Interaction of free cholesterol and apoproteins of low and high density lipoproteins with isolated rabbit hepatocytes

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Abstract Primary cultures of rabbit hepatocytes were incubated with rabbit high density (HDL) and low density (LDL) lipoproteins in order to compare the surface transfer of free cholesterol with the uptake of apoproteins. Hepatocytes were maintained for various intervals with either LDL or HDL which contained both ^{125}I-labeled protein and free [4-^{14}C]cholesterol. After a 3-hr incubation with an LDL concentration equivalent to 25% of the normal rabbit serum level, the percentage of media ^{14}C in hepatocytes was 2.3 times greater than the percentage of ^{125}I; cells that had been incubated with HDL showed an eight-fold selectivity for ^{14}C. Although the influx of free cholesterol from HDL was greater than that from LDL, there was no difference between the uptake of LDL protein and of HDL protein. The degradation of lipoproteins labeled with [^{3}H]-leucine or ^{125}I was compared. Hepatocytes incubated with lipoproteins labeled with [4-^{14}C]cholesterol showed a greater influx of cholesterol from HDL_2 than from LDL. The efflux of labeled cellular cholesterol was also greater to HDL_2 than to LDL, whether the cellular cholesterol was labeled by prior exchange with labeled HDL_2 or by endogenous synthesis of cholesterol from [2-^{3}H]mevalonic acid lactone. **—O'Malley, J. P., P. A. Soltys, and O. W. Portman.** Interaction of free cholesterol and apoproteins of low and high density lipoproteins with isolated rabbit hepatocytes. *J. Lipid Res.* 1981. **22:** 1214–1224.

Supplementary key words cholesterol exchange · cholesterol influx · ^{125}I-LDL · ^{125}I-HDL · lipoprotein metabolism

Cellular cholesterol is derived partially from in situ synthesis and partially from plasma lipoproteins. Cholesterol is taken up from plasma lipoproteins during the process of apoprotein binding, internalization, and degradation, as has been shown to occur in several cell types (1). The free cholesterol of lipoproteins also exchanges with cellular free cholesterol (surface transfer). This phenomenon occurs rapidly between lipoproteins and erythrocytes (2), where the specific activities of labeled cholesterol in the two components eventually become equal (3). This exchange is probably universal for all cell types and appears to be more rapid than the uptake of lipoprotein protein by nucleated cells. While it has been established that the receptor-mediated uptake of low-density lipoproteins (LDL) is required for cell growth in ovary cells (4), the relative importance of lipoprotein uptake and free cholesterol exchange in determining the cholesterol composition of hepatocytes and in contributing to the cholesterol required for such functions as bile and lipoprotein secretion has not yet been determined.

Exchange of free cholesterol between lipoproteins and cells has been shown to result in a net transfer of cholesterol in cases where either the cholesterol/phospholipid ratios (5, 6) or the free cholesterol/esterified cholesterol ratios (7, 8) in the lipoproteins or in the cells have been altered. This net transfer is thought to result from a shift in the equilibrium in molecular exchange between serum lipoproteins and cell membranes (7). Net loss of cholesterol from fibroblasts and smooth muscle cells to serum containing high density lipoproteins (HDL) and no LDL has also been shown to occur in the absence of lecithin:cholesterol acyl transferase (LCAT) activity (9). Both HDL and LDL have been shown to participate in exchange of free cholesterol with cells (4, 7, 8, 10). Wu and Bailey (10) have compared the rates of LDL and HDL free cholesterol exchange in fibroblasts, but no comparative study of the two lipoproteins with respect to their rates of free cholesterol exchange with hepatocytes has been done.

Abbreviations: LDL, low density lipoprotein (d 1.019–1.063 g/ml); HDL, high density lipoprotein (d 1.063–1.21 g/ml); HDL_2, high density lipoprotein, subfraction #2 (d 1.063–1.125 g/ml); LCAT, lecithin:cholesterol acyltransferase; TCA, trichloroacetic acid; Hanks' BSS, Hanks' balanced salt solution.

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The study being reported here was undertaken to compare the rates of lipoprotein free cholesterol flux to apoprotein uptake in monolayers of rabbit hepatocytes, and to examine the relative activities of LDL and HDL with respect to the rates of free cholesterol flux into and efflux from hepatocytes.

