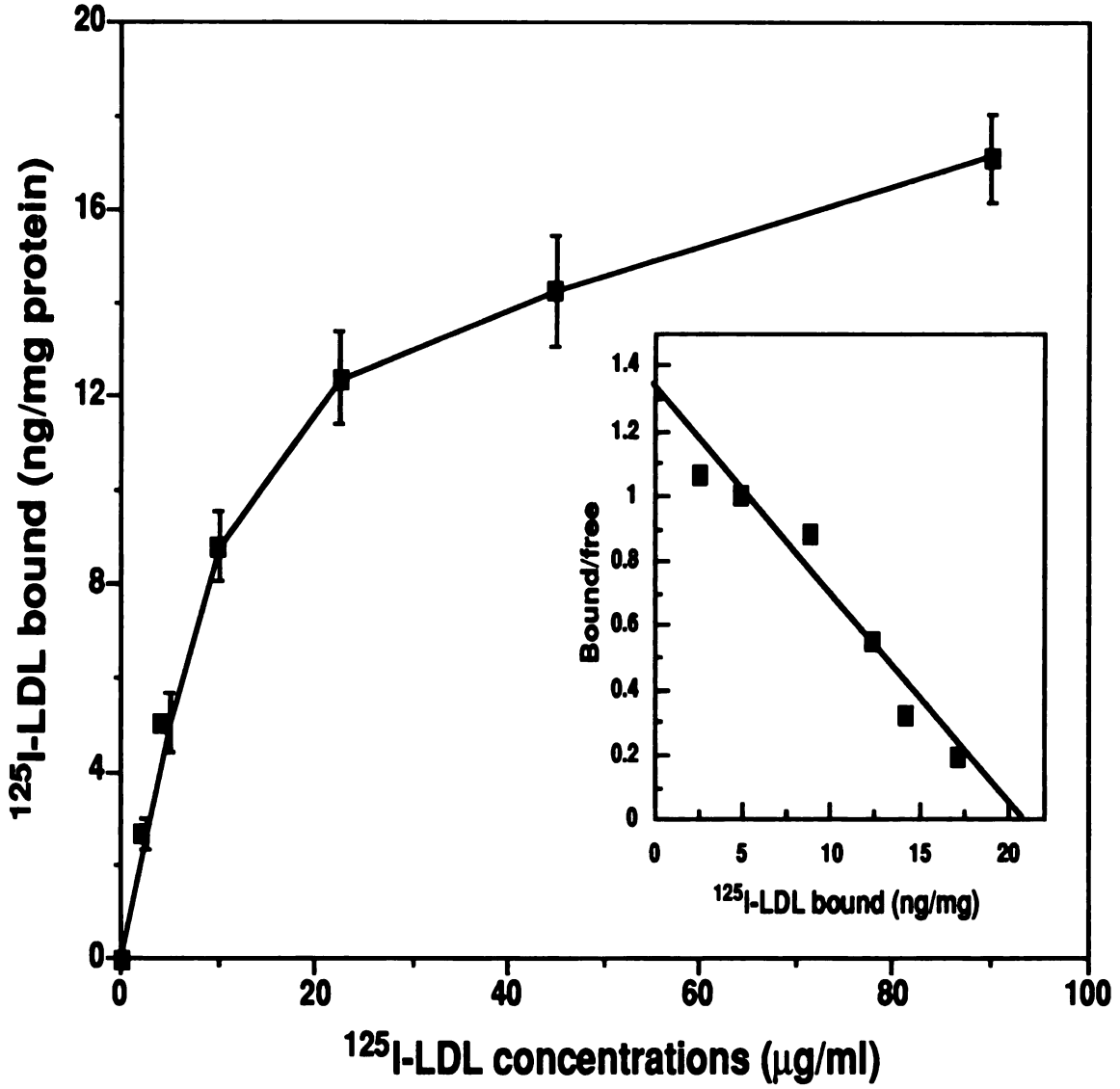

Figure 17: Binding of radiolabeled bovine LDL to bovine liver homogenate as a function of ^{125}I-LDL concentration. Liver homogenates (3 mg proteins) were incubated in 150 μl of buffer B with the indicated concentrations of ^{125}I-LDL for 2 h on ice. Specific bindings were determined by measuring the heparin-sensitive bindings. Each data point represents the means ± SE 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).

