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The metabolic fate of cholesterol delivered to rat hepatocytes by rat plasma lipoproteins was determined. Binding and degradation of rat low and high density lipoproteins (LDL and HDL) in rat hepatocytes were studied. ^{125}I-labelled LDL and HDL were incubated with cells in the presence of varying concentrations of unlabelled lipoproteins for two hours at 37°C. The amount of ^{125}I-LDL and ^{125}I-HDL binding and degradation decreased by increasing concentrations of respective unlabelled lipoproteins. The presence of 50-fold excess of unlabelled LDL or HDL resulted in a reduction of ^{125}I-LDL and ^{125}I-HDL bindings by 66-82%, and degradations by 63-88%, respectively.

Equilibrium dissociation constants (K_d) determined by Scatchard analysis for HDL (.15 x 10^{-8}M) and LDL (1.04 x10^{-8}M) revealed that HDL have approximately 7-fold higher binding affinity for receptors on cell surface than LDL.

Specific use of LDL and HDL-cholesterol for bile acid synthesis by rat hepatocytes was investigated. When LDL and HDL labelled with ^{3}H-free cholesterol were incubated with hepatocytes for four hours at 37^{o}C, 3.4% of ^{3}H-HDL cholesterol was converted to bile acids, mainly primary bile acids, whereas, 1.1% of ^{3}H-LDL cholesterol was transformed to bile acids mostly as lithocholic, chenodeoxy and deoxycholic acids.

A technique developed for isolation of hepatocytes from rat liver was described. Once isolated by the technique most cells retained their microscopic structural integrity, and excluded trypan blue. The viability was 93%, which decreased to 86% after four hours of incubation.

The presented data demonstrated that both HDL and LDL bind to specific receptors on hepatocytes and undergo proteolytic degradation in rats. The study also showed that the binding affinity of HDL to hepatic receptors was much greater than that of LDL but in total binding LDL uptake was four times greater than HDL, suggesting the presence of two specific binding sites for HDL and LDL.

The first direct evidence for the preferential utilization of HDL-cholesterol for biosynthesis of bile acids *in vivo* is presented. This finding is compatible with the current concept of HDL as the protective lipoprotein against developing coronary heart disease.

The Metabolic Fate of Lipoprotein Cholesterol in Isolated Rat Liver Parenchymal Cells

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Chapter I

INTRODUCTION

Recent studies (1-5) indicated that the liver is the most important organ for the metabolism of cholesterol and lipoproteins. It is now well established that the liver participates in cholesterol metabolism in several ways. First; the liver is the major organ involved in endogenous biosynthesis of cholesterol (6-8). Second, the liver is the site of cholesterol catabolism, namely the formation of bile acids (9-11). Third; the liver is involved in cholesterol excretion through the entrohepatic circulation of cholesterol (9). Fourth; the liver plays an important role in the circulating lipoprotein metabolism, regulating serum cholesterol concentration (3, 12).

The objective of the present study was to determine the metabolic fate of cholesterol delivered to rat hepatocytes by rat low density and high density lipoproteins in vitro. The hypothesis was that the metabolic fate of cholesterol delivered by high density lipoprotein (HDL) to hepatocytes may be different from that of lower

density lipoproteins (LDL). To be compatible with the current concept of HDL as the protective lipoprotein agains the risk of developing coronary heart disease, HDL-cholesterol may be selectively utilized for de novo synthesis of bile acids and/or for free cholesterol source of the biliary cholesterol. The present study was designed to investigate three specific aims. The first aim (Chapter II) deals with binding and degradation of low and high density lipoproteins in rat liver parenchymal cells. The second aim (Chapter III) was to determine the metabolic fate of LDL-free cholesterol and HDL-free cholesterol in rat hepatocytes after binding and degradation. The last aim (Chapter IV) was to develop a technique for isolation of rat liver hepatocytes by perfusing isolated liver with the enzyme collagenase and Ca^{2+}.

The pertinent literature for the study was reviewed and presented in the following sections.

A. PLASMA LIPID TRANSPORT SYSTEMS

1. Transport of exogenous lipids by chylomicrons:

After intestinal absorption, fatty acids from dietary fat and dietary cholesterol are reesterified in the endoplasmic reticulum of mucosal cells to form nonpolar triglycerides and cholesteryl esters. These

are packaged to form chylomicrons together with the intestinal B apolipoprotein (B-48), A apolipoproteins, and polar lipids (phosopholipids and cholesterol). The chylomicron particles are concentrated in secretory vesicles of the Golgi apparatus and then secreted from the cell (2). The apolipoproteins and polar lipids form a monomolecular film that surrounds the nonpolar lipids in the core of the chylomicron. In the absence of B-48, secretion of chylomicrons does not occur (13). The recent chylomicrons enter lacteals in the intestinal villus and are transported through the thoracic duct into the blood (14). In the lymph and blood, the chylomicron particles acquire additional apolipoproteins (mainly apoprotein E and the several C apoproteins) from high density lipoproteins (HDL) (3). The intact chylomicrons then interact with lipoprotein lipase, an enzyme bound to the endothelial surface of blood capillaries in many extrahepatic tissues, resulting in rapid hydrolysis of most of the triglycerides that compose the core of the particles (3). As the triglycerides are removed, much of the surface lipids and C apoproteins, together with virtually all of the A apoproteins, are transferred to HDL (3). Loss of one of the C apoproteins (apo C-II), an essential cofactor for lipoprotein lipase, eventually reduces the affinity

of the particle for lipoprotein lipase, such that it cannot compete effectively with other particles for the enzyme (14). At the same time, the apoprotein E, which is retained at the surface of the chylomicron "remnant" is altered by the loss of C apoproteins in such a way that the particle is now recognized by an apoprotein E receptor on the surface of hepatic parenchymal cells (2). The bound remnant particle is rapidly taken up into the cell by endocytosis and transported to the region of the bile canaliculus. There, lysosomal catabolism of the lipid and protein components occurs. The dietary cholesterol esters are hydrolyzed to free cholesterol which in turn can be excreted in the bile (as such or after oxidation to bile acids) or can be incorporated into hepatogenous lipoproteins (2).

2. Endogenous transport from the liver:

Triglycerides are continuously synthesized in the liver from fatty acids (derived from plasma free fatty acids and non-lipid precursors), in amounts of about 40 to more than 100 gm daily (2). As the amount of fatty acids handles by the liver exceeds their oxidation capacity for energy needs, a large fraction of these triglycerides must be secreted from the liver to prevent

steatosis. This process is subserved by very low density lipoproteins (VLDL). VLDL synthesis and secretion occur by processes analogous to those for chylomicrons. A distinct apolipoprotein B (B-100) is required for secretion of nascent VLDL, which also contain C apoproteins and apoprotein E (13). After acquisition of more C apoproteins from HDL, the VLDL interact with lipoprotein lipase in a similar mode as in the catabolism of chylomicrons, and the remnant particles are formed called intermediate density lipoproteins (IDL). The excess surface materials, mostly phospholipids and cholesterol, are transferred to HDL (2-3). The HDL particles interact with the plasma enzyme, lecithin-cholesterol acyltransferase (LCAT) which esterifies the free cholesterol with fatty acids derived from the 2-position of lecithin, the major phospholipid of plasma (15). The newly synthesized cholesteryl ester is transferred back to the IDL particles from HDL, apparently through the action of a plasma cholesteryl ester exchange protein (3). The net result of the coupled lipolysis and exchange reactions is the replacement of most of the triglyceride core of VLDL with cholesteryl esters. After lipolysis, the IDL particles are released from the capillary wall into the circulation. They are further modified by poorly defined processes to form low density

lipoproteins (LDL). These processes involve loss of most of the remaining triglycerides and all protein components except B-100.

Unlike VLDL, which are normally metabolized in a few hours, LDL are metabolized slowly over a couple of days (2). The metabolism of LDL has been studied in various types of cultured cells. Studies in human fibroblasts elucidated a receptor-mediated pathway in which LDL is bound to a specific cell surface receptor, internalized, and degraded as previously reported (16-17). In extrahepatic tissues the "LDL pathway" provides cholesterol needed for membrane synthesis in dividing cells and synthesis of steroid hormones in the adrenal cortex and gonads. LDL are also metabolized by other pathways. Some of these pathways involve other lipoprotein receptors; the remainder may occur during bulk fluid endocytosis (16).

3. HDL and "Reverse Cholesterol Transport":

Unlike chylomicrons and VLDL, HDL are not secreted in a near final form from the tissues that synthesize their component proteins. The liver, and possibly the small intestine secrete nascent HDL in the form of discoidal particles composed of a bilayer of phospholipids surrounded by apoproteins (A and B). HDL present in the circulation arises by the action of LCAT

upon "nascent" HDL particles. During the conversion of the discs into mature spherical HDL particles by LCAT, the cholesterol content of the particles increases markedly by esterification of free cholesterol continuously taken up by the particle from other plasma lipoproteins (2, 14). Thus, most of the cholesteryl esters found in VLDL, LDL are produced in this way (2). While human LDL carries the bulk of plasma cholesterol from plasma to tissues where LDL undergoes the receptor mediated cellular metabolism (16-17), HDL particles pick up free-cholesterol from membrane surface of peripheral cells and deliver it to the liver where HDL-cholesterol may be preferably utilized for bile acid synthesis and/or biliary excretion (18). This is the major pathway by which cholesterol is excreted out of the body. Therefore, this cholesterol transport route has been called "reverse cholesterol transport" (14).

B. HEPATIC LIPOPROTEIN METABOLISM

The liver is the major source of plasma lipoproteins, secreting mainly very low density lipoproteins (VLDL) and high density lipoproteins (HDL). Most of the LDL in plasma in normal man is derived from VLDL by the action of lipoprotein lipase and other undefined processes (1).

The role of the liver in lipoprotein metabolism has been elucidated over the past decade (1). Most or all

of the "remnants" generated by the action of lipoprotein lipase on chylomicrons are removed by the liver. In rats, at least, the VLDL "remnants" share the same fate (19) and, therefore, only a small amount of apolipoprotein B from VLDL enters the LDL fraction. The low levels of LDL in rats may be, in part, attributable to this. In man, much more VLDL is converted to LDL before it is removed from the plasma component (14). In some cases, essentially all of VLDL apoprotein B, the characteristic apoprotein for both VLDL, and LDL, is converted to LDL apoprotein B (20); in some subjects, as much as 50% or more VLDL apoprotein B leaves the plasma before entering the LDL fraction (21). It is generally assumed that this direct loss occurs in the liver by removal of VLDL "remnants" as in experimental animals, but some uptake by extrahepatic tissues cannot be ruled out.

The liver is important in the catabolism of LDL (22-27), although the mechanisms by which LDL may interact with hepatic cells are not entirely clear. The liver accumulates injected LDL (22, 24, 28, 20). Studies in rabbits (22), and estradiol-treated rat, (29) suggest that approximately half the uptake of LDL is mediated by a mechanism similar to the LDL receptor. High-affinity uptake and degradation of LDL have been observed in

suspended rat (30-31) and rabbit hepatocytes (32-33). Pangburn et al (34) recently presented evidence in cultured swine hepatocytes for the degradation of ^{125}I-LDL by an LDL receptor mediated mechanism.

Less is known about the metabolism of HDL. HDL participates in the esterification of cholesterol during the initial stages of the catabolism of triglyceride-rich lipoproteins (3) and may also function in part to transport cholesterol to the liver. In some cultured cells, HDL has been found to stimulate the removal of cholesterol (35-39). The role of the liver in the catabolism of HDL is unclear. Several studies (40-42) suggested that most of the catabolism of HDL apoproteins occurs extrahepatically. Other studies indicate that the liver may contribute significantly to the catabolism of HDL. The liver accumulates injected HDL (43-46), and HDL associates primarily with parenchymal cells (48). Suspended rat hepatocytes and nonparenchymal cells apparently bind HDL to high-affinity sites in the cell membrane, and binding is associated with the lysosomal degradation of HDL-protein (30-31, 43, 48-49).

Studies in liver microsomes suggest the existence of more than one kind of high-affinity site for lipoproteins. Rat liver microsomes bound little to LDL, but LDL receptor like binding activity was stimulated in

rat treated with 17-∝-ethinylestradiol (50-51). Liver microsomes from young dogs bound LDL primarily have been shown to bind through LDL receptor-like sites (52-53). These sites were absent in adult dogs but were induced by cholestyramine treatment (53). Liver microsomes from immature and adult dogs contain a second site that recognized apo-E-containing lipoproteins but not LDL (53). Much of the LDL binding in rabbit liver microsomes was to an LDL receptor-like site, but LDL also bound to a second site with different properties (54). Bachorik et al. (55), found that most of the high-affinity LDL binding in pig liver plasma membranes was to sites whose properties differed from those of the LDL receptor of fibroblasts. The peripheral LDL receptor recognizes apolipoprotein B (apo B) and apo E (17, 56-60), but not apo A-I or apo A-II (61). Binding requires Ca^{2+} and intact lysine and arginine residues in the apoproteins, and the receptor is inactivated by proteolytic enzymes (62-64). In pig liver membranes, the high-affinity ^{125}I-LDL binding site recognized apo-E-free HDL, was not inactivated by pronase (65-66) and did not require intact arginine or lysine residue in LDL (55). In view of its unusual lipoprotein specificity, the authors called the site "lipoprotein binding site."

More recently, Bachorik et al. (67), studied the relation between the uptake and degradation of LDL and HDL in cultured swine hepatocytes. Evidence shows at least two kinds of high-affinity sites. One site was similar to the peripheral LDL receptor and mediated the lysosomal degradation of LDL. The other site resembled the lipoprotein binding site in that it recognized LDL and Apo-E-Free HDL. This site mediated little if any LDL degradation but appeared to account for all of the high-affinity HDL degradation that occurred in the cells.

1. Lipoprotein receptors in the liver

Exogenous (dietary) cholesterol is delivered to the liver in chylomicron remnants (3-5), which are derived from intestinal chylomicrons through the action of lipoprotein lipase. The remnants rapidly enter the liver by receptor-mediated endocytosis after binding to specific remnant receptors (53, 68-69). Endogenous cholesterol transport begins when the liver secretes cholesterol into plasma together with triglycerides in very low density lipoproteins (VLDL). After the triglycerides of VLDL are removed by lipoprotein lipase, the resultant cholesterol-rich particle is designated intermediate density lipoprotein (IDL). IDL particles

bind with high affinity to hepatic LDL receptors (3, 70). Some of the particles are rapidly cleared from plasma by this route; other IDL particles are converted to LDL. The mechanism of this conversion is unknown. LDL is removed relatively slowly from the plasma by binding to LDL receptors in the liver and extrahepatic tissues (71). These receptors are genetically and immunologically identical to the LDL receptors of cultured fibroblasts (68, 72). In rabbits, rats, and hamsters, more than half of the total LDL receptors are located in the liver (73-74). However, the precise distribution of these receptors in man is unknown.

Appreciation of the separate fates of endogenous and exogenous cholesterol is reinforced by new knowledge concerning apoprotein (apo) B, the major structural protein of cholesterol-carrying lipoprotein (13). The apo B synthesized by the intestine (designated apo B-48) is only 48% as large as the apo B synthesized by the liver (apo B-100). Chylomicrons and chylomicron remnants contain apo B-48. VLDL, IDL, and LDL contain apo B-100, but no apo B-48 (13). Hence, these three particles must arise from endogenous hepatic sources.

