Preferential Utilization of Free Cholesterol from High-Density Lipoproteins for Biliary Cholesterol Secretion in Man

Abstract. *High- and low-density lipoproteins carrying free cholesterol labeled with ^{3}H or ^{14}C were administered to a patient with a bile fistula. The free cholesterol from high-density lipoproteins was more rapidly incorporated into biliary cholesterol than the free cholesterol from low-density lipoproteins. These findings show that the liver in man selectively utilizes and secretes the free cholesterol from a particular lipoprotein.*

Epidemiological data indicate that there is a negative correlation between the concentration of cholesterol carried by high-density lipoprotein (HDL) in the plasma and coronary artery disease (CAD) and, by contrast, that there is a positive correlation between the concentration of cholesterol carried by low-density lipoprotein (LDL) in the plasma and CAD (*1*). Present concepts of the metabolism of the cholesterol moieties in the lipoprotein fractions in man are based on studies (*2, 3*) with isolated tissue culture cells (fibroblasts, arterial smooth muscle cells) and the lipoprotein alterations induced in vitro by lecithin acyltransferase (E.C. 2.3.1.43) in the plasma (*4*). The bulk of the cholesterol in the blood is contained in the LDL fraction which is thought to be taken up and degraded by extrahepatic tissues. Deposition of cholesterol in tissues could result from failure to eliminate efficiently the cholesterol derived from LDL. The beneficial effect of HDL may relate to its possible role as a vehicle for the transport of cholesterol from peripheral tissues to the liver. Tissue cholesterol may be removed in the free form and added to the reservoir of free cholesterol carried by the HDL; lecithin acyltransferase could then esterify this excess cholesterol. The cholesterol-laden HDL would finally be deposited in the liver where the lipoprotein would be degraded and esterified cholesterol would be hydrolyzed. However, no evidence has been obtained in vivo to support the postulated role of HDL as a carrier of cholesterol from the tissues to the liver in man. Since the liver is the only organ in the body that can catabolize cholesterol, we investigated whether this tissue acts selectively on the cholesterol carried by LDL or HDL.

We have shown (*5, 6*) that there are definitive hepatic cholesterol precursor sites associated with the synthesis of bile acids and the secretion of biliary cholesterol; these sites derive a substantial proportion (70 percent) of their cholesterol from the plasma; 60 percent of this cholesterol is free (unesterified) and 10 percent is esterified (after hydrolysis). The observation that a major portion of the biliary cholesterol precursor originated from the free cholesterol and not the esterified cholesterol provided the impetus for the present study. We simultaneously administered HDL and LDL carrying free radioactively labeled cholesterol to a patient with a bile fistula, and measured the hepatic uptake of the ^{14}C- and ^{3}H-labeled cholesterol.

For this study we used a 68-year-old black female patient who had undergone a cholecystectomy and common bile duct exploration 14 days previously. Blood (80 ml) was withdrawn from the patient and maintained at 5° to 7°C; red blood cells (RBC's) were separated and portions of the plasma were incubated with Whatman No. 1 filter paper impregnated with either [1,2-^{3}H]cholesterol or [4-^{14}C]cholesterol. All samples were incubated at 7°C for 3 hours. The LDL (1.019 to 1.063) and HDL (1.063 to 1.21) fractions were separated from each portion by preparative ultracentrifugation at 5°C. The four labeled lipoproteins ([^{14}C]LDL, [^{14}C]HDL, [^{3}H]LDL, and [^{3}H]HDL) were dialyzed overnight against saline with 0.01 percent EDTA at *p*H 7.4. The lipoproteins were then passed through a 0.22-μm Millipore filter to remove particulate cholesterol and for sterilization. Analysis of the lipoproteins showed that (i) they were virtually devoid of labeled cholesterol esters (< 2 percent); (ii) the electrophoretic mobilities of the HDL and LDL were unchanged, and the label migrated with the appropriate lipoprotein band; and (iii) the label appeared in the appropriate hydrated density fraction upon repeated ultracentrifugation. The experiment was carried out in two stages. In stage 1, the patient received [^{3}H]HDL (46×10^{6} dis/min) intravenously over 2 minutes, followed immediately by [^{14}C]LDL (1.22×10^{6} dis/min). Bile and blood were then obtained at frequent time intervals as indicated in Figs. 1 and 2. After 3 days, stage 2 of the experiment was carried out: the patient received [^{3}H]LDL (33.5×10^{6} dis/min) followed immediately by [^{14}C]HDL (0.95×10^{6} dis/min). Again, blood and bile were obtained at frequent time intervals. The RBC's were immediately separated from the plasma and washed with saline. The RBC's, biliary free cholesterol mass, and radioactivity were determined as described earlier (*6, 7*). There were only small amounts of radioactivity remaining in the bile from stage 1; this radioactivity was subtracted out from the data obtained from stage 2.