MATERIALS AND METHODS

$Na^{125}I$, L-[4,5-^{3}H]leucine, [4-^{14}C]cholesterol and [7(n)-^{3}H]cholesterol, and DL-[2-^{3}H]mevalonic acid lactone were obtained from Amersham/Searle, Des Plaines, IL. Fresh rabbit serum was provided by Grand Ronde Rabbitry, Grand Ronde, OR. The Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, obtained from Gibco, Grand Island, NY, also contained 0.5% (w/v) bovine serum albumin; insulin, 0.055 mg/dl; penicillin, 7.0 mg/dl; and streptomycin, 11.0 mg/dl. Ham's F-12 medium, prepared by Microbiological Associates, Los Angeles, CA was supplemented to contain 20% (v/v) lipoprotein-deficient rabbit serum, insulin, 0.055 mg/dl; penicillin, 7.0 mg/dl; and streptomycin, 11.0 mg/dl. Collagenase (Type II) and hyaluronidase were purchased from the Sigma Chemical Co., St. Louis, MO. Silica gels G & H (E. Merck, Darmstadt) were obtained from Brinkman Instruments, Burlingame, CA. Celite 545 was obtained from Fisher Scientific, Fair Lawn, NJ.

Cell culture

Rabbit hepatocytes were isolated and cultured by a method we have described elsewhere (11). This method is a modified version of one described by Bonney for rat hepatocytes (12). Rabbit livers were continuously perfused at 37°C with 100 ml of oxygenated balanced salt solution (supplemented as indicated above) containing 50 mg of collagenase and 80 mg of hyaluronidase. After the liver had been perfused for 15 to 30 min with oxygenated Hanks' BSS, the cells were combed away from the remaining connective tissue, filtered through nylon gauze (253 and 64 μm mesh), and washed twice with warm, oxygenated Hanks' BSS. Cell viability was determined by a trypan blue exclusion test, and preparations in which less than 90% of the cells excluded trypan blue were not used. Cells (6×10^6) were plated in 60-mm tissue culture dishes (Falcon) containing 3 ml of medium supplemented with 20% (v/v) lipoprotein-deficient rabbit serum. The medium, with any unattached cells, was removed after 20 hr and 3 ml of fresh medium were added. All experiments were carried out during the subsequent 48 hr.

Preparation of lipoprotein and lipoprotein-deficient serum

Lipoproteins were isolated from 180 ml of fresh, pooled rabbit serum by sequential ultracentrifugation (13). The following density ranges were used: LDL, 1.019 g/ml < d < 1.063 g/ml; HDL_2, 1.063 g/ml < d < 1.125 g/ml; HDL, 1.063 < d< 1.21 g/ml; and lipoprotein-deficient serum, 1.25 g/ml < d. The LDL from each 180-ml serum pool was mixed with equal volumes of KBr solution of d = 1.063 g/ml and concentrated by a second ultracentrifugation. The lipoproteins and lipoprotein-deficient serum were exhaustively dialyzed against isotonic saline before use. This lipoprotein-deficient rabbit serum has been shown to contain only 4–5% of the original HDL and no detectable LDL (11). The level of lecithin:cholesterol acyltransferase (LCAT) activity was very low in our lipoprotein-deficient serum (1 ml had a maximum activity of 0.9 μg of cholesterol esterified/hr with LDL as substrate and 0.5 μg/hr with HDL_2 as substrate). This is in contrast to the studies of cholesterol metabolism by hepatocytes by Ray et al. (8) in which the effect of LCAT modification of lipoproteins was studied. The concentrations of apolipoprotein E were 1.9% of LDL protein and 2.2% of HDL_2 protein.