2. Hepatic LDL receptors and chylomicron remnant receptors:

Hepatic LDL receptor recognizes apo B-100, the only apoprotein of LDL. The same LDL receptor also recognizes

apo E, a constituent of chylomicrons and chylomicron remnants as well as of VLDL and IDL. Much of the work has been performed with apo E-HDL_c, a lipoprotein that accumulates in plasma of cholesterol-fed dogs (75). Although named as an HDL, apo E-HDL_c differs from typical HDL in that it contains little apo A; its major apoprotein is apo E. The affinity of the LDL receptor for apo E-HDL_c is 20-fold greater than that for LDL (which contains only apo B) (59). Lipoproteins such as IDL that contain both apo B-100 and apo E bind to LDL receptors with higher affinity than LDL, suggesting that apo E is the preferential ligand. Chylomicron remnants, which contain apo E and apo B-48, also bind to LDL receptors (68, 78).

Certain lipoproteins that contain apo E do not bind to LDL receptors. In particular, newly circulating chylomicrons and VLDL bind poorly despite their content of apo E (76-77). This poor binding has been attributed to the presence on these triglyceride-rich particles of another family of apoproteins, apo C (3). The apo C's are believed to play a dual metabolic role: they mask the receptor binding site on apo E and they also activate lipoprotein lipase. After the triglycerides have been hydrolyzed by lipoprotein lipase, the apo C's leave the particles and the particles are rapidly cleared from plasma.

Although chylomicron remnants can bind to LDL receptors in vitro, evidence indicates that most of these particles enter the liver by binding to a separate class of unregulated receptors that do not recognize apo B-100 containing lipoproteins. Sherrill et al. (78) found that apo E-HDL_c competes with ^{125}I-labelled chylomicron remnants for uptake in perfused rat livers. Moreover, apo E-HDL_c binds to liver membranes in vitro at a time when LDL receptors have been metabolically suppressed (53). In view of these binding specificities, hepatic LDL receptors have been called "apo B, E receptors" and chylomicron remnant receptors have been called "apo E receptors."

The binding of apo E to both the remnant receptor and LDL receptor creates a paradox. In certain metabolic and genetic situations, the LDL receptor is suppressed or absent, but the remnant receptor functions normally. Under these conditions the plasma accumulates large amounts of lipoproteins, such as IDL and so-called "B-VLDL" that contain both apo-100 and apo E (69). If apo E-containing particles can bind to the remnant receptor, why are they not removed from the circulation in vivo? The answer is that apo E cannot bind to the remnant receptor when it is present on a lipoprotein particle together with apo B-100. Somehow apo B-100 may

mask the ability of apo E to bind to the remnant receptor and thereby direct it to the LDL receptor (3, 70).

In summary, apo E is an important determinant of the lipoprotein affinity for both the chylomicron remnant receptor and the LDL receptor. The decision as to whether a given lipoprotein binds to the remnant receptor or the LDL receptor is controlled by the interaction of apo-E with its neighboring apoproteins and perhaps lipids (70). Through these interactions, exogenous apo B-48 containing lipoproteins are directed to remnant receptors and endogenous apo B-100 containing lipoproteins are directed to LDL receptors.

C. HEPATIC CHOLESTEROL METABOLISM

Cells require cholesterol for synthesis of new membranes, and as a substrate for steroid hormone synthesis. Under normal conditions, most extrahepatic tissues do not synthesize cholesterol but rather depend on plasma lipoprotein sources of cholesterol to meet metabolic requirements. Cholesterol in the plasma is present in spherical lipoprotein particles, either as free cholesterol in the surface coat, or as esterified cholesterol in the core of the particle (79). Approximately 60% of the cholesterol in the plasma of normal man is associated within LDL. High affinity binding of

LDL to extrahepatic tissues followed by cellular uptake and lysosomal degradation of the LDL particle results in the release of free cholesterol within the cell (3).

Under certain conditions, it may be desirable for cells to reduce their cholesterol content. Nongrowing cells which acquire cholesterol via LDL uptake must also dispose of cholesterol in order to maintain a steady state. The process by which cholesterol is removed from extrahepatic tissues has not been completely elucidated, but HDL may be involved in tissue removal of cholesterol. Norum and Glomset et al. showed that patients with familial lecithin cholesterol acyltransferase (LCAT) deficiency were unable to form cholesterol ester and that their plasma became filled with disc-like particles made up of lecithin and free cholesterol and some apoproteins (80-84). Based on the above findings, Glomset (15) suggested that high density lipoprotein was probably secreted from the liver as a disc which picked up free cholesterol. Free cholesterol was then converted to esters and returned to the liver for excretion. Further, the work of several groups have shown that free cholesterol may be removed from cell cultures by high density lipoprotein (37, 85-86).

Ultimately all cellular cholesterol, including that absorbed from liver can eliminate cholesterol out of the

body. The synthesis of steroid hormone from cholesterol in the adrenals and gonads contributes an insignificant fraction to total daily cholesterol (87). In the liver, cholesterol can be disposed of in two ways: (1) transformation of the two primary bile acids, cholic acid, and chenodeoxycholic acid, which are secreted in the bile and are eventually lost in the feces (11). Approximately 500 mg of cholesterol is converted daily to bile acids (3). This biotransformation occurs only in the liver, and cholesterol is the only substrate for primary bile acid synthesis. (2) The liver can also secrete free cholesterol directly into bile (1-2 g/day), a portion of which is reabsorbed in the small intestine (87).

The generally accepted view of how cholesterol is metabolically transformed by the liver cell into the primary bile acids is presented in Fig. 1 (page 19). Recent studies have shown that in man, there are definitive hepatic precursor sites associated with the secretion of biliary cholesterol and the synthesis of bile acids (10). These compartments derive approximately 70% of their cholesterol from lipoprotein-free cholesterol, 0-10% from plasma esterified cholesterol, and 25% from newly synthesized hepatic cholesterol. However, these findings are different from studies in rats in which

Figure I.1. The current view of hepatic cholic acid and chenodeoxycholic acid biosynthesis based on studies primarily in the rat. (Taken from Vlahcevic, Z.R. et al. 1980. Biosynthesis of bile acids in man. J. Biol. Chem. 256: 2925-2933.)

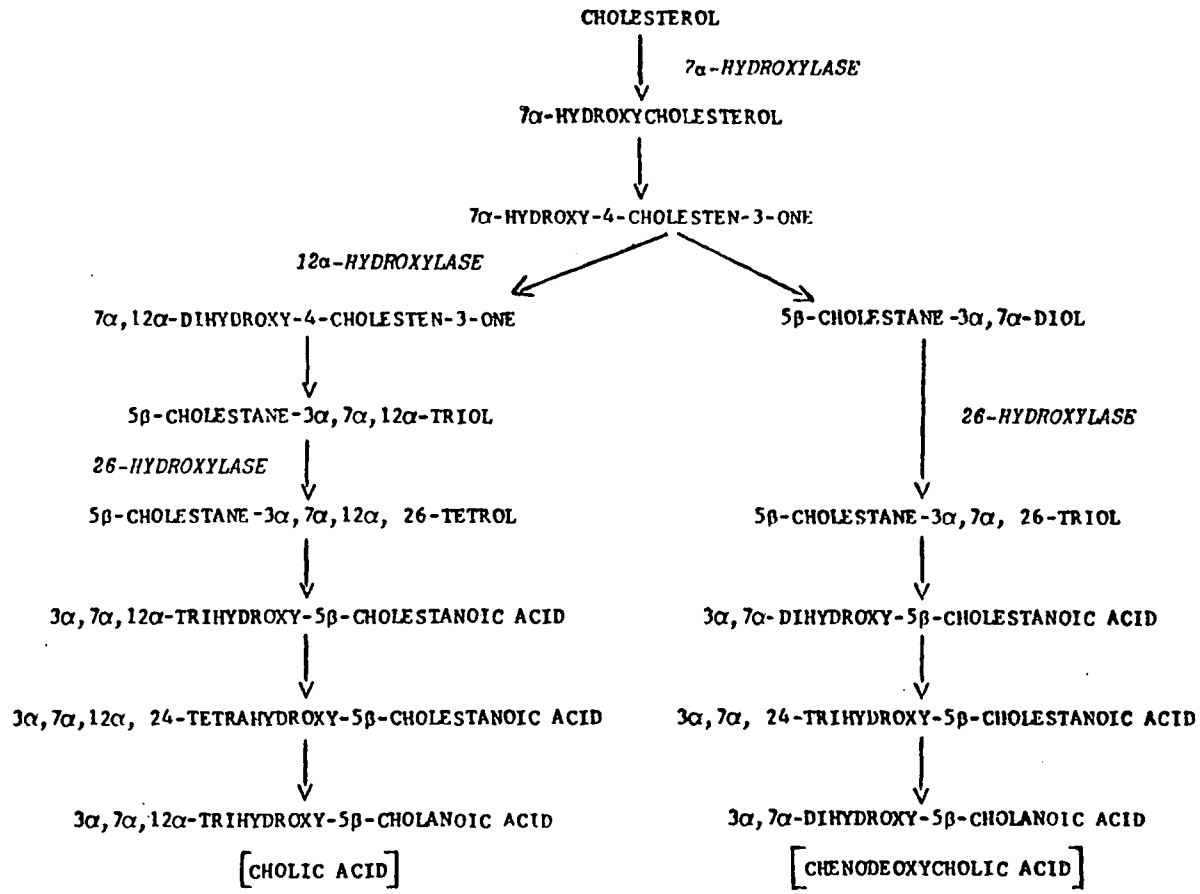
CHOLESTEROL
7α-HYDROXYLASE
7α-HYDROXYCHOLESTEROL
7α-HYDROXY-4-CHOLESTEN-3-ONE
12α-HYDROXYLASE
7α,12α-DIHYDROXY-4-CHOLESTEN-3-ONE
5β-CHOLESTANE-3α,7α-DIOL
5β-CHOLESTANE-3α,7α,12α-TRIOL
26-HYDROXYLASE
26-HYDROXYLASE
5β-CHOLESTANE-3α,7α,12α, 26-TETROL
5β-CHOLESTANE-3α,7α, 26-TRIOL
3α,7α,12α-TRIHYDROXY-5β-CHOLESTANOIC ACID
3α,7α-DIHYDROXY-5β-CHOLESTANOIC ACID
3α,7α,12α, 24-TETRAHYDROXY-5β-CHOLESTANOIC ACID
3α,7α, 24-TRIHYDROXY-5β-CHOLESTANOIC ACID
3α,7α,12α-TRIHYDROXY-5β-CHOLANOIC ACID
[CHOLIC ACID]
3α,7α-DIHYDROXY-5β-CHOLANOIC ACID
[CHENODEOXYCHOLIC ACID]

Figure I.1

hepatic newly synthesized cholesterol makes up a major contributor to bile acid synthesis (89-91). On the other hand study by Quarfordt et al. (92) reported that in the rat, esterified cholesterol of HDL is transported to the liver for catabolism as bile acids and biliary cholesterol. They showed that hepatic uptake of HDL rich in E-apoprotein (apo E) was about 10 times more than the bulk of the HDL rich in the A-I protein, indicating the important role of E-rich HDL_s in the return of cholesterol to the liver for subsequent metabolism. Furthermore, Sniderman et al. (93) showed that human LDL esterified cholesterol rather than HDL is removed from splanchnic circulation and in swine, Pittman et al. (25) reported that 40% LDL degradation is attributed to the liver.

These findings were not supported by the data of Schwartz et al. (10) who found that less than 10% of the biliary cholesterol arose from plasma esterified cholesterol, and suggested that lipoprotein esterified cholesterol is not earmarked for excretion from the body as biliary cholesterol. Other studies have shown that free cholesterol associated with HDL is used preferentially over LDL-free cholesterol by the liver as a substrate for bile acids synthesis and biliary cholesterol secretion (18, 79, 94). The mechanism by

which HDL-free cholesterol is preferentially selected for hepatic metabolism to bile acid is unknown. Free cholesterol could be bound more loosely to the HDL particle and therefore, could exchange more readily with other membranes (10). Hepatocellular degradation of the entire HDL particles does not seem likely, because only a small percentage of the total cholesterol secreted daily by a normal individual could be by this mechanism (10, 18). Therefore, the presence of a hepatocyte surface receptor mechanism which selectively removes surface bound free cholesterol from the HDL particle, leaving it otherwise intact, would be most consistent with HDL shuttling cholesterol from the tissues to the liver for excretion as bile acid and biliary cholesterol.

The risk of developing atherosclerotic coronary heart disease has been related directly to the plasma cholesterol concentration (95), which mainly reflects LDL cholesterol and has been found to be inversely related to HDL-cholesterol concentration (96). HDL-cholesterol concentration, however, is a three-fold better predictor than total cholesterol concentration of the future risk of coronary heart disease in both men and women (95). The protective effect from coronary heart artery disease which may be provided by a high

level of HDL could be linked to the more efficient removal of tissue cholesterol via the HDL's free cholesterol shuttle system.

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CHAPTER II

BINDING AND DEGRADATION OF LOW AND HIGH DENSITY LIPOPROTEINS IN RAT LIVER PARENCHYMAL CELLS

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ABSTRACT

The binding and degradation of rat low density lipoprotein (LDL; $1.006 < d < 1.063$ g/ml) and high density lipoprotein (HDL; $1.063 < d < 1.21$ g/ml) in rat liver parenchymal cells were studied. At 37^0C, ^{125}I-labelled LDL and ^{125}I-HDL were incubated with cells in the presence of varying concentrations of unlabelled lipoproteins. The amounts of ^{125}I-LDL and ^{125}I-HDL binding and degradation were decreased by increasing the concentrations of respective unlabelled lipoproteins. The presence of a 50-fold excess of unlabelled HDL resulted in a reduction of ^{125}I-HDL binding to 82% of total amount bound and unlabelled LDL in 50-fold excess reduced the binding of ^{125}I-LDL to 66%. When the specificity of LDL and HDL degradation in rat hepatocytes was assessed, the 50-fold excess amount of unlabelled HDL inhibited ^{125}I-HDL degradation by 88% whereas much less inhibition of ^{125}I-LDL degradation by unlabelled LDL occurred (63%). These data demonstrate that rat liver parenchymal cells showed similar binding affinities for LDL and HDL but the capacity for binding and for degrading HDL was much higher than that for LDL. The large proportion of HDL degraded by rat parenchymal cells suggest a specific role of the liver in HDL catabolism and in the rat, hepatic cholesterol may be derived from circulating HDL.

Equilibrium binding studies were conducted at $4^{o}C$ to determine the mechanism of the binding affinities for LDL and HDL. Binding of LDL and HDL to rat parenchymal cells were characterized by saturable (specific) and nonsaturable (nonspecific) process. Binding and degradation appeared to be simple bimolecular receptor interaction and no heterogeneity of binding sites. Equilibrium dissociation constants determined by Scatchard analysis of the equilibrium binding data for HDL ($.15 \times 10^{-8}M$) and LDL ($1.04 \times 10^{-8}M$) revealed a 7-fold greater affinity of HDL for receptors. The Scatchard plots also confirmed that saturation of the receptors occurred at much lower levels for HDL than LDL. The B_{max} were 90 and 375 ng of lipoprotein protein per mg cell protein for HDL and LDL, respectively. These data suggest that rat liver parenchymal cells are more suited and preferable for binding and degradation of HDL than LDL, in which HDL can be the transport vehicle for cholesterol from peripheral tissues to the liver for excretion as bile acid and biliary cholesterol.

Supplementary key words: LDL, HDL, rat liver parenchymal cells, binding and degradation.