The rate of appearance of radioactivity in biliary cholesterol and RBC cholesterol after the administration of labeled lipoproteins is shown in Fig. 1. When either [^{3}H]HDL or [^{14}C]HDL were given with [^{14}C]LDL or [^{3}H]LDL there was rapid appearance of radioactivity in the biliary cholesterol fraction. However, the label associated with the HDL fraction appeared much more rapidly in biliary cholesterol than the label from the LDL fraction. After the administration of labeled HDL, the peak specific activity was reached in 80 to 100 minutes, whereas the labeled cholesterol derived from LDL did not reach its peak until 120 to 140 minutes. The curves for specific activity merged at approximately 180 minutes and thereafter assumed the same specific activity which would be expected when the ^{3}H- and ^{14}C-labeled free cholesterol had exchanged sufficiently between the plasma lipoprotein classes. The labeled cholesterol from HDL was preferred by the RBC over the labeled cholesterol in LDL, but the differences were much smaller than were observed for the biliary cholesterol. The curves for the specific activity for RBC cholesterol became identical at approximately 200 minutes and merged with those for the biliary cholesterol at approximately 400 minutes.

The ratios of either [^{3}H]HDL or [^{14}C]HDL to [^{14}C]LDL or [^{3}H]LDL in biliary cholesterol and RBC (Fig. 2) clearly illustrate the marked selectivity with respect to the labeled cholesterol from HDL. A ratio of one is equivalent to the ratio of the injected labels. The ratios of labeled HDL to LDL in biliary cholesterol at the early time periods greatly exceeded the ratio in the RBC.

Because of the complete exchange of free cholesterol among the lipoprotein classes, which occurs during the isolation of lipoproteins from plasma, studies of the metabolism of the free cholesterol fractions have been hampered. The design of the present study circumvented these difficulties in that the liver could selectively extract the free cholesterol of HDL or LDL and we could then examine the labeling pattern of biliary cholesterol. Also, in order to obviate an isotope effect or other unknown factors, we con-

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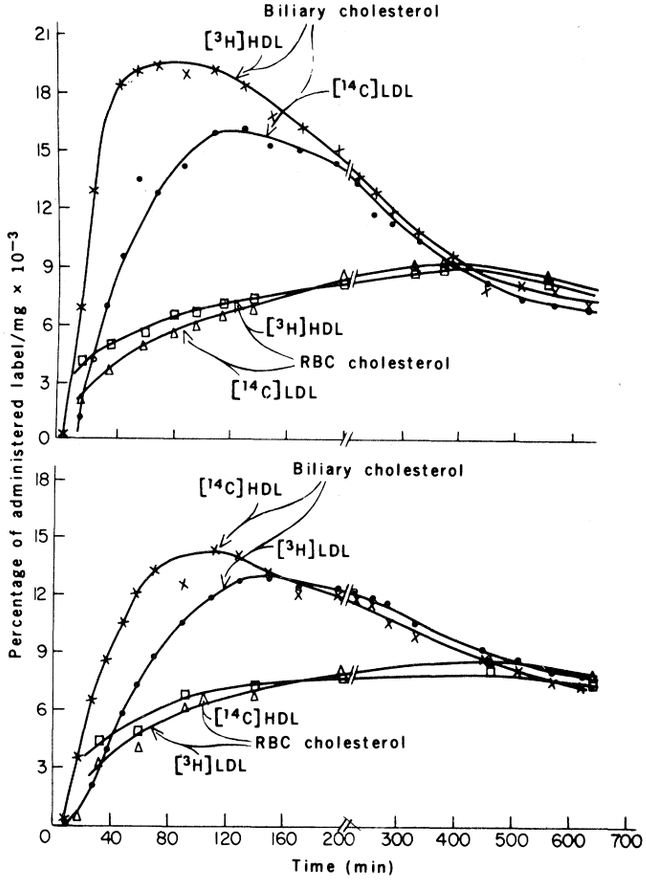
ducted the experiments first with [^{14}C]LDL and [^{3}H]HDL, and then with [^{3}H]LDL and [^{14}C]HDL, and obtained similar results. The present findings can not be solely attributed to simple exchange processes because the RBC cholesterol data which are indicative exclusively of exchange between RBC and the lipoprotein fractions were vastly different from the biliary cholesterol data. Also, the data derived from biliary cholesterol are representative of net movement of lipoprotein cholesterol mass from the blood to the bile.