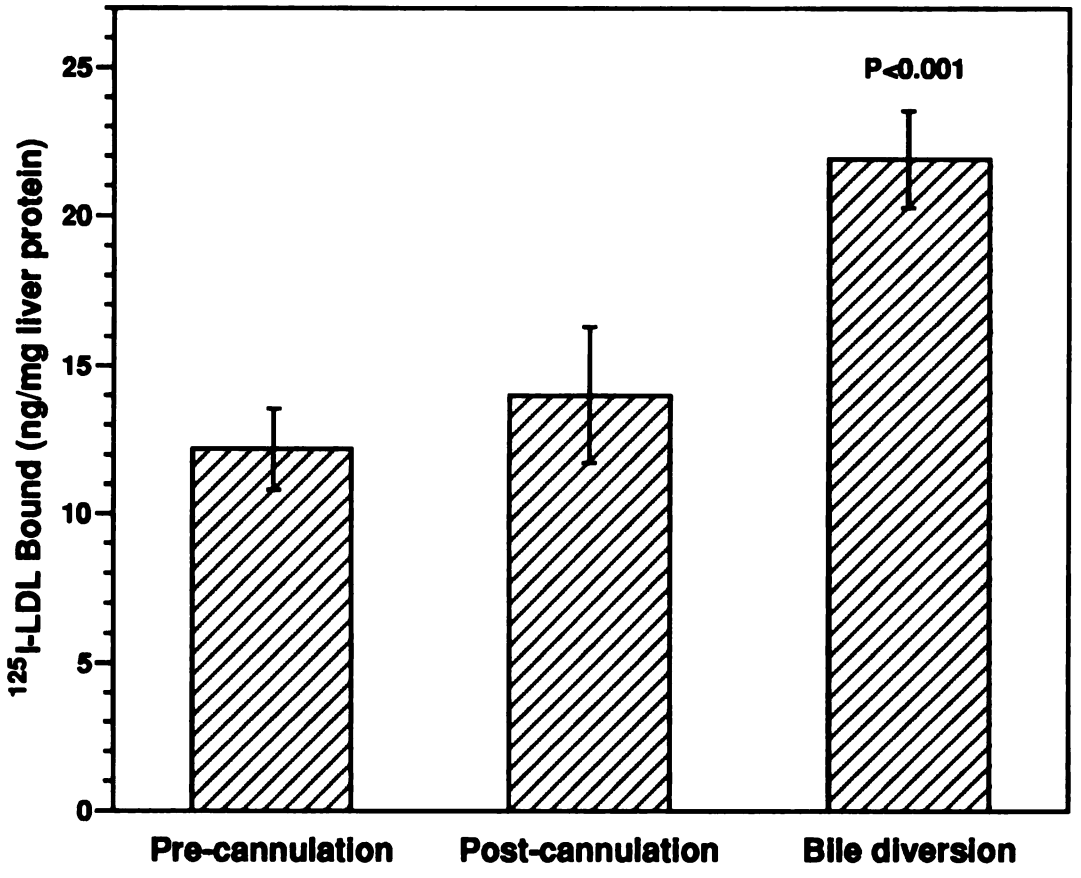

Figure 18: Effects of bile diversion on hepatic LDL receptor binding activity. LDL receptor binding assays were carried out as described in Experimental Procedures. Each value represents the mean ± SEM of five cows.

Two control periods were employed in this study in order to ascertain the effects of the cannulation procedure *per se* on hepatic cholesterol synthesis rates and the expression of hepatic LDL receptors. It was assumed that the samples taken at the time of the cannula placement (pre-cannulation controls) represented the completely natural state, while the samples taken after post-surgical stabilization, but prior to bile diversion (post-cannulation controls) would reveal direct effects due to the presence of the cannulas. The lack of differences in either cholesterol

synthesis rate or LDL receptor-binding activity between these two control periods indicates that there was no detectable effect of the cannulation procedure on the variables of interest.

The importance of ruminant liver to total body cholesterol production has not been known with certainty. Previous studies (7, 8) have suggested that ruminant liver was not a major site of body cholesterol synthesis, but this judgment was based on experiments with ^{14}C-labeled acetate, a substrate which is poorly utilized by ruminant liver (25). In the present study, by using ^{3}H-water as labeled substrate, we confirmed the conclusions from these earlier studies (7, 8). We found that the normal cholesterol synthesis rate in the bovine liver was barely detectable, while the small intestinal cholesterol synthesis rate was relatively high. We further 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. This demonstrates that, in this species, the small intestine is a much more important site for cholesterol production than the liver.