Labeling of lipoprotein protein and cholesterol

^{3}H-labeled LDL and ^{3}H-labeled HDL were prepared by injecting 15 mCi of L-[4,5^{3}H]-leucine into the marginal ear vein of a 4-kg rabbit in three 5-mCi doses at 0, 30, and 60 min. Each 5-mCi dose contained 2% (v/v) ethanol in a total volume of 5 ml of sterile saline. Three hours after the final injection, the animal was exsanguinated via a catheter placed in the femoral artery. In order to obtain as much blood as possible, a total of 65 ml of saline was infused as 150 ml of blood was withdrawn. ^{3}H-labeled LDL and ^{3}H-labeled HDL were isolated from the serum by sequential ultracentrifugation and diluted with unlabeled lipoproteins to specific activities of 1320 dpm/μg of protein for LDL and 192 dpm/μg of protein for HDL. ^{125}I-labeled lipoproteins were prepared by labeling isolated LDL and HDL with ^{125}I by the method of McFarlane (14) as modified by Fidge and Poulis (15). Iodinated lipoproteins were purified by passing the preparation through a Sephadex G-50 column and by dialysis as described elsewhere (11), and they were diluted to the desired specific activity with unlabeled lipoproteins. The specific activities of three preparations of lipoproteins were LDL: 220, 7500, and 2000 cpm/μg of protein; HDL: 169, 2800, and 550 cpm/μg of protein. Eleven percent of the LDL and 3% of the HDL radioactivity was in the lipid portion. For each mole of LDL particles, there was 0.04 mole of ^{125}I and for each

mole of HDL particles there was 0.01 mole of ^{125}I. In the experiments in which both the lipoprotein protein and cholesterol were labeled, ^{125}I-labeled lipoproteins were incubated with [4-^{14}C]cholesterol dispersed on acid-washed Celite 545 for 4 hr at 37°C with shaking (16). Sixty micrograms of cholesterol containing 5 μCi of [4-^{14}C]cholesterol were repurified by thin-layer chromatography on silica gel G plates developed in cyclohexane–ethyl acetate 75:25. The cholesterol was then eluted with chloroform–methanol 2:1 (v/v). The solution was concentrated and added to 20 mg of acid-washed Celite 545. After the chloroform had been allowed to evaporate for 30 min in a hood, the lipoprotein preparation was added and incubated for 4 hr at 37°C. The Celite was then removed by centrifugation and washed with buffered isotonic saline (pH 7.4). The [4-^{14}C]cholesterol-labeled lipoproteins were diluted to the desired specific activity with unlabeled lipoproteins. The specific activities for the two preparations of lipoproteins used were 130 and 568 dpm ^{14}C/μg of free cholesterol for LDL and 424 and 2790 dpm ^{14}C/μg of free cholesterol for HDL. In experiments in which only the lipoprotein cholesterol was labeled, the isolated lipoproteins were incubated as desired with either 5 μCi of [4-^{14}C]cholesterol or 100 μCi of [7(n)-^{3}H]cholesterol dispersed on 20 mg of acid-washed Celite or on 1 cm^2 of acid-washed Whatman #1 filter paper. The range of specific activity for the five preparations of [4-^{14}C]cholesterol-labeled lipoproteins was 79–516 dpm ^{14}C/μg of free cholesterol for LDL and 919–3238 dpm ^{14}C/μg of free cholesterol for HDL or HDL_2. The specific activities of two preparations of [7(n)-^{3}H]cholesterol-labeled lipoproteins were 1550 and 3210 dpm/μg of free cholesterol for LDL and 21590 and 25170 dpm/μg of free cholesterol for HDL_2.

When the lipoprotein lipids were examined by thin-layer chromatography, more than 99% of the label was recovered in the free cholesterol region and less than 0.1% was recovered in cholesteryl ester. The recovery of radioactivity in the appropriate density ranges after recentrifugation of the labeled lipoproteins was 88.8% for HDL and 83.2% for LDL. Agarose gel electrophoresis of LDL and HDL showed no alteration in mobility or appearance of extraneous bands as a result of the labeling procedures. The free cholesterol/protein and cholesterol ester/protein ratios of the lipoproteins used are given in **Table 1.** The composition of the preparations that contained [4-^{14}C]cholesterol was not significantly different from that of native lipoproteins. All lipoproteins were used within 2 weeks of the collection of serum and within 3 days of isotopic labeling.