The present observations could be attributed to three possibilities. (i) The free cholesterol on the surface of HDL is more loosely bound than the free cholesterol on the LDL particle and is therefore more readily exchangeable. Comparison of the biliary and RBC cholesterol data indicate that this type of exchange probably occurs to some extent. However, this is probably quite minimal because of the large differences between the RBC cholesterol and bile cholesterol. (ii) The HDL particle could be attached to hepatocyte plasma membrane receptors, internalized, and degraded (*3, 8*). This would imply that the cholesterol in bile originated from the complete catabolism of the HDL particle. Several lines of evidence argue against this mechanism. It can be estimated that hepatic degradation of HDL is insufficient to account for the daily excretion of cholesterol from the body. For example, a 70-kg man has approximately 50 mg of HDL cholesterol (of which two-thirds is esterified) per 100 ml of plasma. The $t_{1/2}$ (time for 50 percent to be removed) of HDL is approximately 5 days (*9*). Complete degradation of HDL would therefore supply 200 mg of cholesterol per day which is only 20 percent of the 1000 mg of sterols secreted each day by a normal individual. In all probability, direct degradation of HDL in the hepatocyte does not even contribute 200 mg of cholesterol per day since the esterified fraction contributes very little to the bile acid and biliary cholesterol precursor sites (*6*). In addition, a recent study (*10*) suggests that some HDL degradation may occur in extrahepatic tissues. (iii) The liver extracts free cholesterol by a cell surface receptor mechanism without internalizing the HDL particles. In this manner, the HDL could act as a shuttle for the transport of free cholesterol from the tissues to the liver. This mechanism would be consistent with recent studies of hepatic cholesterol metabolism (*6*), which indicate that free cholesterol from the plasma is the major precursor of bile acids and biliary cholesterol, and with tissue culture studies (*11*) which have shown that HDL selectively binds free cholesterol, whereas LDL does not have this property. Since HDL ester probably contributes very little to the cholesterol degradative pathway, it seems likely that most of the free cholesterol which is removed from the tissues by mature HDL particles is not esterified in vivo by lecithin acyltransferase. Our studies are also consistent with the present concepts of the metabolism of LDL (*2*). However, we could not determine whether any free cholesterol of LDL is directly taken up by the liver. Our data for bile acids show a labeling pattern virtually identical with biliary cholesterol after the administration of the labeled lipoproteins (*12*).

The present report provides a basis for exploring the possible protective effect of HDL in heart disease. High levels of



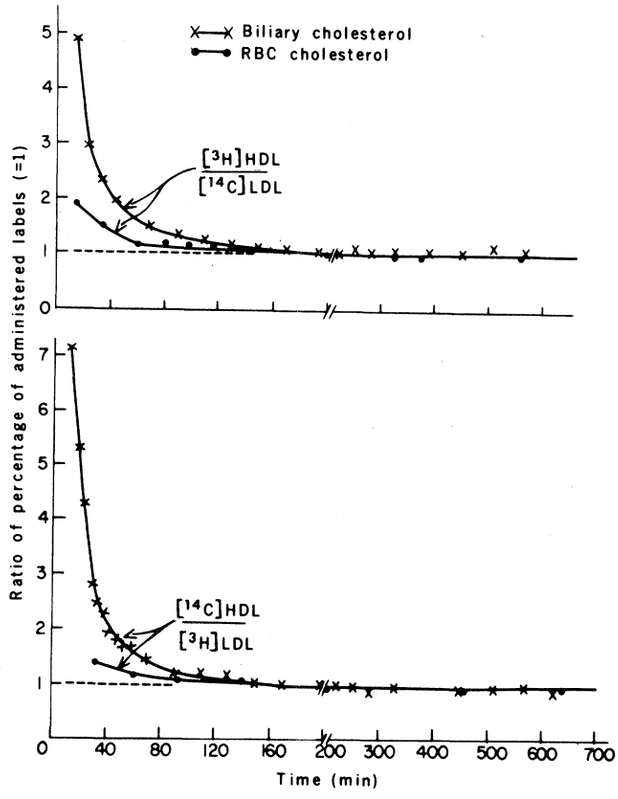


Fig. 1 (left). Curves for the specific activity of biliary and RBC cholesterol after the administration of [^{3}H]HDL plus [^{14}C]LDL (top) and [^{14}C]HDL plus [^{3}H]LDL (bottom). Fig. 2 (right). Ratios of the percentages of administered labels found in biliary and RBC cholesterol after administration of labeled lipoproteins. A ratio of 1 is equivalent to the proportion of the injected labels.

HDL would foster the efficient removal of tissue cholesterol and its subsequent elimination from the body by the liver and, by contrast, low levels of HDL could lead to excessive accumulation of cholesterol in tissue. Additional studies are needed to delineate the mechanism of hepatic uptake of free cholesterol, and to determine whether other lipoproteins contribute substantially to the bile acid and biliary cholesterol hepatic precursor sites, and whether one particular HDL subfraction is more active than another.