An important finding in this study was that the inherently low rate of cholesterol synthesis in the bovine liver can be augmented by bile diversion, indicating that the bovine liver has the capacity to increase cholesterol synthesis when cellular cholesterol is depleted. This is consistent with results of studies with other species (6, 26). However, since the basal rate of bovine hepatic cholesterol synthesis is so low, the absolute magnitude of this increase is negligible. At the new steady-state, during bile diversion, the bovine hepatic cholesterol synthesis rate was increased by more than 10-fold, with a total estimated synthesis rate of 1.2 mmoles cholesterol/day. This amount of cholesterol was far less than the cholesterol required to account for the sterols lost due to bile diversion, which was at

least 25 mmoles/day. Furthermore, the hepatic cholesterol store was almost completely depleted at this time period. Taken together, these data suggest that bovine liver primarily relies on extrahepatic tissues to provide cholesterol as substrate for increased bile acid synthesis during bile diversion. Alternatively, it might be possible that, in our study, the *in vivo* cholesterol synthesis rates in these animals might have been underestimated by the *in vitro* measurements. However, based on extensive studies of a variety of tissues from several species, a previous study (26) has reported that the *in vitro* cholesterol synthesis rate obtained by using ^{3}H-water as labeled substrate represented 10 to 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 loss and the hepatic cholesterol synthesis during bile diversion.

It appears that, due to the low capacity of ruminant liver to increase its cholesterol synthesis rate, interruption of EHC in ruminants produces an effect analogous to the combined administration of an HMG-CoA reductase inhibitor drug and bile acid sequestrants to humans or other species. In each case, there is a net increase in sterol elimination from the body combined with relatively low rate of cholesterol synthesis in the liver. The combination of these effects is known to cause a large reduction in plasma cholesterol concentration (6, 27). This reduction in plasma cholesterol appears to occur as a result of increased LDL receptor expression in the liver, which in turn leads to an increase in the clearance of circulating LDL cholesterol (27).

The increased hepatic LDL receptor-binding activity observed in this study further supports the enhanced catabolism of lipoprotein as a

mechanism of plasma cholesterol reduction during bile diversion in cattle. This finding also provides evidence that bovine hepatic LDL receptor expression is regulated metabolically. Hepatic LDL receptor plays a key role in regulating plasma cholesterol catabolism in the body (5, 28). A significant negative relationship between liver LDL receptor-binding activity and plasma cholesterol concentration in cattle has also been observed by other investigators (12), suggesting a possible physiological role of the hepatic LDL receptor in ruminants. However, the LDL receptor-dependent pathway might only play a relatively minor role in plasma lipoprotein catabolism in our animal model. This is because that LDL cholesterol only accounts for a very minor fraction of total plasma cholesterol in cattle (29) and hence, an increase in the hepatic uptake of LDL alone might not cause a significant change in plasma cholesterol concentration. In fact, we have previously shown that even a 50% reduction in both VLDL and LDL cholesterol did not result in any appreciable decrease in plasma cholesterol in our animal model (13). Furthermore, high density lipoprotein (HDL) particles of ruminant animals do not appears to contain apoE (30, 31), which is a ligand for hepatic lipoprotein receptors, including the LDL receptor (32). In addition, bovine plasma possesses very low levels of cholesterol ester transfer activity (33), which mediates the transfer of HDL cholesterol to LDL and for clearance by the liver (34). Thus, it appears that LDL receptor does not participate in the hepatic clearance of HDL cholesterol in cattle.

In view of the large reduction in HDL cholesterol observed in this study, it is intriguing to speculate that mechanisms of hepatic uptake of HDL cholesterol might not involve the LDL receptor. In addition to the LDL receptor, HDL cholesterol can be taken up by the hepatocytes through two

other pathways, which are independent of the LDL receptor-mediated process. The first pathway involves the uptake and internalization of the whole HDL particle and is known to be mediated by the cell surface HDL receptor (35, 36), whereas the second one appears to involve selective uptake of cholesterol esters from HDL by the cell without the HDL particle being internalized (35, 37). It is not clear whether HDL receptor is also involved in the latter pathway, but the role of hepatic lipase has been proposed (38, 39). However, previous studies (40-42) have shown that ruminant liver is almost devoid of hepatic lipase. This suggest that the pathway of selective uptake of cholesterol ester might not be fully functional in bovine liver. Presumably, the HDL receptor-mediated pathway might play an important role in the uptake of HDL cholesterol by the liver. In fact, high affinity binding sites for HDL have been identified in bovine liver plasma membrane (43). Because the preponderance of bovine plasma cholesterol is in the HDL fraction, understanding HDL metabolism is important for the complete understanding of our animal model.

In conclusion, the present study shows that bovine liver responds to interruption of EHC with augmented cholesterol synthesis and enhanced capacity for lipoprotein uptake. However, due to the inherently low rate of cholesterol synthesis, bovine liver must rely on uptake of lipoprotein cholesterol for bile acid synthesis during bile diversion. Thus, bile diversion causes a dramatic decline in plasma cholesterol in this species.

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