Analytical techniques

Protein was determined by the method of Lowry et al. (17). Cells and isolated lipoproteins were extracted twice with chloroform–methanol 1:1 (v/v) according to the method of Bligh and Dyer (18). The protein was removed by centrifugation, and the solvent was separated into chloroform and methanol–water fractions by adding an additional volume of chloroform and enough water to form two phases. Free cholesterol and cholesteryl ester concentrations were determined on measured aliquots of the chloroform fraction after thin-layer chromatography on silica gel H (19, 20). Electrophoresis was performed on 1.0% agarose gel in 0.05 M barbital with 0.001 M EDTA (pH 8.6) (21). Radioactivity was measured in a Packard 526 scintillation counter after 1 ml of water and 10 ml of mixture 950A (New England Nuclear Corp., Boston, MA) were added to the sample, or by direct assay of ^{125}I in a Searle 1185 gamma counter.

Processing of cells and media containing only labeled cholesterol

In order to determine the uptake of cholesterol by hepatocytes, we removed the medium from each dish and washed the cells twice with phosphate-buffered saline (pH 7.4) containing 0.2% bovine serum albumin and once with phosphate-buffered saline without added albumin. Then 0.5 ml of water was

TABLE 1. Composition of rabbit lipoproteins

	$\frac{\mu g \text{ Free Cholesterol}}{\mu g \text{ Protein}}$	$\frac{\mu g \text{ Esterified Cholesterol}}{\mu g \text{ Protein}}$	$\frac{\text{Esterified Cholesterol}}{\text{Total Cholesterol \%}}$
LDL			
Native	0.445 ± 0.065	0.746 ± 0.102	63.8 ± 2.4
Labeled	0.408 ± 0.040	0.663 ± 0.140	59.8 ± 4.2
HDL_2			
Native	0.117 ± 0.012	0.192 ± 0.013	62.0 ± 1.6
Labeled	0.146 ± 0.027	0.203 ± 0.037	59.1 ± 1.9

Values are the means ± standard errors for six preparations of rabbit lipoproteins before and after labeling with [7(n)-^{3}H]cholesterol or [4-^{14}C]cholesterol as described in the text.

added to the dish, and the cells were scraped from the dish with a rubber policeman. The lysed cells and water were transferred to test tubes, and the dish was rinsed with an additional 0.5 ml of water which was combined with the cells. The cells were frozen and thawed twice to ensure complete cell disruption. Lipid was removed from the lysed cells by precipitating the cellular protein with chloroform–methanol 1:1 (v/v) in conical tubes. The precipitate was removed by centrifugation and washed with an additional aliquot of chloroform–methanol. The quantity of [4-^{14}C]-cholesterol was determined on dried aliquots of the chloroform fractions of the chloroform–methanol–water extracts. The extracted protein precipitates were dried, digested in 0.1 N NaOH, and the protein contents were determined by the method of Lowry et al. (17).

Simultaneous measurements of the movement of free cholesterol to and from cells were made by first incubating plated hepatocytes in medium containing 20% (v/v) lipoprotein-deficient rabbit serum and enough [4-^{14}C]cholesterol-labeled HDL_2 (specific activity of 6270 or 7800 dpm ^{14}C/μg free cholesterol) to give a free cholesterol concentration of 1.8 μg/ml. After 3 hr, the labeling medium was removed and the cells were washed three times with unsupplemented medium. The cells were then incubated with supplemented medium containing [7(n)-^{3}H]cholesterol labeled LDL or HDL_2. After this incubation, the cells and medium were processed as described above.