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Tunicate Eggs Utilize Ammonium Ions for Flotation

Abstract. *Unlike most solitary ascidians,* Corella willmeriana *retains its eggs and embryos well past hatching. The early stages float to the top of the enlarged atrium from which they cannot escape. Ammonium ions replace other more dense substances in the cell sap of the float cells surrounding the embryo. Energy derived from glycolysis but not mitochondrial processes supports this process.*

Among the many different adaptations to flotation in the marine environment, the substitution of ammonium ions for more dense substances is at present known to occur only in certain phytoplankton cells (*1*) and a few pelagic cephalopods (*2*). *Corella willmeriana*, from the U.S. Pacific Northwest, is one of the few ascidians that produce floating eggs. In all ascidians the eggs and embryos are surrounded by two cellular layers and a noncellular chorion. The outer cellular layer consists of follicle cells that are especially large in *C. willmeriana* and in the European species *C. parallelogramma* and *Ascidiella aspersa*, which also have floating eggs (*3*). In *C. willmeriana* these cells are responsible for egg flotation; if they are removed, the eggs rapidly sink (*4*). The floating eggs are an adaptation to brooding, as they are incubated in an enlarged atrial chamber in which the siphonal opening is never uppermost. After hatching the tadpoles swim actively upward, which may ensure their retention in the atrium until they are quite advanced, an optimal strategy for a fugitive species such as *C. willmeriana* (*5*). We have determined, by chemical analysis, that the low density of the follicle cells results from the fact that ammonium ions replace other, more dense elements of the vacuolar sap. Inhibitor studies implicate glycolysis but not mitochondrial processes in providing the energy to maintain the asymmetry of ammonium concentration across the thin peripheral cytoplasm of the follicle cells.

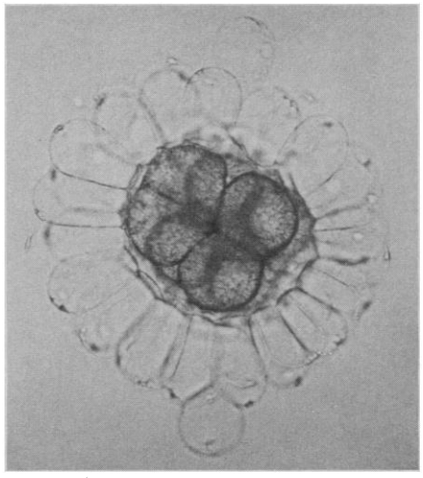

Fig. 1. Embryo of *Corella willmeriana*, showing large flotation cells surrounding the chorion.

Each embryo is surrounded by an average of 89 flotation cells which are 65 μm in diameter (Fig. 1), giving a flotation volume of 1.31×10^{-5} cm^3 per embryo. The chorion has a diameter of 170 μm with an enclosed volume of 2.57×10^{-6} cm^3, yielding a fivefold excess of flotation space to embryonic space. Naturally spawned eggs float in seawater with a salinity of 28.0 per mil (density, 1.023 g/cm^3) and in dilutions of seawater with salinities as low as 19.5 per mil (calculated density, 1.02 g/cm^3). However, the eggs rise at a rate of 2.41×10^{-2} cm/sec in seawater with a salinity of 28.0 per mil, giving a calculated density of 1.018 g/cm^3 (*6*). Eggs and embryos that are deprived of their float cells as a result of being shaken in a test tube were found by isopycnic centrifugation in sucrose-seawater solutions to have a density of about 1.08 g/cm^3. Thus the follicle cells decrease the density by about 0.06 g/cm^3 in the intact embryo.

The follicle cells are composed of a thin rind of cytoplasm with a single large central vacuole; obviously, the vacuolar contents are responsible for the observed decrease in density. One appealing possibility would be that the follicle cells are filled with lipid, which would make it possible for them to float. Since formalin-fixed eggs sink rapidly, lipids are probably not involved. In addition, the lipophilic stain sudan black B fails to stain the vacuolar sap. Since ammonium ions have been implicated in the flotation of other marine forms, it seemed reasonable to analyze the ammonium content of the follicle cells. We shook eggs and embryos gently in test tubes to remove the follicle cells. The embryos were allowed to settle; then the follicle cells in the supernatant were lysed, and we determined their ammonium content by the colorimetric analysis of indophenol blue produced from ammonia with commercially prepared reagents (Boehringer Manheim). The follicular fraction contained large amounts of ammonium. However, no ammonium ions were detected in the eggs and embryos. By counting the number of embryos extract-

Table 1. The effect of metabolic inhibitors on the flotation of *Corella* eggs.

Inhibitor	Concentration (m*M*)	Effect on	
		Flotation	Development
Sodium azide	3	Float	Inhibits
Dinitrophenol	2	Float	Inhibits
Malonic acid	1	Float	
Sodium fluoride	3	Sink	Supports
Iodoacetic acid	3	Sink	Inhibits

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