INTRODUCTION

Plasma low density lipoprotein (LDL) is an end product of the catabolism of VLDL (1), and constitutes an important source of cholesterol for many tissues. The metabolism of LDL has been studied in various types of cultured cells. Studies in human fibroblasts elucidated a receptor mediated pathway in which LDL is bound to specific receptors, internalized, transported to lysosomes, and degraded (2, 3). During this process, cholesterol released from the lipoprotein is available for membrane synthesis. Less is known about the metabolism of HDL. HDL participates in the esterification of cholesterol during the initial stages of the catabolism of triglyceride-rich lipoproteins (4) and may also function in part to transport cholesterol to the liver. In some cultured cells, HDL has been found to stimulate the removal of cholesterol (5-9).

The liver is important in catabolism of LDL (10-17), although the mechanisms by which LDL may interact with the liver are not entirely clear. The liver accumulates injected ^{125}I-LDL (10-12, 18). Studies in rabbits (10) and estradiol-treated rats (19) suggest that approximately half the uptake of LDL is mediated by a mechanism similar to LDL receptor. High-affinity uptake and degradation of

LDL have been observed in suspended rat (16, 20, 21) and rabbit hepatocytes (22, 23). Pangburn et al. (24) recently presented evidence in cultured swine hepatocytes for the degradation of ^{125}I-LDL by an LDL receptor-mediated mechanism.

The mechanism by which the apoproteins of HDL are catabolized and the role of the liver in this process are unclear. Several studies indicated that the liver may contribute significantly to the catabolism of HDL. The liver accumulates injected ^{125}I-HDL (25-28) and HDL associates primarily with parenchymal cells (29). Suspended rat hepatocytes and non-parenchymal cells apparently bind HDL to high-affinity sites in the cell membrane, and binding is associated with the lysosomal degradation of HDL-protein (20, 21, 25, 30-31).

In this report we present data on the binding and degradation of ^{125}I-LDL and ^{125}I-HDL in freshly isolated rat liver parenchymal cells prepared by liver perfusion with collagenase. Rat parenchymal cells were able to bind and degrade both ^{125}I-LDL and ^{125}I-HDL, but with much higher capacity for HDL as compared to LDL.

METHODS

Chemicals

Collagenase (type I), bovine serum albumin (fraction V), and EDTA were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Sodium ^{125}I (iodine) (carrier-free) was purchased from Amersham International Ltd. (Arlington Heights, Ill., USA). Iodine monochloride was purchased from (Aldrich Chemical Co.). Minimum essential medium (MEM) was purchased from GIBCO Laboratories (Grand Island, N.Y., USA).

Lipoprotein isolation

All rats were fasted overnight for at least 16 hours prior to the isolation of the plasma lipoproteins. LDL ($1.006 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) were obtained from 140-150 ml of blood collected in centrifuge tubes contained EDTA, 1 mg/ml of blood. Lipoproteins were prepared from rats' plasma-pool by differential ultracentrifugation according to Havel et al (33). Isolated fractions were extensively dialyzed for at least 24 hours at $4^{o}C$ against buffer containing 0.15 M NaCl and 0.3 mm EDTA, at pH 7.4. Following dialysis, lipoprotein fractions were concentrated to small volume (approximately 3 ml). Protein content of lipoprotein fractions were determined by the Lowry method (34).

Analysis of lipoproteins

For the chemical composition of isolated plasma lipoprotein -- lipids, total cholesterol was measured by an enzymatic method (35), triglycerides by a colorimetric procedure (36), and phospholipids by phosphorus analysis (37).

Iodination

^{125}I-labelled LDL and HDL were prepared by the iodine monochloride method of McFarland (38). Free iodine was removed by extensive dialysis against buffer containing 0.1 M KI, 0.15 NaCl, and 0.01% EDTA, pH 7.0 over 35 hours at 4^{o}C. The labelling efficiency was 21-35%. The specific activity in the lipoproteins' preparation was 117-262 count per minute (cpm) per ng of protein. A Packard Gamma Counter (model 5230) was used to count radioactivity.

Animals and cells

Adult male Spargue Dawly rats about 200-300 g were purchased from Bantin and Kingman, Inc. (Fremont, Ca. 94538). Rats were fed ad libitum with a cereal based diet prepared by OSU standard for experimental animals.

Liver cell suspensions were prepared by a modification of the method described by Berry and Friend (39). Rats were fasted for 24 hours prior to isolation of liver

parenchymal cells. Each rat used for liver cell isolation was anesthetized with ether. The abdomen was opened and the liver was perfused by cannulating the portal vein and the inferior vena cava just above the level of the renal vein was ligated. The initial perfusion medium was Hank's bicarbonate buffer (27.8 mM Na CO_3), pH 7.4 and contained no calcium or collagenase. This buffer was gassed with 95% O_2/ 5% CO_2 at 37°C for 1 hour. The liver was perfused at a rate of 35 ml/min for 3 minutes by the use of a Varistaltic pump (Manostat, VWR Scientific, Denver, Col.). The liver was then removed and transferred to a beaker containing 100 ml Hank's solution. Subsequently, 50 mg of collagenase (type I) were added to the perfusion volume of 100 ml (0.05% collagenase) in the reservoir. Five minutes after adding collagenase, .3 ml of calcium chloride ($CaCl_2$ 1 mM) was added to 100 ml of perfusion liquid. Four minutes after adding $CaCl_2$ (total perfusion time with medium containing collagenase was 9 minutes), the liver became markedly soft. The liver was then transferred to a petri dish containing minimal essential medium (MEM) with 1.5% bovine serum albumin (fraction V, fatty acid free). The liver cells were dispersed by gentle swirling, followed by filtration through cheese cloth into a 50 ml centrifuge tube. This tube was centrifuged at 400 rpm on an International

Centrifuge for 2 minutes. The supernatant, which contained the non parenchymal cells, was removed by aspiration and the parenchymal cell pellet was suspended in the same medium. After recentrifugation, the supernatant was aspirated and the cells were resuspended in a volume of 30-40 ml which gave approximately 5 x 10^6 cells per 1 ml of cell suspension. More than 90% of the isolated parenchymal cells excluded trypan blue.

Incubation study of ^{125}I-labeled LDL and HDL with isolated rat liver parenchymal cells

For the study of ^{125}I-labeled LDL and ^{125}I-labeled HDL binding, and degradation by rat parenchymal cells, 15 (25 ml Erlenmeyer flasks) for each lipoprotein fraction received 2 ml of medium (MEM) which consisted of 10 x 10^6 cells and 1.5% fatty acid free bovine albumin, pH 7.4, 10 ug/ml of ^{125}I-LDL or ^{125}I-HDL, and unlabeled lipoprotein at concentrations of 10, 50, 100, 300, and 500 ug/ml. All cells were incubated for 2 hours in a water-bath shaker and oxygenated (95% O_2 / 5% CO_2) at 37°C.

Determination of ^{125}I-LDL and ^{125}I-HDL binding in the cells

After 2 hours of incubation, flasks were placed on ice and their contents were poured into chilled test tubes. Medium and cells were separated immediately by centrifugation (2000 rpm, 5 minutes) on an International

Centrifuge. Cells were washed three times with 2 ml of ice cold Buffer A containing 2.0 g bovine albumin, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter. Then the cells were washed once with Buffer A containing no albumin and received 2 ml of 6 N NaOH. Cell aliquots were transferred to Lowry tubes for protein determination. Aliquots of cells solutions were counted for the measurement of radioactivity by a gamma counter. Counted radioactivity (cpm) was converted to ng of ^{125}I-LDL or ^{125}I-HDL protein and the results were expressed per mg of cell protein.

Determination of ^{125}I-LDL and ^{125}I-HDL degradation by liver parenchymal cells (determination of acid soluble material)

Bound ^{125}I-Labeled lipoprotein protein is known to be hydrolyzed by the lysosomal enzymes and degraded to a product which is soluble in trichloracetic acid (TCA), whereas free ^{125}I-lipoprotein precipitates with the acid (Goldstein and Brown, (40)). After incubation, the medium was separated from cells and mixed with 0.5 ml 50% (W/V) TCA and then incubated for 30 minutes on ice to precipitate unhydrolyzed proteins. To remove the precipitate, the entire solution was centrifuged for 10 minutes at 3000 rpm. One ml of supernatant was mixed with 10 ul of 40% KI and 40 ul of 30% H_2O_2 and incubated at room temperature for 5 minutes. The mixture was mixed with

2 ml $CHCl_3$ using a Vortex and centrifuged for 5 minutes at 2500 rpm to remove free iodine which had been present in the medium before the lipoprotein binding. An aliquot from the aqueous phase was used on a gamma counter to determine the radioactivity of iodine associated with tyrosine.

Determination of equilibrium constants

The affinity constants for specific binding of LDL and HDL to cell surface receptors of rat hepatocyte cells were determined from data obtained in direct binding studies with the iodinated lipoproteins. Iodinated LDL at concentrations of 2-10 ug/ml and iodinated HDL at concentrations of 1-5 ug/ml were incubated with 2 ml of hepatocyte cell suspension at $4^{o}C$. Receptor-bound lipoprotein, the saturable or specific component of binding, was determined at each concentration by subtracting the quantity of nonspecifically bound lipoprotein from the total cell-bound lipoprotein. Nonspecific binding was measured by the addition of rat LDL or HDL (500 ug/ml). Under these conditions the quanity of lipoprotein bound to the cell represents the nonspecifically bound lipoprotein. The equilibrium dissociation constant (Kd) was determined from the Scatchard plot (41). The slope of the graph plotting the ratio of receptor-bound lipoprotein to free lipoprotein versus receptor-bound lipoprotein is

equal to -1/Kd. The x intercept is the maximum amount of lipoprotein bound per mg cell protein. To convert from nanograms of lipoprotein protein to moles, the molecular weights of 2.5×10^6, 20% protein for LDL, and 6×10^5, 50% protein for HDL were used in the calculation (42).

Statistical analysis

Analysis of variance was conducted as previously described (43), to determine differences between binding and degradation, and student's "t" test was performed.

RESULTS

Chemical composition of rat plasma lipoproteins

The chemical composition of rat plasma lipoproteins used for the present study is presented in Table II.1. There were marked differences in total cholesterol content of LDL and HDL in that, unlike in humans, HDL carries the bulk of cholesterol along with greater amounts of phospholipids and protein than LDL. About 72% of total plasma cholesterol is carried by HDL, whereas LDL carries about 21% of it. These observations confirmed previous findings that rats are a HDL species (44).

Binding and degradation of ^{125}I-labelled LDL and HDL by rat liver parenchymal cells

The competitive binding and proteolytic degradation of ^{125}I-labelled LDL and HDL by isolated hepatocytes are shown in Fig. II.1. A and B. Rat unlabelled LDL and HDL effectively displaced ^{125}I-labelled LDL and ^{125}I-HDL from the cell surface receptors of normal rat hepatocytes. The logarithmic decreases of binding activities of ^{125}I-LDL or ^{125}I-HDL as a function of the amounts of unlabelled LDL or HDL mean competitive binding between the unlabelled LDL or HDL and the labelled LDL or HDL respectively. The presence of a 50-fold excess of unlabelled HDL resulted in a reduction of ^{125}I-labelled HDL binding to 82% of the total amount bound and the unlabelled LDL in 50-fold excess reduced the binding of ^{125}I-labelled LDL to 66%. These

TABLE II.1

Distribution of plasma lipids and lipoproteins in rats.[a]

Plasma Lipoproteins	Total Cholesterol mg/dl	TG[b] mg/dl	PL[c] mg/dl	Protein mg/dl
Plasma	57.0 ± 4.6	73.9 ± 4.6	63.7 ± 6.7	
VLDL[d] (d=1.006 g/ml)	3.5 ± 0.7	40.7 ± 0.2	7.7 ± 1.3	8.7 ± 0.88
LDL[d] (d=1.006-1.063 g/ml)	10.2 ± 2.3	20.1 ± 1.9	9.8 ± 1.4	5.8 ± 1.65
HDL[d] (d=1.063-1.21 g/ml)	35.1 ± 7.9	7.7 ± 0.6	36.7 ± 3.0	38.6 ± 5.27

[a] Values are mean ± SD (n=3).

[b] TG = Triglycerides.

[c] PL = Phospholipids.

[d] VLDL = Very low density lipoprotein; LDL = Low density lipoprotein; and HDL = High density lipoprotein.

observations suggest that rat parenchymal cells bind more ^{125}I-HDL than ^{125}I-LDL. However, there were notable differences in the proportion of LDL and HDL bound and degraded by rat hepatocytes with relatively more HDL being degraded compared with LDL (Fig. II.1.A and B). When the specificity of LDL and HDL degradation in rat hepatocytes was assessed, the 50-fold excess amount of unlabelled HDL (500 ug/ml) inhibited ^{125}I-HDL degradation by 88% whereas much less inhibition of ^{125}I-LDL degradation by unlabelled LDL occurred. Unlabelled LDL in 50-fold excess (500 ug/ml) reduced the degradation of ^{125}I-LDL to 63% (Fig. II.1.A). These data show that excess unlabelled HDL almost completely inhibited ^{125}I-labelled HDL degradation (Fig. II.1.B) whereas excess unlabelled LDL inhibited degradation of ^{125}I-labelled LDL by only 63% (Fig. II.1.A). However, statistical analysis did not show a significant difference between binding and degradation for both LDL and HDL in rat liver parenchymal cells.

Equlibrium Studies

Since the competitive binding experiments demonstrated the presence of a specific binding site for both LDL and HDL to normal rat hepatocytes, the binding capacity of rat parenchymal cells was studied by incubating the cells with increasing concentrations of ^{125}I-LDL or ^{125}I-HDL for 2 hours at 4^{o}C. In this experiment the

Figure II.1.A and B. Binding and degradation of rat ^{125}I-LDL (117 cpm/ng protein) and ^{125}I-HDL (173 cpm/ng protein) to normal rat hepatocytes plotted against unlabelled rat LDL and HDL. ^{125}I-LDL or ^{125}I-HDL (10 ug/ml) was added to the flasks containing 2 ml of cell suspension and concentration unlabelled of LDL or HDL indicated. Incubation for 2 hr. at 37^{o}C. Each point represents the average of triplicate determinations. The mean cellular protein was 1.5 $\pm$ .093 mg/flask (1.0 x 10^{7} cells per flask).