Processing of cells and media containing labeled apolipoproteins

In one experiment, cellular uptake of ^{3}H-labeled apoprotein and ^{125}I-labeled apoprotein was compared in parallel series. In other experiments, lipoproteins containing ^{125}I-labeled apoprotein and [4-^{14}C]cholesterol were studied. The cells were washed three times with phosphate-buffered saline (pH 7.4) and incubated with trypsin for 5 min according to the method of Bierman, Stein, and Stein (22) to release surface-bound protein. The trypsin solution was removed by centrifugation and the cells were washed once with phosphate-buffered saline. The cells were lysed by freezing and thawing in water and an aliquot was taken for protein determination. In the experiments in which the lipoproteins contained both cholesterol and protein labels, the trypsin supernatants and the lysed cells were extracted twice with chloroform–methanol 1:1 (v/v) in order to separate the lipid-associated from the protein-associated radioactivity. The protein precipitates were isolated by centrifugation and hydrolyzed with 10 N NaOH, and the alkaline hydrolyzates were neutralized to the colorless phenolphthalein endpoint with concentrated HCl. The protein-associated radioactivity was determined from the ^{125}I in the neutralized protein hydrolyzates plus that in the methanol–water fraction of the chloroform–methanol extract. (The ^{125}I in the methanol–water fractions was assumed to represent proteolysis by cellular enzymes; that in the tryptic digest resulted from proteolysis by trypsin. Cells incubated with Na ^{125}I and analyzed by the above procedure did not take up radioactivity.) The cholesterol-associated radioactivity was determined from the ^{14}C in the chloroform fraction isolated from the chloroform–methanol extract. The ^{14}C windows were set to exclude interference from ^{125}I, and quenching was determined by reassay of the samples after addition of an internal standard. Degraded apolipoprotein in the incubation medium was determined by adding 50% trichloroacetic acid to give a final concentration of 10%. The mixture was heated to 100°C for 10 min, the precipitate was removed by centrifugation, and an aliquot of the trichloroacetic acid-soluble fraction was taken for direct radioassay in the gamma or beta counter. When the protein had been labeled with ^{125}I, another aliquot was treated with hydrogen peroxide and extracted with chloroform as described by Bierman et al. (22). This extraction removes free iodide from the acid-soluble fraction, but does not remove tyrosyl-iodide. This allows differentiation of tyrosyl-iodide derived from lipoprotein proteolysis from free iodide derived from intact apolipoprotein or tyrosyl-iodide by liver deiodinases.

Hepatocytes were labeled with endogenously synthesized [^{3}H]cholesterol by plating them in medium supplemented with 0.043 mM DL-[2-^{3}H]mevalonic acid lactone (155 mCi/mmole). The cells were incubated for 20 hr in this medium, and the medium and any unattached cells were discarded. The cells were then rinsed with 1 ml of unsupplemented medium, and 3 ml of fresh medium supplemented with [4-^{14}C]-cholesterol-labeled LDL or HDL_2 was added per dish. After a 3-hr incubation at 37° the cells were washed, incubated with trypsin, and analyzed as described above.

RESULTS

Uptake of ^{125}I-labeled apoproteins and free [^{14}C]cholesterol from LDL and HDL

The uptake of ^{125}I-labeled apoprotein and free [4-^{14}C]cholesterol from LDL and HDL was compared in two preparations of hepatocytes. After incubation in medium supplemented with 20% (v/v) lipoprotein-deficient serum plus labeled lipoproteins, the