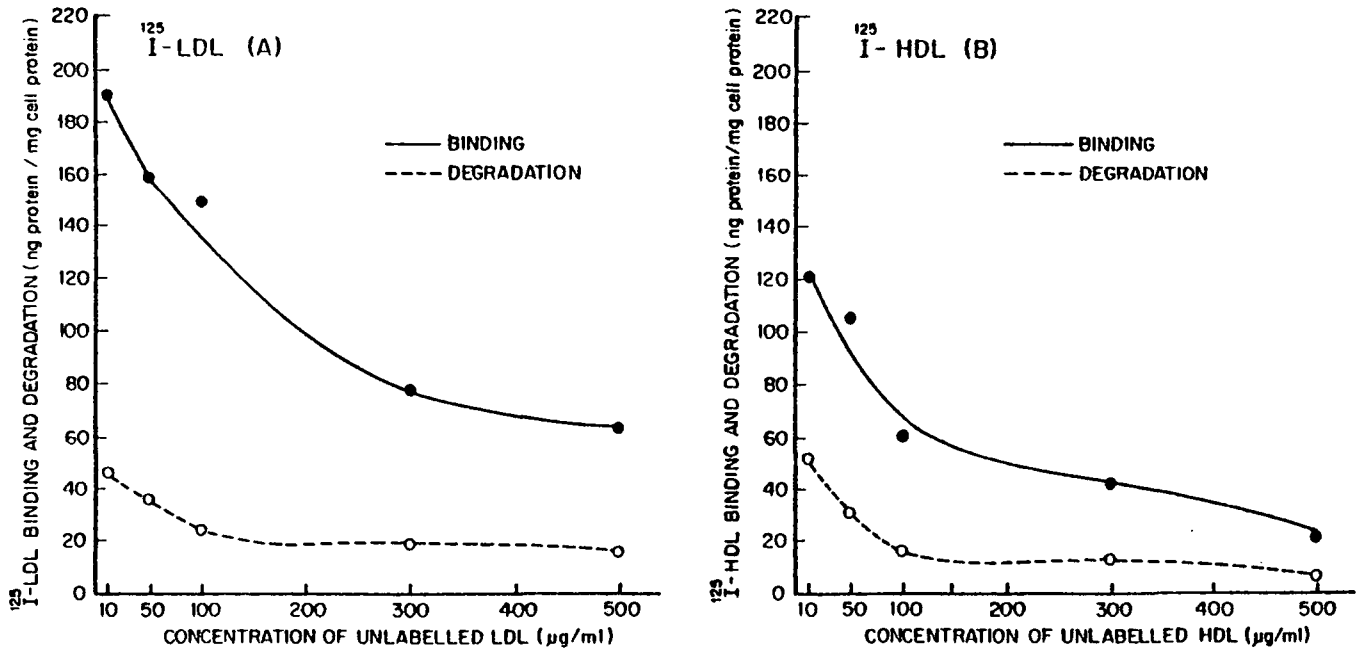
125 I-LDL (A)
BINDING
DEGRADATION
125 I-LDL BINDING AND DEGRADATION (ng protein / mg cell protein)
CONCENTRATION OF UNLABELLED LDL (µg/ml)
125 I- HDL (B)
BINDING
DEGRADATION
125 I-HDL BINDING AND DEGRADATION (ng protein/mg cell protein)
CONCENTRATION OF UNLABELLED HDL (µg/ml)

Figure II.1

binding capacity of LDL or HDL to a specific receptor was assessed as the difference between ^{125}I-labelled LDL or HDL bound in the absence and in the presence of a 50-fold excess of unlabelled lipoprotein during a 2 hour incubation at 4°C. The binding activity of rat ^{125}I-LDL and ^{125}I-HDL as a function of lipoprotein concentration in the medium is shown in Fig. II.2.A and B. The amount of ^{125}I-labelled LDL and HDL specifically bound to rat hepatocytes was increased in a linear fashion with increasing amounts of ^{125}I-labelled LDL and HDL in the incubation mixture. It rose to a 351 ng LDL protein bound/mg cell protein and 90 ng HDL protein bound/mg cell protein (Fig. II.2.A and B). These values were in good agreement with the maximum binding capacity (B_{max}) of 375 ng LDL protein/mg cell protein and 90 ng HDL protein/mg cell protein, obtained from Scatchard plot analysis (Fig. II.3). About 40% of total bound LDL appeared to be nonspecifically bound and, by contrast, 25% of rat HDL binding was non-specific at the saturating concentration (Fig. II.2.A and B).

As shown in Fig.II.3 both LDL and HDL gave linear Scatchard plots. Linearity of the Scatchard plots indicates that only one class of receptor exists (homogeneity of the receptor sites) and that there is no cooperativity among receptor sites (20). Furthermore, the slope of the HDL plot was greater than that of the LDL plot, indicating that HDL have a higher affinity for the receptor (Fig.II.3).

Figure II.2.A and B. Concentration-dependent binding of rat ^{125}I-LDL (141 cpm/ng protein) and ^{125}I-HDL (262 cpm/ng protein) to normal rat hepatocytes. Two ml of cell suspension were incubated with the indicated concentration of ^{125}I-labelled lipoproteins for 2 hours at 4^{o}C in the absence (O) and presence (▲) of 500 ug/ml of unlabelled lipoproteins. The amount of ^{125}I-labelled lipoprotein bound specifically to the cell surface receptors (●) was obtained by subtracting the ^{125}I-labelled lipoprotein bound in the presence of excess unlabelled lipoprotein from that bound in the absence of unlabelled lipoprotein. Each point represents the average of duplicate determinations. The mean cellular protein was 1.5 ± .19 mg/flask (1.0 x 10^{7} cells per flask).

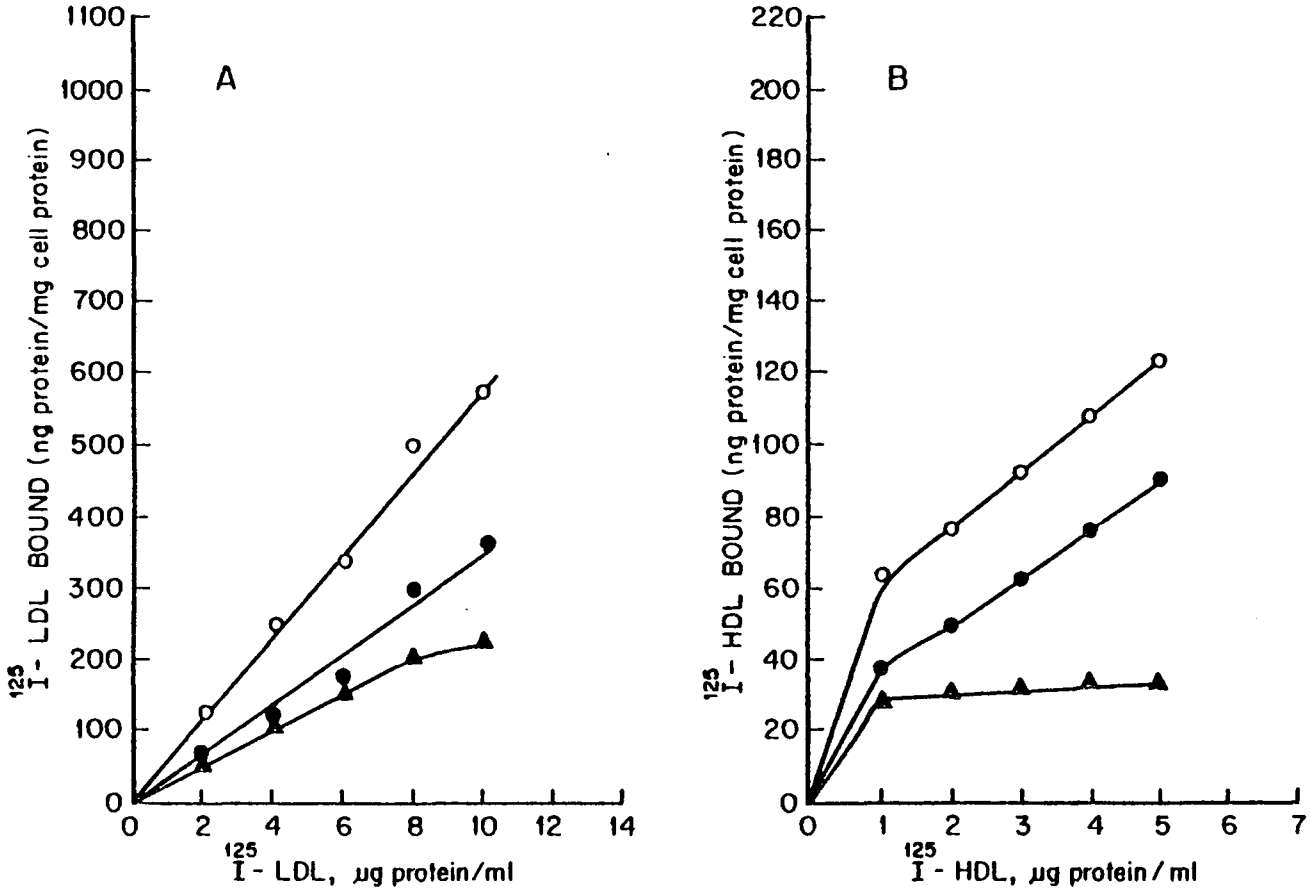
A
B
^{125}I- LDL BOUND (ng protein/mg cell protein)
^{125}I- HDL BOUND (ng protein/mg cell protein)
^{125}I- LDL, µg protein/ml
^{125}I- HDL, µg protein/ml

Figure II.2

Figure II.3. Scatchard plots for specific bindings of LDL (O) and HDL (●) to normal rat hepatocytes. "Bound/free" represents the total amount of ^{125}I-lipoprotein bound (ng of protein/mg cell protein) divided by the amount of unbound (free) in the media (ng of protein/mg cell protein).

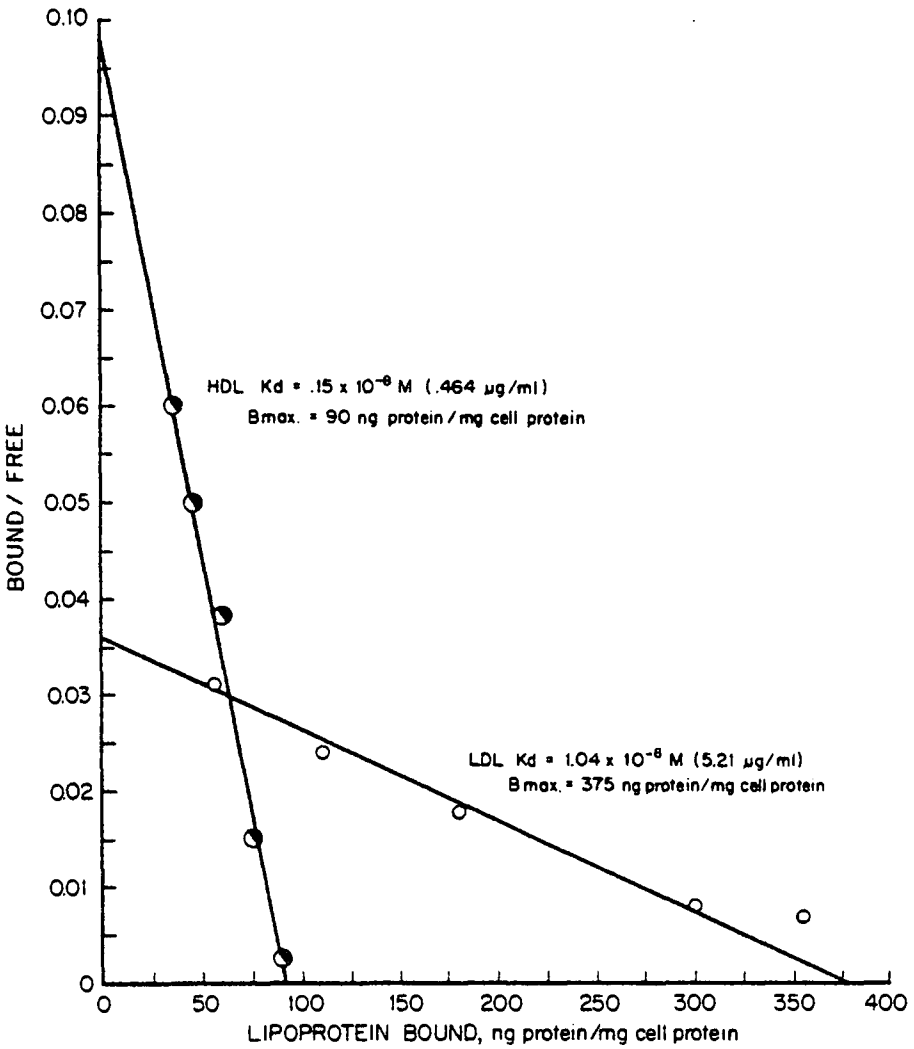
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
BOUND / FREE
HDL Kd = .15 x 10⁻⁸ M (.464 µg/ml)
Bmax. = 90 ng protein / mg cell protein
LDL Kd = 1.04 x 10⁻⁸ M (5.21 µg/ml)
Bmax. = 375 ng protein/mg cell protein
0
50
100
150
200
250
300
350
400
LIPOPROTEIN BOUND, ng protein/mg cell protein

Figure II.3

The K_d for HDL and LDL derived from the slopes of the Scatchard plots were .15 x 10^{-8}M and 1.04 x 10^{-8}M, for rat HDL (mol. wt., 6 x 10^5, 50% protein), and rat LDL (mol. wt., 2.5 x 10^6, 20% protein) respectively. This means that HDL have approximately 7-fold higher binding affinity for the receptors than LDL. The Scatchard plots also confirmed that saturation of the receptors occurred at much lower levels of HDL than LDL. The values, as determined from the X-intercept in Fig. II.3 were 90 and 375 ng of lipoprotein protein per mg of protein cell content for HDL and LDL respectively.

DISCUSSION

The present study was conducted to compare the binding and degradation of two different types of rat plasma lipoproteins (LDL and HDL) in rat liver parenchymal cells. The binding of ^{125}I-labelled LDL and HDL to isolated rat liver parenchymal cells at 37^{o}C was decreased with increasing concentrations of respective unlabelled lipoproteins, suggesting the presence of a specific binding sites for both LDL and HDL to normal rat hepatocytes. It was found that a fraction of ^{125}I-labelled LDL or HDL binds to liver parenchymal cell membrane even in the presence of high concentrations of native LDL or HDL. This non-specific binding at saturation concentration ranged from 37-40% for LDL and 12-20% for HDL (Fig. II.1 and 2). These observations suggest that isolated rat liver parenchymal cells have a saturable (specific) component and a nonsaturable (nonspecific) component for binding LDL and HDL. Several studies have reported that there are specific and nonspecific binding sites for rat LDL and HDL in isolated hepatocytes from various species (20, 21, 23, 24, 28).

Evidence for high-affinity binding for HDL or LDL has been presented by others in freshly suspended rat liver parenchymal and nonparenchymal cells (16, 20, 21,

25, 32) and in cultured rabbit and pig hepatocytes (22, 24). Binding sites with properties of the peripheral LDL receptor have been detected in liver microsomes from several species (45-48), suggesting the presence of a site(s) similar to the lipoprotein binding site as well. Our data show that HDL did compete with ^{125}I-labelled HDL for binding sites but was even more effective at reducing the degradation of ^{125}I-labelled HDL (Fig. II.1,2). On the other hand, unlabelled LDL was less effective in reducing ^{125}I-labelled LDL binding and degradation (Fig. II.1,2). Our observations show that rat parenchymal cells bind and degrade both LDL and HDL with a higher capacity for HDL as compared to LDL.

When present in excess, unlabelled HDL almost completely inhibited ^{125}I-labelled HDL degradation during 2 hours incubation at 37^{o}C, whereas binding was only partly inhibited and a small amount was found in the cells (Fig. II.1.B). The interpretation of this phenomenon is that, in the presence of excess unlabelled HDL, a large amount of ^{125}I-labelled HDL nonspecifically binds to hepatocytes at 37^{o}C and some is subsequently internalized. The small number of specific binding sites, however, are nearly all occupied by unlabelled HDL, and during the short incubation we observed only HDL that has been bound to cell binding sites is degraded, hence

the almost complete inhibition of observable degradation. The present data suggest that the large amount of HDL degradation by rat parenchymal cells reflects a specific physiological role for the liver in HDL catabolism.

The liver plays a vital role in cholesterol metabolism. Since large amounts of cholesterol are required for the formation of very low density and high density lipoproteins for fat transport (49), it is important to establish its source. There are two possible sources of cholesterol in the liver, these include cholesterol newly synthesized in the liver, and free and esterified cholesterol derived from plasma lipoproteins (after intrahepatic hydrolysis of plasma lipoprotein origin) (50, 51). Schwartz et al. (52) found that in patients with a bile fistula, hepatic cholesterol was largely derived from plasma lipoprotein free cholesterol, with little contribution from plasma lipoprotein esterified cholesterol or hepatic newly synthesized cholesterol. The present study supports the possibility that a significant amount of hepatic cholesterol may be derived from HDL which were shown to bind with high affinity and capacity to hepatocytes. Since, in rat, most circulating cholesterol is associated with HDL, the liver may be a major site of HDL removal and catabolism.

At $4^{o}C$, HDL bound to hepatocytes with a higher (7-fold) affinity (K_d = .15 x 10^{-8}M) than LDL

(K_d = 1.04 x 10^{-8}M). The K_d value of HDL agrees with those Ose et al. (31), and Soltys et al. (23) who reported .11 x 10^{-8}M and .14 x 10^{-8}M as K_d values for HDL in hepatocytes of rat, rabbit, respectively. Our K_d of 1.04 x 10^{-8}M for LDL agrees with that 1.48 x 10^{-8}M of Soltys et al. (23), but not with Ose et al. (31) who reported a K_d value of 6.22 x 10^{-9}M for LDL in rat hepatocytes. In the present study, analysis of the binding equilibrium data by the method described by Scatchard (41) also revealed the linearity of the plots for both LDL and HDL indicating the existence of only one class of binding sites. Saturation of the receptors occurred at much lower levels of HDL (B_{max} = 90 ng HDL protein/mg cell protein) than LDL (B_{max} = 375 ng LDL protein/mg cell protein). Therefore, it can be concluded that rat HDL preferably binds to rat liver parenchymal cells and is also degraded at a higher rate than LDL. The present findings are compatible with the previous hypothesis (8) that HDL may be the reverse transport vehicle for cholesterol to be removed from peripheral tissues to the liver. HDL-free cholesterol is known to be the preferred precursor for bile acid synthesis and biliary cholesterol excretion (53). This may be the mechanism by which HDL cholesterol renders protection for coronary heart disease. It remains to be

seen what is the fate of the bound HDL cholesterol on the cell surface? The hepatocytes may catabolize the HDL cholesterol to bile acid synthesis or transfer it to other classes of lipoproteins which are secreted.

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CHAPTER III

EVIDENCE OF PREFERABLE UTILIZATION OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL FOR BILE ACID SYNTHESIS BY RAT LIVER PARENCHYMAL CELLS

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Utilization of Cholesterol by Rat Liver Cells

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ABSTRACT

Specific use of low and high density lipoproteins cholesterol for bile acid synthesis by normal rat liver parenchymal cells was investigated. Plasma low and high density lipoproteins (LDL; $1.006 < d < 1.063$ g/ml and HDL; $1.063 < d < 1.21$ g/ml) from rats were labelled with ^{3}H-free cholesterol and incubated individually with rat liver parenchymal cells for 4 hours at 37°C. After incubation, media were separated from cells and tritium radioactivity was counted. Bile acids were extracted from media and cells, and radioactivity in the residue was determined. Identification of bile acids was made by thin layer and gas liquid chromatographic analysis by comparison to the purified standards of bile acids. It was found that 3.4% of ^{3}H-free cholesterol labelled HDL was converted to bile acids mainly as lithocholic, chenodeoxycholic, deoxycholic and cholic acids, whereas, 1.1% of ^{3}H-free cholesterol labelled LDL was converted to bile acids as lithocholic, chenodeoxy and deoxycholic acids. These data indicate a more preferred utilization of HDL-cholesterol to LDL-cholesterol for bile acid synthesis by rat liver parenchymal cells.

INTRODUCTION

The liver plays an important role in lipoprotein catabolism (1) and is the only organ where cholesterol is degraded to bile acids (2, 3). Several studies have been conducted to elucidate the source of precursor cholesterol for bile acid synthesis in animals' models (4-6). Cholesterol present in remnant particles may be one source for the bile acid biosynthesis. After hydrolysis of the triacylglycerol in VLDL and chylomicrons by lipoprotein lipase, cholesterol ester-rich "remnant particles" are formed (7) and they are rapidly removed by the liver (8, 9). Cholesterol ester present in HDL, might be taken up and degraded in the liver as postulated by Glomset (10). Furthermore, the work of several groups has shown that HDL picks up free cholesterol from cell membranes and delivers it to the liver (11-13). Other studies have demonstrated that free cholesterol of HDL is used preferentially over LDL-free cholesterol by the liver as substrate for bile acid synthesis and biliary cholesterol secretion (14-17).

However, not all experimental evidence is consonant with the preferential utilization of HDL-cholesterol for bile acid synthesis. Sniderman et al (18) showed that human LDL esterfied cholesterol rather than HDL is removed

from splanchnic circulation. In swine, Pittman et al (19) reported that 40% of LDL degradation is attributed to the liver. Recently, in the perfused liver, Quarfordt et al (20) reported that, in the rat, esterified cholesterol of HDL was transported to the liver for catabolism as bile acids and biliary cholesterol.

In the present study, we compared the rate of conversion of ^{3}H-labelled HDL-cholesterol with that of ^{3}H-labelled LDL-cholesterol into bile acids in normal rat liver parenchymal cells. We found that HDL cholesterol was preferred precursor for bile acid synthesis to LDL-cholesterol in rat liver parenchymal cells.

METHODS

Materials

1, 2α(n)-^{3}H-cholesterol, specific activity 44 curies/mmole, was purchased from Amersham Radiochemical Center (Amersham, UK). Cholesterol Standard, bovine serum albumin (fraction V, fatty acids free), Collagenase (Type I), EDTA, 5,5-dithiobis-(2-nitrobenzoic acid) and mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, Mo. USA). Analytical grade bile acids standard, lipopure methanol and instant methanolic HCL kit (acetyl chloride) wer acquired from Alltech Associates, Inc. (Applied Science Lab.,
Minimum Essential Medium (MEM) was purchased from GIBCO Laboratories, Grand Island, N.Y., USA. Precoated Thin Layer Chromatography Plates (Silica gel G soft layer 0.500 mm, 20 cm x 20 cm) was bought from Supelco Inc., Supelco Park, Bellefonte, Pa.

Animals

Adult male Spargue Dawly rats about 200-300 g were purchased from Bantin and Kingman, Inc. (Fremont, Ca., USA). Cereal based diet prepared at OSU for experimental animals and water were fed ad libitum.

Lipoprotein isolation

All rats were fasted overnight for at least 16 hours prior to taking blood from the abdominal aorta after anesthesia with diethyl ether. Plasma was prepared from 120 ml of blood collected in centrifuge tubes containing disodium ethylene diamine tetra-acetic acid (EDTA), 1 mg/ml of blood. Lipoproteins were isolated from rats' plasma-pool by differential ultracentrifugation as described by Havel et al (21). Isolated lipoprotein fractions were dialyzed for 24 hours at $4^{o}C$ against buffer containing 0.15 M NaCl and 0.3 mM EDTA, at pH 7.4. Following dialysis, lipoprotein fractions were concentrated to small volumes (approximately 3 ml) with Diaflo ultrafiltration membranes (Amicon Co., Lexington, Mass. 02173). Protein and total cholesterol content of lipoprotein fractions were determined by the Lowry method (22) and enzymatic cholesterol assay (23).

Isolation of hepatocytes

Hepatocytes were prepared by the procedures of Berry and Friend (24) with some modifications as described in detailes previously (unpublished paper). Preparations were considered suitable when the viability of isolated hepatocytes was greater than 90% by trypan blue exclusion test, and structural integrity of the cells was verified microscopically for expected morphology.

Preparation of cholesterol labelled lipoproteins

1,2α(n)-^{3}H cholesterol was purified on precoated thin layer chromatograph (TLC) plates (Silica gel G, 0.500 mm). The plates were cleaned by running in distilled chloroform and activated by heating at 100^{o}C for one hour before use. Fifty ul of 1,2α-^{3}H cholesterol containing 50 uCi, was spotted into TLC plate along with 100 ul of standard free cholesterol (100mg%) on another column. After the plate stood for about 1 hour in a developing tank containing the solvent system (petroleum ether (bp 60-70^{o}C); diethyl ether; acetic acid; 80:20:2, V/V) and the standard cholesterol was localized by iodine vapor, the area corresponding to cholesterol standard was scraped off from the plate and eluted with isopropanol. Radioactivity was determined using a liquid scintillation solution (T-cocktail) consisting of 4 g of 2,5 diphenyloxazole (ppo) and 50 mg of 4-di-2(5-phenyloxazolyl)-benzene (PoPo) in 1 liter toluene. The recovery of the ^{3}H-cholesterol activity from the purification was 37.2% and the total activity was 22.3 uCi in 1.7 ml isopropanol. The volume was divided into two conical tubes (each containing 0.85 ml (24571630 cpm)). Isopropanol was dried under a stream of nitrogen and redissolved in 100 ul acetone. To label lipoprotein cholesterol pool, the acetone solution containing ^{3}H-cholesterol was slowly

added, using 100 ul syringe into LDL or HDL solutions which was constantly stirred by a magnetic stirring bar and spine. To the incubation tube, 100 ul of 5,5-dithiobis-nitrobenzoic acid (DTNB), 14 mM in 0.2 M phosphate buffer pH 7.1 was added to inactivate lecithin: cholesterol acyltransferase during 2 hours in incubation at 35-37^{o}C in shaking water bath. After incubation, the labelled lipoprotein fractions with ^{3}H-cholesterol were extensively dialyzed for at least 24 hours at 4^{o}C against buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. The dialysis was aimed to remove free ^{3}H-cholesterol which might not be incorporated in the lipoprotein cholesterol pool. Following dialysis, the radioactivity of labelled lipoprotein fractions was determined using a liquid scintillation solution (aquasol, New England Nuclear, Boston, Mass. 02118). The labelling efficiency was about 85%.

Incubation of rat liver parenchymal cells with ^{3}H-cholesterol labelled LDL and HDL

Four 25 ml Erlenmeyer flasks were used for each incubation experiment and each flask contained:

1. 8 ml cell suspension + 1.0 ml of ^{3}H-cholesterol labelled LDL solution + 10ul mercaptoethanol.

2. 8 ml cell suspension + 1.0 ml of ^{3}H-cholesterol labelled HDL solution + 10 ul mercaptoethanol.

3. 8 ml cell suspension which was previously incubated at 55°C for 2 hours + 1.0 ml ^{3}H-cholesterol labelled LDL + 10 ul mercaptoethanol as a blank control for LDL.

4. 8 ml cell suspension which was previously incubated at 55°C for 2 hours + 1.0 ml ^{3}H-cholesterol labelled HDL + 10 ul mercaptoethanol as a blank control for HDL.

All flasks were incubated for 4 hours in a water bath shaker at 37°C and oxygenated with 95% O_2 and 5% CO_2 during the incubation. After incubation, flasks were placed on ice and their contents were poured into chilled test tubes. Medium and cells were separated immediately by centrifugation at 2000 rpm for 5 minutes in a centrifuge (IEC, Needham Heights, MA 02194). Cells were washed three times with 2 ml of ice cold buffer solution containing 2.0 g bovine albumin, 8.0 g NaCl, 0.2 g KCL, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter. Then, 4 ml of 6 NaOH was added to the cells. Aliquots of media and cell solution were counted for the measurement of radioactivity in aquasol as described above.

Extraction of cholesterol and bile acids from media and cells

Cholesterol and bile acids in media and cells were extracted using the method described by Grundy et al (25), with slight modifications. Five ml of media or cell

solution, 100 ul of hyodeoxy cholic acid as an internal standard in methanol (1 mg/ml), and 7.0 ml of 1 N NaOH in 95% ethanol were added to 40 ml polyethylene centrifuge tubes (Oak Ridge type) with caps and saponified for 1 hour at 80°C in a water bath. Cholesterol was extracted three times with 20 ml of petroleum ether after centrifugation at 1000 rpm for 5 minutes. 100 ul of aliquots of the petroleum ether extract was taken into scintilation vials containing 10 ml of T-cocktail to be counted for radioactivity. After extraction of cholesterol 1 ml of 10 N NaOH was added to each tube, sealed and autoclaved for 3 hours at 116°C. After hydrolysis of bile acids, the pH of the solution in the tube was adjusted to 2.0 using concentrated HCl, then free bile acids were extracted three times with 10 ml of ethyl acetate.

Separation of bile acids of samples (media and cells) on thin layer chromatography

Bile acids extracts were dried under nitrogen gas and redissolved in 200 ul of ethyl acetate. A quarter of the total bile acid extract was applied on a column of thin layer chromatographic plate (0.500 mm) along with bile acid standard mixture on an adjacent column of the TLC plate. The plates were put in a developing tank containing a solvent system of benzene:isopropanol:acetic acid (30:10:1) (26) until the solvent front migrated to

about 2 cm from the top edge of the plate. After the chromatographic separation, bile acid standards were identified by spraying with 10% phosphomolybdic acid in ethanol and the unsprayed portion corresponding to standard bands were scraped off the plate. Tritium radioactivity of bile acids bands was determined directly after dissolving it in 10 ml of T-cocktail and counting in a Scintillation Spectrophtometer (Beckman LS-3133P Liquid Scintillation System).

Gas liquid chromatographic analysis of bile acids

150 ul of extracted bile acids in ethyl acetate was dried under N_2 gas and methylated with 3 ml of fresh methanolic-HCL (3%), then dried again and redissolved in 200 ul of ethyl acetate.

A glass U-tube, 3 ft x 2 mm column, was packed 3% of SP-2250 on 120-100 mesh (Supelco, Inc., Bellefonte, Pa.) and conditioned at 280°C for 24 hours. A Hewlett Packard, S 710 Gas Liquid Chromatograph was used for analysis of bile acids in samples.

Operating conditions were as follows: oven temperature, 270°C; injection port, 300°C; detector, 300°C and helium carrier gas 40 ml/min. Ethyl acetate was used to dissolve samples. Bile acids were identified by comparison with standard bile acid methyl esters by gas chromatographic retention time.

RESULTS

Uptake of ^{3}H-cholesterol labelled lipoprotein by rat liver parenchymal cells

Rat hepatocytes were incubated with ^{3}H-cholesterol-labelled lipoprotein fractions for 4 hours at 37°C. Our results (Table III.1) showed that the uptake of LDL cholesterol by isolated rat hepatocytes was greater than that of HDL. The percentage of tritiated cholesterol activity in cells taken up from LDL was higher (44% for LDL-cholesterol) than that of HDL (37% for HDL-cholesterol). On the other hand, there was a relative abundance of the labelled ^{3}H-cholesterol HDL in the media (63%) over labelled ^{3}H-cholesterol LDL (55%). These observations suggest that the total binding activity of LDL is greater than that of HDL in rat liver parenchymal cells.

Characterization of ^{3}H-cholesterol in incubation media

The dialysis data of the media recovered from the incubation mixture showed that nearly all of the ^{3}H-cholesterol activity was bound to undialyzable macromolecules, possibly LDL or HDL (Table III.2). After dialysis 10% of LDL-^{3}H-cholesterol was dialyzed out as free cholesterol or its catabolic products, bile acids, while 4.2% of HDL-^{3}H-cholesterol was slipped out of the dialysis bag. Prior to the use for the incubation experiment with

TABLE III.1

Uptake of ^{3}H-cholesterol by rat liver parenchymal cells from the labelled LDL or HDL with ^{3}H-cholesterol.