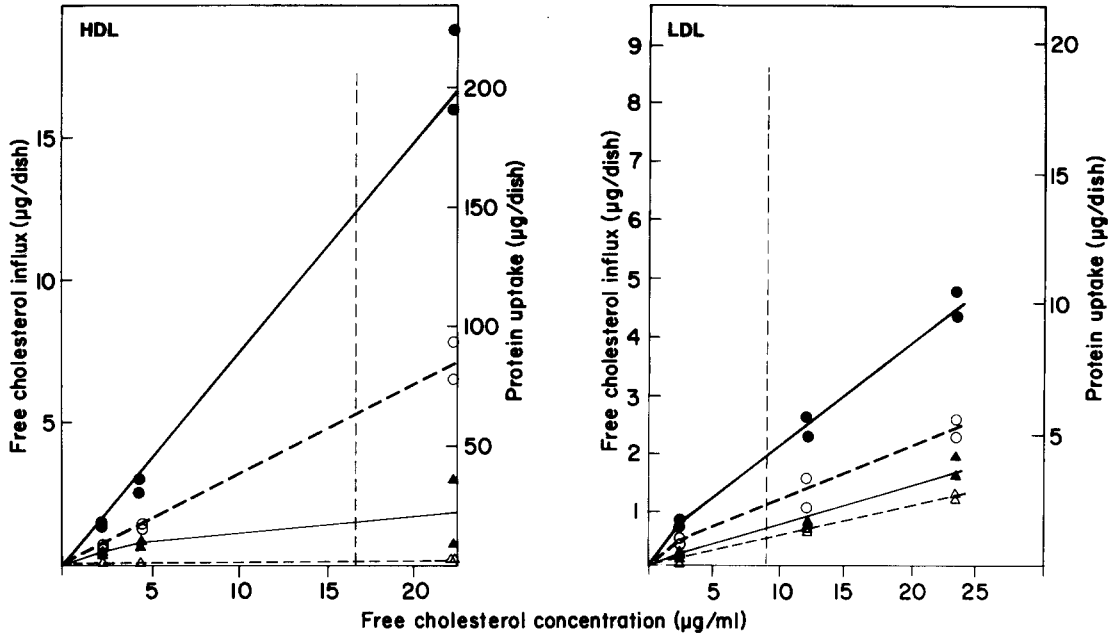

Fig. 1. Comparison of the uptake of free cholesterol and apoprotein from LDL and HDL by hepatocytes. The total cellular uptake of ^{125}I-labeled apoprotein (▲——▲) and free [^{14}C]cholesterol (●——●) from LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) during a 3-hr incubation are shown above. The quantities of protein (△-----△) and cholesterol (○ – – – ○) released by a 5-min incubation with trypsin are also shown. The values shown are for individual tissue culture dishes from one hepatocyte preparation. Uptake of lipoproteins varied between two hepatocyte preparations but the ratios of free cholesterol influx to protein uptake were similar. The total cellular uptake of apoprotein was determined from the sum of the internalized apoprotein plus the trypsin-releasable apoprotein plus the increase in degraded apoprotein in the medium. The ordinants for free cholesterol influx and protein uptake have been arranged to reflect their ratio in the lipoprotein particles; thus uptake of intact lipoprotein particles with no independent free cholesterol exchange would result in the superimposition of the free cholesterol influx and protein uptake lines.

hepatocytes were treated with trypsin to release apoprotein bound to the cell surfaces (22). The total cellular uptake of [4-^{14}C]cholesterol and ^{125}I-labeled apolipoprotein in one of the preparations of hepatocytes after a 3-hr incubation is illustrated in **Fig. 1** (solid lines). The total cellular uptake of apoprotein shown in Fig. 1 includes the degraded apoprotein (^{125}I-labeled tyrosine) released into the medium. A lipoprotein concentration equal to 25% of the level in normal rabbit serum (the mean values for normal serum are 80 µg LDL protein/ml, 800 µg HDL protein/ml) is indicated on the graphs to facilitate comparison of the uptake of LDL and HDL free cholesterol and protein. Fig. 1 shows that at concentrations equivalent to 25% of the normal serum levels, the hepatocytes took up 12.2 µg of free cholesterol and 18.1 µg of apoprotein/tissue culture dish from HDL and only 1.9 µg of free cholesterol and 2.0 µg apoprotein/tissue culture dish from LDL. This is equivalent to 18.5% of the free cholesterol and 2.3% of the apoprotein from HDL and to 5.3% of the free cholesterol and 2.4% of the apoprotein from LDL. Thus at a physiological LDL/HDL ratio, the fraction of apolipoprotein taken up by hepatocytes is almost equivalent for the two classes of lipoproteins, whereas the fraction of free cholesterol taken up from HDL is greater than that from LDL.

Release of free cholesterol and cholesteryl ester from hepatocytes by incubation with trypsin

We examined the proportion of the total cellular uptake of cholesterol and apoprotein released by a 5-min incubation with trypsin since trypsin treatment is a method frequently used to release protein bound to cell surfaces. As indicated in Fig. 1 (broken lines), trypsin-treatment also causes the release of a large amount of cholesterol. The ratio of [4-^{14}C]cholesterol/^{125}I-labeled apoprotein released by incubation with trypsin is too large to be accounted for simply by the release of intact lipoproteins bound to the cell surface. The ratio of trypsin-released cholesterol/apoprotein was 47 times the ratio of free cholesterol to HDL apoprotein and 2.5 times the ratio of free cholesterol to LDL apoprotein in the lipoproteins that were added to the medium.