Lipoprotein Fractions	^{3}H-cholesterol activity in incubation mixture		Uptake of ^{3}H-cholesterol by cells (cpm)	% of ^{3}H-cholesterol in cells (cpm)	^{3}H-cholesterol in media (cpm)	% of ^{3}H-cholesterol in media
	Total Activity (cpm)	Specific Activity (cpm/mg lipoprotein cholesterol				
LDL ($1.006 < d < 1.063$)	4,610,333	6251299[a]	2,053,333	44.5%	2,557,000	55.5%
HDL ($1.063 < d < 1.21$)	4,290,000	1941176[b]	1,590,000	37.0%	2,703,000	63.0%

Plasma containing ^{3}H-cholesterol-labelled lipoprotein fractions were added to rat liver parenchymal cells and incubated for 4 hours at 37°C. After incubation, media were separated from cells. Cells were washed three times with cold buffer solution and dissolved in 4 ml 6N NaOH. The radioactivities were counted in both media and cell solutions. The amounts of (^{3}H) cholesterol taken up by cells and media were calculated by using the amount of radioactivity present in media or cells divided by the total activity of ^{3}H-cholesterol in media and cells times 100:

$$\left(\frac{\text{activity present in media or cell}}{\text{total activity present in media and cells}} \times 100 \right)$$

[a]concentration of LDL-cholesterol = .74 mg LDL cholesterol/ml LDL solution.

[b]concentration of HDL-cholesterol = 2.21 mg HDL cholesterol/ml HDL solution.

TABLE III.2

Characterization of ^{3}H-cholesterol activities in the incubation media with rat liver parenchymal cells.

Fraction	Before Dialysis cpm	After Dialysis cpm	Recovery %
LDL (1.006 < d < 1.063 g/ml)	2,557,000/5.0ml	2,301,000/5.0ml	90%
HDL (1.063 < d < 1.21 g/ml)	2,703,000/5.0ml	2,590,200/4.5ml	95.83%

Rat hepatocytes were incubated with ^{3}H-cholesterol labelled lipoprotein fractions for 4 hours at 37°C. After incubation, media were separated from cells and tritium radioactivity was counted. Then media were dialyzed overnight against buffer solution containing 0.9% NaCl and 0.01 EDTA at pH 7.4 and radioactivity of ^{3}H-cholesterol was counted.

isolated hepatocytes, both, ^{3}H-cholesterol labelled LDL and ^{3}H-cholesterol labelled HDL were dialyzed for 24 hours at 4^{o}C against the same kind of dialysis solution. This means that the tritiated cholesterol activities remained in the media for LDL (55.5%) and HDL (63.0%) were still associated with LDL and HDL.

Conversion of LDL-^{3}H-cholesterol and HDL-^{3}H-cholesterol to ^{3}H-bile acids

After 4 hours of incubation of the labelled lipoprotein cholesterol with freshly isolated rat liver parenchymal cells, the incubation media were separated from the cells and bile acids were extracted from the cells and the recovered media for determination of the tritiated radioactivity in the bile acid fractions. The ^{3}H-activities measured from bile acid extract and the percentages of conversion from ^{3}H-cholesterol to ^{3}H-bile acid extract are presented in Table III.3. The percentages of conversion from HDL-^{3}H-cholesterol activity to the activity of the bile acid extract was 3.75%, while that of LDL-^{3}H-cholesterol was 1.42%. After subtracting the activities of the blank control in which heat treated cells (at 55^{o}C for 2 hours) were used instead of the viable cells, the net conversion was 3.4% and 1.1% for HDL and LDL, respectively. These net conversion values were in close agreement with the values obtained from

TABLE III.3

Conversion of LDL-^{3}H-cholesterol and HDL-^{3}H-cholesterol to ^{3}H-bile acids as determined in the media after incubation with rat liver parenchymal cells.

Fraction	Volume of Media (ml)	Initial Activity of ^{3}H-cholesterol (cpm)	Activity in Bile Acid Extracts (cpm)	% of Conversion into Bile Acids[a]	
				% of gross conversion	% of net conversion
LDL ($1.006 < d < 1.063$) + viable cells	4.0	2045600	29200	1.42	1.12[b]
LDL + cells (killed)	4.0	1436800	4128	0.29	
HDL ($1.063 < d < 1.21$) + viable cells	4.0	2162400	81333	3.75	3.40[b]
HDL + Cells (killed)	4.0	819200	2848	0.35	

Rat plasma LDL-^{3}H-cholesterol and HDL-^{3}H-cholesterol were incubated with rat liver parenchymal cells for 4 hours at 37°C. Media were separated from cells and saponified for 1 hour with 7.0 ml 1N NaOH in 95% ethanol. Cholesterol was extracted from the residue three times with 20 ml petroleum ether. The bile acids in the residue was hydrolyzed for 3 hours at 116°C with 1 ml of 10 N NaOH and the pH was adjusted to 2.0 with concentrated HCl. Then free bile acids were extracted three times with ethyl acetate and assayed for radioactivity. For blank control, 8 ml of cell suspensions were pre-incubated for 2 hours at 55°C in water bath before they were replaced with the incubation media used for treatment groups.

[a]The amount of ^{3}H-free cholesterol labelled lipoprotein fractions that is converted to bile acids into the media was calculated from this formula:

$$\frac{\text{radioactivity in bile acid extraction (cpm)}}{\text{Initial activity of } ^{3}\text{H-cholesterol in media (cpm)}} \times 100$$

[b]Percentages of the net conversion of ^{3}H cholesterol labelled lipoprotein fractions to ^{3}H-bile acids, after subtracting the control values from the treatment data.

TABLE III.4

Distribution of ^{3}H-activities of individual bile acid bands on thin layer chromatogram (TLC).

Fractions	Distribution of ^{3}H-activities of bile acid bands								Total Activity of ^{3}H-bile acids (from ¼ of extract)	Initial Activity of ^{3}H-cholesterol (¼ of total activity)	% of[a] conversion of ^{3}H-cholesterol to ^{3}H-bile acids
	Cholic		Chenodeoxy-cholic		Deoxy-cholic		Litho-cholic				
	NCPM	%	NCPM	%	NCPM	%	NCPM	%	NCPM		
LDL (1.006<d<1.063 g/ml)	64	1.0	1480	23.0	1018	15.8	3884	60.2	6448	511400	1.26%
HDL (1.063<d<1.21 g/ml)	1262	7.6	5894	35.5	1214	7.3	8226	49.6	16596	540600	3.07%

After plasma LDL-^{3}H-cholesterol and HDL-^{3}H cholesterol were incubated with rat liver parenchymal cells for 4 hours at 37°C, the media were separated from cells and bile acids were extracted with ethyl acetate as described earlier. Bile acid residues were dried under N_2 stream and redissolved in 200 ul of ethyl acetate. One-quarter of the bile acid solution (50 ul) was applied on silica gel G thin layer chromatographic plate (0.5 mm). The mixture of standard bile acids (cholic, chenodeoxycholic, deoxycholic, and lithocholic acid) was also applied on the next column of the same TLC plate. After chromatographic separation, bile acids standards were identified by spraying the TLC plate with 10% phosphomolybdic acid in ethanol, then heated. The unsprayed bands on the plate corresponding to standard bile acid bands were scraped off. Tritium radioactivity was directly determined after dissolving it in 10 ml of T-Cocktail, then counting in a Scintillation Photo Spectrophotometer.

[a] % of ^{3}H-cholesterol labeled lipoproteins fractions converted to ^{3}H-bile acids represent:

$$\frac{\text{total activity of } ^{3}H\text{-bile acids in 50 ul}}{\text{initial activity of } ^{3}H \text{ cholesterol in 50 ul}} \times 100$$

counting data of TLC bands of individual bile acids as shown in Table III.4. The data demonstrated that HDL-^{3}H-cholesterol was three times more effectively used for bile acid biosynthesis than LDL-^{3}H-cholesterol. There were also quantitative differences in the bile acids synthesized from HDL and LDL cholesterol. HDL-^{3}H-cholesterol was converted into more primary bile acids (cholic and chenodeoxy cholic acids) while LDL-^{3}H-cholesterol was mostly transformed into secondary bile acids.

Fig. III.1 shows a gas chromatogram of a standard mixture of five bile acids methyl esters. Hyodeoxycholic acid was added at the beginning of extraction and chromatographic profiles of the major bile acid isolated from the media of the incubation mixture with ^{3}H-cholesterol HDL or ^{3}H-cholesterol LDL are shown in Figs. III.2, 3. Major bile acids produced by the hepatocytes using HDL-cholesterol were lithocholic, chenodeoxycholic, cholic acids, with smaller amounts of deoxycholic acid (Fig. III.2). As shown in Fig. III.3, the majority of the bile acids synthesized from the media containing LDL were lithocholic and chenodeoxycholic acids with a very small amount of deoxycholic acid. The concentrations of individual bile acids were calculated from the areas under peaks by comparing to the area of hyodeoxycholic acid of which the concentration

Figure III.1. Gas liquid chromatogram of methylated bile acid standards. Column temperature, 270^{o}C; 1) cholesterol; 2) lithocholic acid; 3) deoxycholic acid; 4) chenodeoxycholic acid; 5) hyodeoxycholic acid; and 6) cholic acid.

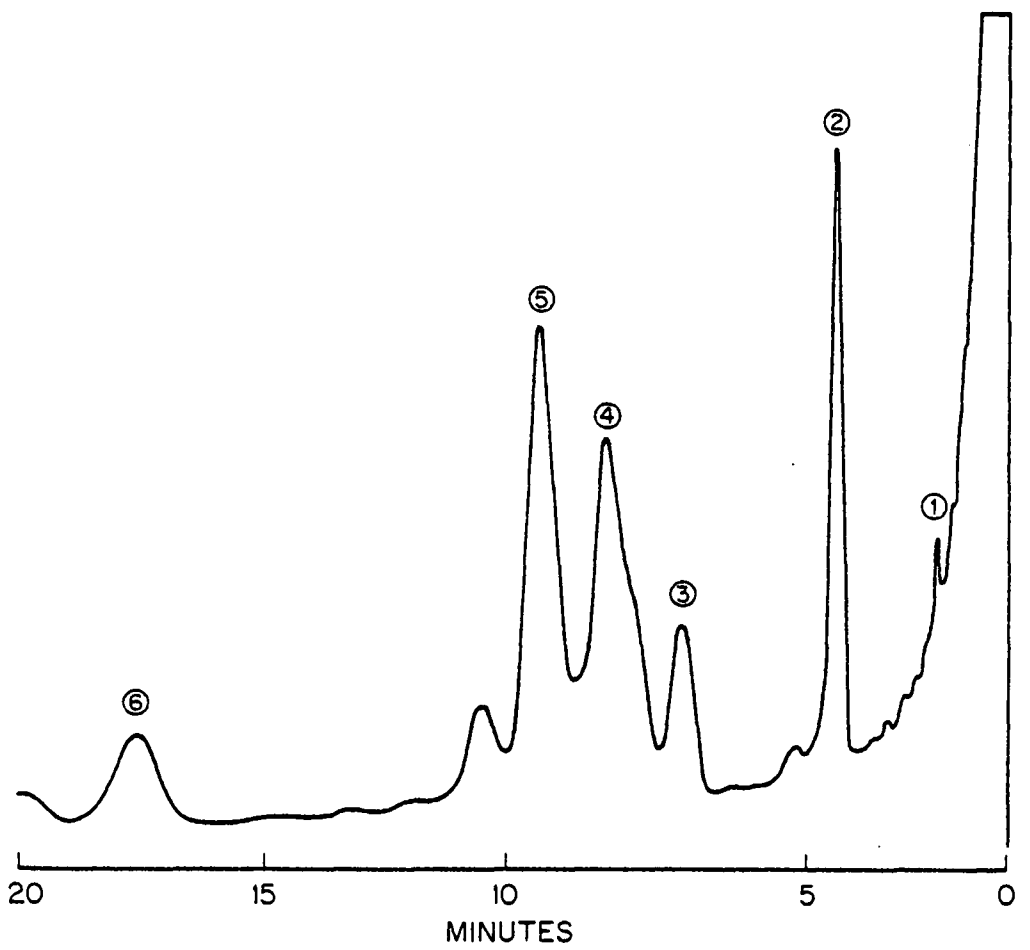

Figure III.1

Figure III.2. Gas liquid chromatogram of bile acids extracted from the incubation media after the isolated rat liver parenchymal cells were incubated for 4 hours at 37°C in culture media containing rat plasma HDL. Conditions are the same as in Fig. III.1: 1) cholesterol; 2) lithocholic acid; 3) deoxycholic acid; 4) chenodeoxycholic acid; 5) hyodeoxycholic acid; and 6) cholic acid.

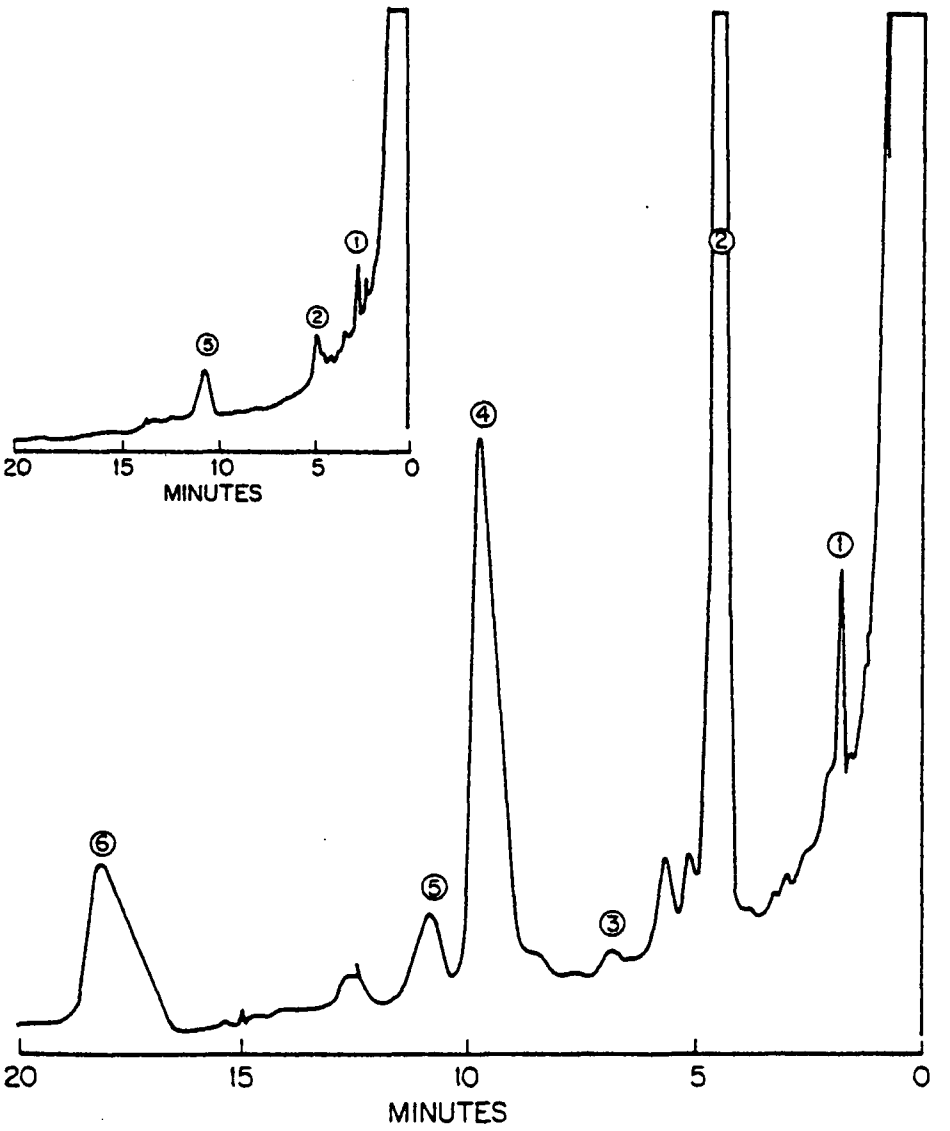

Figure III.2

Figure III.3. Gas liquid chromatogram of bile acids extracted from the incubation media after the isolated rat liver parenchymal cells were incubated for 4 hours at 37°C in culture media containing rat plasma LDL. Conditions are the same as in Fig. III.1: 1) cholesterol; 2) lithocholic acid; 3) deoxycholic acid; 4) chenodeoxycholic acid; and 5) hyodeoxycholic acid.

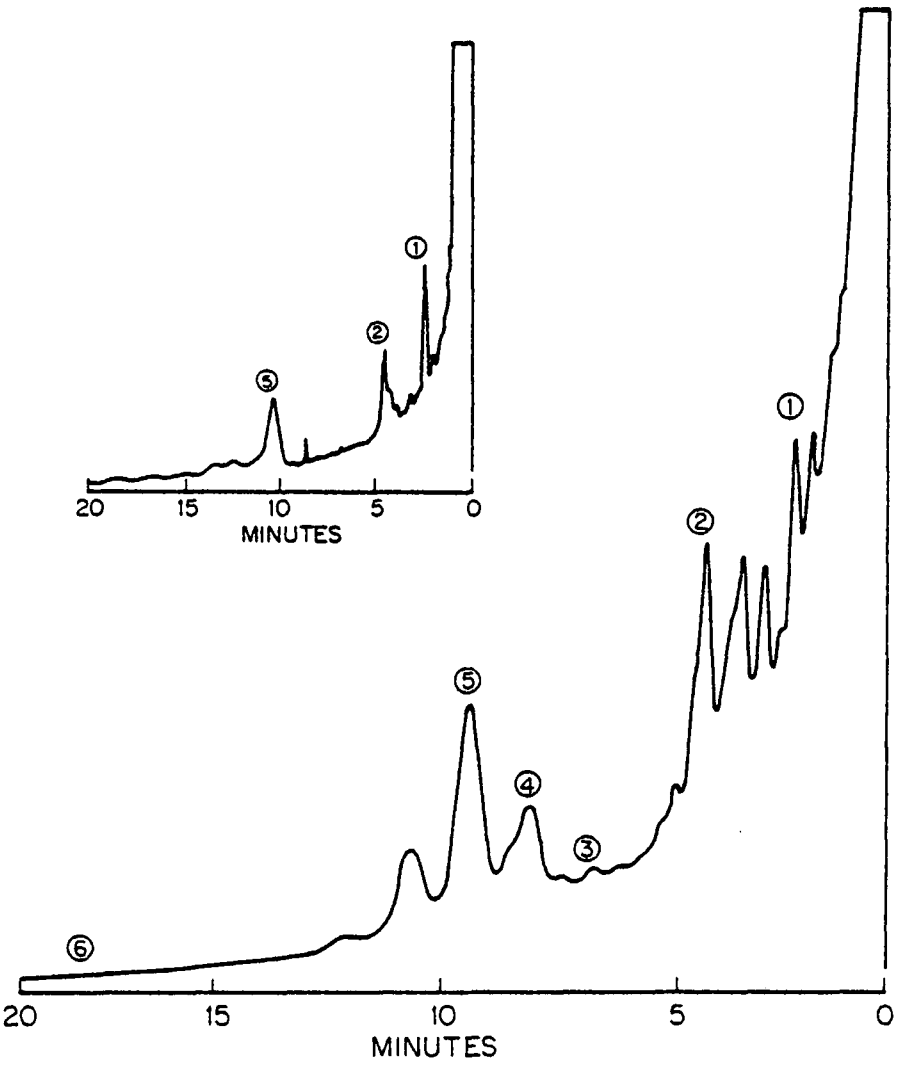

Figure III.3

is known (Table III.5). The relative amounts of individual bile acids calculated from the chromatograms are comparable with the percentage of ^{3}H-bile acid activities of individual bile acid bands on TLC (Table III.4). HDL-cholesterol produced more (53%) primary bile acids, cholic and chenodeoxycholic acids, while LDL-cholesterol synthesized mostly (66%) secondary bile acids, lithocholic and deoxycholic acids.

TABLE III.5

Distribution of bile acids in recovered media as determined quantitatively by gas liquid chromatography after ^{3}H-cholesterol labelled lipoprotein fractions were incubated with rat liver parenchymal cells.

Fractions	^{3}H-bile acids in recovered media								Total amount of bile acids
	cholic		chenodeoxycholic		deoxycholic		lithocholic		
	ug	%	ug	%	ug	%	ug	%	ug
LDL (1.006 d 1.063) g/ml	-	-	71.43	34.27	13.77	6.61	123.21	59.12	208.41
HDL (1.063 d 1.21) g/ml	208.64	16.93	444.44	36.10	101.64	8.25	477.36	38.74	1232.08

After plasma LDL-^{3}H-cholesterol and HDL-^{3}H-cholesterol were incubated with rat liver parenchymal cells for 4 hours at 37^{o}C, the media were separated from cells and bile acids were extracted with ethyl acetate as described earlier. Bile acids residues were dried under N_2 stream and redissolved in 3 ml of methanolic HCl (3%) and, dried again, then redissolved in 200 ml ethyl acetate. Five ul of sample was injected in gas liquid chromatograpm and bile acids were identified by comparison with a standard mixture of five bile acids gas chromatographic retention time data of their methyl ester. Hyodeoxycholic acids were used as an internal standard.

DISCUSSION

We have previously shown that the binding and degradation of rat plasma lipoproteins (LDL and HDL) took place in rat liver parenchymal cells. Results indicated that rat hepatocytes were able to competitively bind and degrade LDL and HDL, but they bound HDL with a higher affinity and degraded it more effectively when compared to LDL (unpublished paper). In this paper, we studied further the metabolic fate of cholesterol delivered by rat plasma lipoproteins in rat liver parenchymal cells in vitro. LDL-^{3}H-cholesterol and HDL-^{3}H-cholesterol were incubated with rat liver parenchymal cells for 4 hours at 37^{o}C. After incubation, media were separated from cells and tritium radioactivity of cholesterol and bile acids were counted.

Investigation of the metabolism of free cholesterol in different lipoprotein classes has been hampered by the rapid in vivo and in vitro exchange of free cholesterol between lipoprotein classes. The experimental design of the present study has circumvented this problem by incubating HDL labelled with ^{3}H-cholesterol or LDL-^{3}H-cholesterol with isolated rat liver parenchymal cells. Thus, the relative use of LDL-^{3}H-cholesterol or HDL-^{3}H-cholesterol for bile acid synthesis was determined by

counting ^{3}H-activity incorporation into bile acids.

Present results demonstrate that the free cholesterol associated with HDL was preferentially used by rat liver parenchymal cells as the precursor for bile acid synthesis. When HDL-^{3}H-cholesterol was incubated with rat liver parenchymal cells, 3.4% of HDL-free cholesterol was converted to bile acids within 4 hours, mainly as lithocholic, chenodeoxycholic, cholic and deoxycholic acids, whereas merely 1.1% of LDL-free cholesterol was converted into bile acids as lithocholic, chenodeoxycholic and deoxycholic acids, but not cholic acid, the major primary bile acid (Table III.3, Figs. III.2,3). The proportion of bile acids that were formed from HDL-free cholesterol was more for the primary bile acids such as chenodeoxy and cholic acids while LDL-free cholesterol produced more secondary bile acids (Tables III.4 and 5). These findings are compatible with the previous studies (14-17, 27, 28), showing that HDL-cholesterol is a better precursor for bile acid synthesis than LDL-cholesterol. The mechanism by which HDL-free cholesterol was preferentially utilized for hepatic conversion of cholesterol to bile acids was not known from the present study. Our earlier study demonstrated that rat liver parenchymal cells showed similar binding affinities for LDL and HDL but the capacity for binding and for degrading HDL was

much higher than that for LDL. The present observation of higher conversion of HDL-^{3}H-cholesterol can be explained by our previous findings. The high binding affinity and greater degradation of HDL in the hepatocytes resulted in the higher conversion of HDL-^{3}H-cholesterol to ^{3}H-bile acids. Another explanation may be that free cholesterol could be bound more lossely to the HDL particles and, therefore, could be exchanged more readily with hepatic membranes (14). Therefore, the presence of hepatic membrane receptors which selectively remove surface-bound free cholesterol from HDL particles, leaving the particles otherwise intact, would be most consistent with HDL shuttling of cholesterol from the tissue to the liver for excretion as bile acids and biliary cholesterol. Another mechanism for the preferential utilization of HDL-cholesterol for bile acid formation was reported recently by Miller et al (17) who showed that free cholesterol bound to HDL undergoes greater side-chain oxidation (bile acid formation) than did LDL-cholesterol in rats. Another study by O'Malley et al (29) showed that free cholesterol exchange with rabbit hepatocytes was more rapid with HDL than with LDL.

The reutilization of the free cholesterol released from lipoprotein degradation by rat liver parenchymal cells has not been studied very well. It is unclear if

rat hepatocytes may catabolize the cholesterol that is delivered by lipoproteins to bile acid synthesis or transfer it to other classes of lipoproteins which are secreted. In this study the dialysis data of the media recovered from the incubation mixture showed that nearly all of the ^{3}H-cholesterol activity was bound to undialyzable macromolecules, possibly LDL or HDL (Table III.2). These observations suggest that ^{3}H-free cholesterol labelled lipoproteins bound to cells, internalized and degraded to free cholesterol where it secretes into the medium as newly synthesized lipoprotein labelled cholesterol.

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CHAPTER IV

PREPARATION OF RAT LIVER HEPATOCYTES BY PERFUSION OF ISOLATED LIVER WITH ENZYME COLLAGENASE AND Ca^{2+}

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Preparation of Rat Liver Hepatocytes

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ABSTRACT

A technique for the preparation of high viability rat hepatocyte suspension is described. Rat liver was perfused, in situ, with recirculating Hank's buffer solution containing enzyme Collagenase and Ca^{2+}. The perfusion media consisted of a) 150 ml of calcium-free Hank's solution containing: 0.5 mM ethylene glycol-bis (B-aminoethylether) N, N-tetraacetic acid; b) 100 ml of Hank's solution containing 0.3 ml of 1M $CaCl_2$ and 50 mg collagenase; and c) 100 ml of minimum essential medium (MEM) containing 1.5% fatty acids-free bovine serum albumin, pH 7.4.

Biochemical and morphologic studies indicated that isolated parenchymal cells were highly viable. They retained their microscopic structural integrity and were not stained with trypan blue. The viability of freshly prepared isolated hepatocyte suspensions decreased with time. The initial viability of cells was 93% and after 4 hours of incubation the viability became 86%. Microscopic examinations of these isolated cells showed that they were spherical in shape and found either individually or in small clusters. The best results were obtained when the liver was first perfused for 3 minutes with EGTA, then followed by 9 minutes with Collagenase and Ca^{2+}. This sequential treatment converted the whole liver into a cellular suspension of hepatocytes, of which 93% of cells were viable.

INTRODUCTION

Various methods have been used to prepare isolated cells from rat liver tissue. There were mechanical dispersion (1), chelation of intercellular Ca^{2+} with citrate of EDTA (2), chelation of K^{+} with thionine phosphate (Cocarboxylated) or dissolution of the intercellular matrix with collagenase and hyaluronidase (4) or other enzymes (5, 6).

The enzymatic methods were subsequently improved by Berry and Friend (3) who introduced a recirculating perfusion technique which, however, has been further modified by other investigators. Wagle et al (7) simplified the procedure of Berry and Friend (3) by using collagenase as the sole enzyme for the digestion of liver connective tissue. Later, Seglen (8) was able to decrease perfusion time and increase the yield of viable cells by perfusing the liver with Ca^{2+} removing agent, prior to perfusion with collagenase and Ca^{2+}-containing medium.

Although most of the techniques employed to obtain isolated hepatocytes involve perfusion of the liver with digestive enzymes, attempts have also been made to avoid the perfusion step and achieve hepatocyte isolation simply by incubating cut pieces of liver in enzyme-containing solution (9). Since proper oxygenation of the

liver during the cell isolation procedures appears to be essential for the viability of isolated cells (10), such a procedure not only decrease the total recovery of cells but may also produce fewer viable cells.

Suspension of isolated hepatocytes are now frequently used in biochemical studies. This experimental tool has been successfully employed in various studies on gluconeogenesis, glycolysis, protein, lipid, fatty acids, and urea synthesis, ketone body production, protein metabolism, ethanol oxidation, membrane transport, and response to hormones (11). Isolated hepatocytes have also been used in studies on drug metabolism and have proved to be of value for further elucidation of many aspects of this process (12-16).

In this paper we describe a modified technique of the method of Berry and Friend for isolation of fresh rat parenchymal cells.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (200-300 g) were obtained from Bantin and Kingman, Inc. (Freemont, CA).

Collagenase (type 1), ethylene glycol-bis (B-aminoethylether) N, N tetraacetic acid (EGTA), fatty acids-free bovine serum albumin, fraction V, Heparin, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Minimum essential medium (MEM) was obtained from GIBCO Laboratories (Grand Island, N.Y.)

Isolation of procedures

The present technique was based on liver perfusion with collagenase after removal of Ca^{2+} using a chelator, EGTA. Moderately high yield of viable, structurally intact hepatocytes with spherical appearance and essentially free of nonparnenchymal cells were obtained.

Gas mixture and solutions

All solutions were bubbled with carbogen gas (95% O_2, 5% CO_2) and heated to 37^{o}C for one hour prior to use. The same gas mixture was used during perfusion of the liver and incubation of hepatocytes. Three different buffers were used. Buffer A (150 ml) was a modified Hank's buffer, pH 7.4 (NaCl 80 g; KCl 40 g;

$MgSO_4$ 2.0 g; Na_2HPO_4, .45 g; KH_2PO_4, .60 g; $NaHCO_3$, 2.33 g in a volume of 1 liter) containing .599 mM ethylene glycol-bis (B-aminoethylether) N, N tetraacetic acid (EGTA). Buffer B (100 ml) was the same modified Hank's buffer containing 0.05% collagenase and .3 ml of 1 M $CaCl_2$. Buffer C (100 ml) was minimum essential medium (MEM), pH 7.4 (anhyd)($CaCL_2$, .2g; KCl, .4 g; $MgSO_4.7H_2O$, .2 g; NaCL, 6.8 g; $NaHCO_3$, 2.2 g; NaH_2PO_4, .14 g; D-glucose 1.0 g; phenol red, .01 g; L-arginine HCl, .126 g; L-cystine, 0.024 g; L-glutamine, .292 g; L-Histidine-HCl.$H_2$0, 0,042 g; L-isoleucine, .052 g; L-leucine, .052 g; L-lysine-HCl, 0.072; L-methionine, 0.015 g; L-phenylalanine, 0.032 g; L-threonine, 0.048 g; L-tryptophane, 0.01 g; L-tyrosine, 0.036 g; L-Valine, 0.046 g; D-ca pantothenate, .001 g; choline chloride, .001 g; folic acid, .001 g; inositol, 0.002 g; nicotinamide, .001 g; pyridoxal HCl, .001 g; riboflavin, 0.0001 g; thiamine HCl, .001 g; in a volume of 1 liter).

Surgical procedures

Rats were anesthetized with ether, and the peritoneal cavity was cut open by a midventral incision. 0.15 ml of Heparin (2000 u/ml) was injected into the inferior vena cava. Then the liver was cannulated, via the hepatic portal vein while pumping buffer A at a flow rate of approximately 19 ml/minute. The cannula was tied off and the flow rate was increased to 35 ml/minute.

Perfusion and washing procedure

Perfusion started in situ with buffer A. A sign of adequate perfusion should be that the liver cleared immediately and completely. When the liver had been freed from the body, it was immersed, at once, in the buffer solution in the reservoir and the cannula was fixed to the horizontal bar. After 3 minutes of perfusion with buffer A, the liver was removed from the reservoir beaker and placed in another beaker containing buffer B. Buffer B was recirculated for approximately 9 minutes. At the end of perfusion with buffer B the liver appeared swollen and pale. The liver was then placed in a Petri dish. The outer membrane was gently removed with small and blunt tweezers. 10 to 15 ml of the media (MEM) was added onto the petri dish and the liver was gently swirled to free the cells. The procedure of freeing the cells was repeated three times. Within 2 minutes, at ambient temperature, the dispersed cells were filtered through cotton gauze to remove remaining connective tissues and clumps of cells. The filtrate was collected in a plastic centrifuge tube (40 ml), and spun for 2 minutes at 400 rpm. The supernatant containing non-parenchymal cells was removed by aspiration and cell pellets were resuspended in approximately 20 ml of media (MEM).

Viability of isolated hepatocytes

Cell viability was routinely estimated by trypan blue staining prior to other experiments. During prolonged incubation experiment cell viability was determined periodically (every 2 hours).

The trypan blue examination test is based on the fact that viable cells exclude the dye whereas nonviable cells take up the dye which then stains the nucleus blue. The test is carried out as follows. An aliquot (.20 ml) of the cell suspension was mixed with .90 ml of .14% trypan blue in .9% saline solution containing 20% bovine serum albumin. Numbers of viable and non-viable cells were counted in a hemacytometer counting chamber. From these figures, percentage of viability was calculated.

Incubation procedures

Incubations were started immediately after isolation of the hepatocytes. Long-term incubations ($>$ 1 hour) were performed in Erlynmeyer flask (25 ml) in a shaking water-bath at 37^{o}C. Carbogen gas was applied continuously to the surface of the incubation medium in the flask to insure adequate oxygenation during the incubation.

RESULTS AND DISCUSSIONS

Cell viability of isolated rat hepatocytes was measured by using the trypan blue exclusion test. The initial viability of the cells was found to be 93.3% and decreased with time (Fig. IV.1, and Table IV.1). After four hours of incubation, cell viability was 86%. These findings are compatible with Dalet et al (17) who isolated fresh rat hepatocytes, suspended in a Krebs-Rings bicarbonate (KRb) buffer with 1% BSA, with a cell viability of approximately 85% after four hours incubation. However, as shown in Table IV.1, total number of viable cells in the present study was higher (1.20×10^7 cells/ml) than the values of Dalet et al (17) which were 2×10^6 cells/ml, obtained from rats of a size identical (200-300 gm) to those used in our study.

The previous methods for enzymatic preparation of liver cells employ collagenase and hyaluronidase in a Ca^{2+} free medium (2, 3, 4). However, the results were less impressive, with yields of 60-70% of total number of cells and viability above 75% by the enzymatic perfusion technique (2). In the present study, we used Ca^{2+} with the enzyme collagenase in the media for the perfusion of liver and we were able to obtain cell viabilities above

TABLE IV.1

Viability of isolated hepatocytes as measured by the trypan blue exclusion test.

Time (hrs)	Total Number of Cells Mean ± SD n = 4	Dead Cells Number n = 4	Dead Cells %	Cell Viability %
0	177 ± 10.08	12 ± 2.5	6.7	93.3
2	165 ± 5.90	17 ± 2.88	10.3	89.7
4	145 ± 4.65	20 ± 2.16	13.8	86.0
6	162 ± 13.30	50 ± 5.02	30.8	69.2
8	163 ± 8.98	74 ± 8.54	45.4	54.6

Figure IV.1. Viability of isolated hepatocytes by the trypan blue exclusion test. Cell viability was assessed by the trypan blue (0.14% trypan blue in 2% bovine serum albumin dissolved in 0.9% NaCl solution) exclusion method. Isolated hepatocytes were suspended in minimal essential medium (MEM) containing 1.5% BAS (fraction V) and was incubated in shaking water bath at 37^{o}C and oxygenated. Cells were counted every two hours.

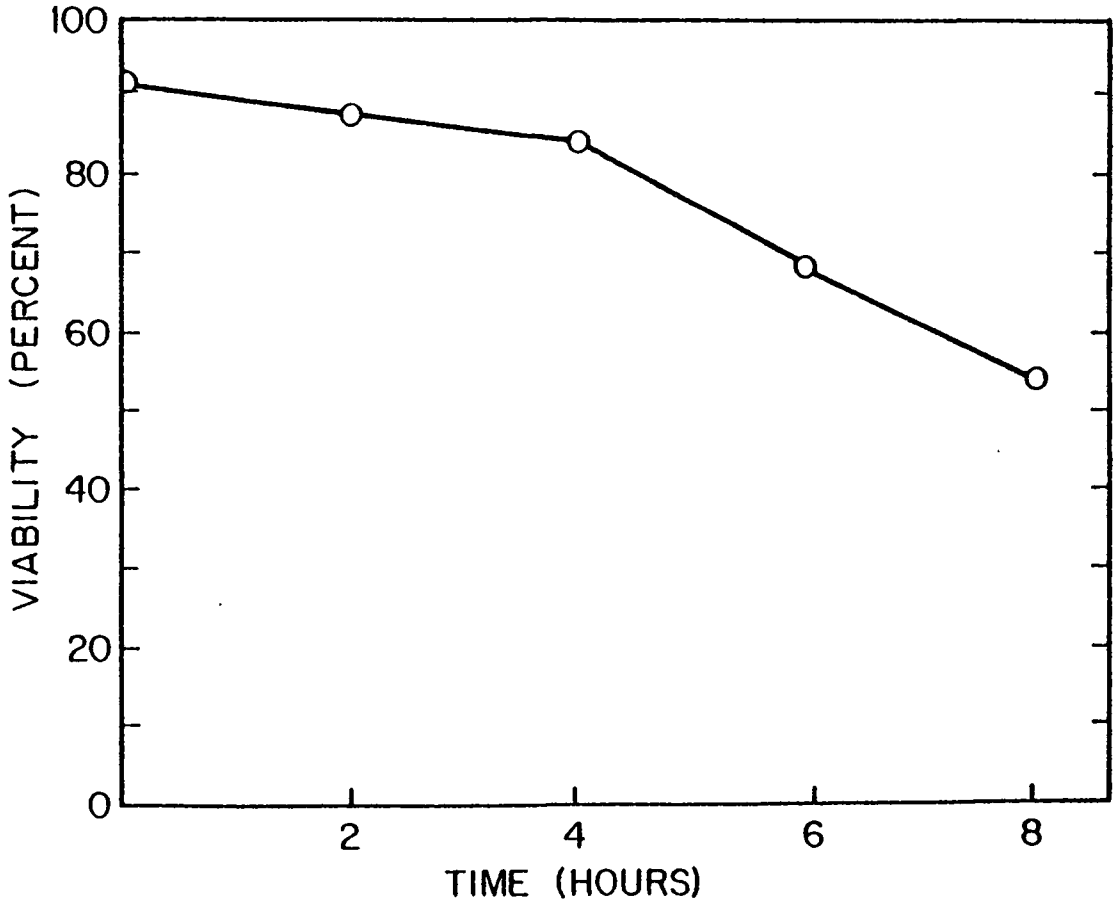

Figure IV.1

90%. We also observed that during the pre-perfusion of the liver with Ca^{2+}-free Hank's buffer solution in the absence of collagenase, the liver did not swell and the perfusate level in the reservoir stayed constant. When collagenase was added in the presence of Ca^{2+}, the liver began to swell after 5 minutes, and the progressive liver swelling was followed by the fall in the perfusate level in the reservoir. These observations demonstrate that addition of Ca^{2+} to the perfusate greatly accelerates enzymatic dispersion. The calcium chelator EGTA on the other hand, inhibited enzymatic dispersion completely. Seglen (8) recommended perfusion of rat liver with the enzyme collagenase in the presence of Ca^{2+}. He showed that the efficiency of enzyme dispersion appeared to increase with Ca^{2+} concentration in the range of .20 - .40 mM in the medium. Accordingly, we found the best results were obtained when the liver was first perfused for 3 minutes with EGTA, then followed by perfusion for 9 minutes with enzyme collagenase and Ca^{2+}. This sequential treatment converted the whole liver into a cellular (mush) suspension, in which 93% of the hepatocytes were intact.

The time needed for the liver cell preparation by this procedure was very short (30 minutes). The yield of parenchymal cells was 1.65×10^7 cells/ml from the

liver of rat weighing about 200 gm and the viability was 93.3% as measured by the trypan blue exclusion test. The representative cell suspension is shown in Fig. IV.2. The suspension consists of a single cells spherical in shape, and were either individually distributed or in small clusters.

Figure IV.2. Liver cell suspension prepared by our pre-perfusion technique with 0.5mM EGTA and the enzyme collagenase in the presence of calcium ions. Three damaged cells, staining darkly with trypan blue are seen in the upper right-hand corner. The cells are in rounded appearance and found either individually or in small clusters. The photograph was taken by a Leitz 35 mm camera (E. Germany) equipped with a Nikon MSD Inverted Microscope (Nikon, Japan). (1/60 sec. exposure, magnifcation X250.)

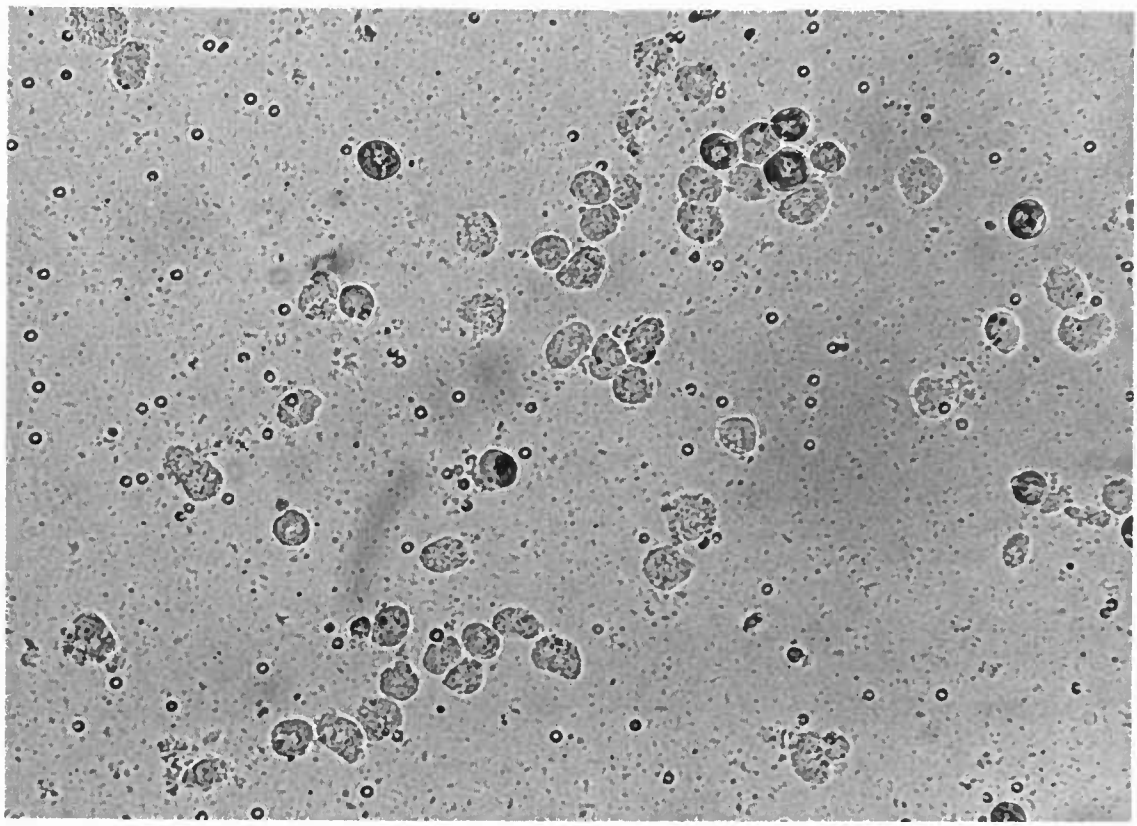

FIG. IV.2

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CHAPTER V

CONCLUSION

Epidemiological studies indicate that there is a negative correlation between the concentration of cholesterol carried by high-density lipoprotein (HDL) in the plasma and the incidences of coronary artery disease (CAD) and, by contrast, that there is a positive correlation between the concentration of cholesterol carried by low-density lipoprotein (LDL) and the risk for coronary artery disease (CAD). Present concepts of the relationship between cholesterol levels in the lipoprotein fractions and the risk for CAD were mostly based on studies with cultured cells such as fibroblasts and arterial smooth muscle cells and with isolated liver cell membranes, hepatocytes or liver perfusion.

The beneficial effect of HDL may be related to its possible role as a vehicle for the transport of cholesterol from peripheral tissues to the liver. Tissue cholesterol may be removed in free form by HDL and transported to the sites where it can be appropriately metabolized. Lecithin cholesterol acyltransferase (LCAT) could then esterify this free cholesterol by its action on the transfer fatty acids from lecithin at position 2 to the free

OH group of cholesterol at position 3. This reaction is catabolyzed by apoprotein A-I as a cofactor which is present in HDL. The cholesterol-laden HDL would finally be taken up in the liver where the lipoproteins would be degraded and esterified cholesterol would be hydrolyzed. However, no evidence has been obtained in vivo to suppor- the postulated role of HDL as a carrier of cholesterol from the tissue to the liver.

Since the liver is the only organ in the body that can catabolize and excrete cholesterol, we studied the metabolic fate of cholesterol delivered to rat liver parenchymal cells by rat plasma lipoprotein fractions in vitro. We have shown that rat low density and high density lipoproteins are bound and degraded in rat liver parenchymal cells. The binding affinity of HDL in rat liver parenchymal cells was 7-fold higher (Kd .15 X 10^{-8}M) than that for LDL (Kd - 1.04 X 10^{-8}M). Further, most of the HDL bound to the cells was degraded by rat liver parenchymal cells (88% of total bound of HDL), which is compared to 63% degradation for LDL.

The specific source of free cholesterol utilized by rat liver parenchymal cells for bile acid biosynthesis was also investigated in the present study. Plasma low- and high-density lipoproteins were labelled with ^{3}H-free cholesterol and incubated with rat liver parenchymal cells

for 4 hours at 37°C. After incubation, media were separated from cells and bile acids were extracted, and tritium activity of bile acids were counted. We found 3.4% of HDL-^{3}H-cholesterol was converted to bile acids mainly in the form of primary bile acids, whereas, 1.1% of LDL-^{3}H-cholesterol was transformed to bile acids mainly in form of secondary bile acids. This trace study was confirmed by data obtained from thin layer and gas liquid chromatographic analysis which showed that 3.07% mass of HDL-cholesterol was actually converted to new bile acids and 1.26% of LDL cholesterol mass was catabolized to bile acids.

Therefore, I report that rat HDLs preferably bound to rat liver parenchymal cells and were degraded at higher rate than LDL. Furthermore, the free cholesterol associated with HDL was preferentially used by the hepatocytes as a substrate for bile-acid synthesis. These findings are compatible with the previous hypothesis that HDL may be the reverse transport vehicle for cholesterol to be removed from peripheral tissue to the liver. This may be the mechanism by which HDL cholesterol renders protection for coronary heart disease. It remains to be seen what proportion of HDL-cholesterol is metabolized by other metabolic routes such as reexcretion into circulation as newly synthesized lipoproteins. Intervention of diet or drugs to alter the rate of bile acid synthesis may lead to a cure or prevention for hypercholesterolemia and atherogenesis.

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