In order to determine whether the trypsin-releasable cholesterol represented a special pool of membrane cholesterol which participates in exchange with medium lipoproteins, we labeled cells with endogenously synthesized [^{3}H]cholesterol by incubating them for 20

hr with [2-^{3}H]mevalonic acid lactone. The cells were then washed and incubated with unsupplemented medium or medium supplemented with [4-^{14}C]-cholesterol-labeled LDL (6.8 μg free cholesterol/ml) or HDL_2 (8.9 μg free cholesterol/ml). The cells were then washed again and treated with trypsin. The percentages of cholesterol and protein released are given in **Table 2.** A somewhat larger proportion of the [^{14}C]cholesterol was released by trypsin in this experiment than in the two described above (71.4% of LDL-derived cholesterol and 66.7% of HDL_2-derived cholesterol compared with an average of 57.5% for LDL and 55.0% for HDL in the two previous experiments); nevertheless, cells treated in this way have appeared intact when examined (320 × magnification) after trypsin treatment and have excluded trypan blue. No cellular debris was apparent when the trypsin digests were examined by microscopy.

Table 2 shows that the proportions of lipoprotein-derived (^{14}C-labeled)-cholesterol and total cellular free cholesterol released by trypsin are not significantly different. This indicates that lipoprotein-derived free cholesterol is not located in a special pool that is particularly susceptible to trypsin-release. Table 2 also shows that a significant proportion of endogenously synthesized (^{3}H-labeled)-free cholesterol and cholesteryl ester are released by trypsin treatment. This proportion is lower than the proportion of total cholesterol released, indicating that some endogenously synthesized cholesterol is in a pool (e.g., in the endoplasmic reticulum or in other intracellular organelles) that is protected from the action of trypsin. It is interesting that, although only about 10% of the [^{3}H]cholesterol was esterified, trypsin released equal proportions of free and esterified [^{3}H]cholesterol. It thus appears that incubation with trypsin releases some cholesterol which has become part of the hepatocyte membrane in addition to the cholesterol bound to lipoprotein receptors as part of intact lipoproteins. Therefore, in our calculation of cholesterol influx to hepatocytes, we used the total of the cholesterol recovered in the trypsin supernatants plus that recovered in the cell pellet.

Comparison of degradation of [^{3}H]leucine labeled lipoproteins and ^{125}I-labeled lipoproteins

Our calculations of proteolytic activity were based on the appearance of ^{125}I-labeled tyrosine in the incubation medium (corrected for no-cell controls). We assumed that the dramatic increase in free ^{125}I that occurs in the medium when ^{125}I-labeled lipoproteins are incubated with rabbit hepatocytes is due largely to a deiodination of ^{125}I-labeled lipoproteins and only minimally due to the deiodination of free ^{125}I-labeled tyrosine. In order to test this assumption about the origin of the free ^{125}I and to determine the overall validity of using ^{125}I-labeled lipoproteins in our studies, we compared the rates of degradation of [^{3}H]leucine-labeled LDL and HDL (prepared in vivo in donor rabbits) with the rates of degradation of ^{125}I-labeled LDL and ^{125}I-labeled HDL in one preparation of hepatocytes. Because of the low specific activities of the [^{3}H]leucine-labeled lipoproteins, we were not able to make the comparison with low levels of lipoproteins in the medium. The results are shown in **Table 3.** The amount of ^{125}I-labeled LDL degradation calculated from the total increase in ^{125}I-labeled tyrosine plus free ^{125}I in the medium was much greater than the amount of [^{3}H]leucine-labeled LDL apoprotein catabolized, while the ^{125}I-labeled LDL degradation based on the increase in medium ^{125}I-labeled tyrosine agrees quite closely with the results obtained with [^{3}H]leucine-LDL. Thus, it appears that very little of the ^{125}I-labeled tyrosine produced by LDL degradation is deiodinated by rabbit hepatocytes. The difference in rates of formation of [^{3}H]leucine and free ^{125}I by hepatocytes cannot be explained by cellular reutilization of the free [^{3}H]leucine as the amount of cell-associated ^{3}H-labeled lipoprotein (calculated from the retained label) is actually somewhat lower than retained ^{125}I-labeled lipoprotein. Therefore, the increase in free ^{125}I in the medium resulted from deiodination of intact lipoproteins and should not be included in the calculation of LDL metabolism. In the case of HDL, the results are not quite so clear-cut. Only one level of HDL was in the range used for the other experiments

TABLE 2. Free cholesterol, cholesteryl ester, and protein released from hepatocytes by trypsin treatment

Incubation Medium	% Free Cholesterol Mass Released	% [^{14}C]Cholesterol Released	% [^{3}H]Cholesterol Released	% [^{3}H]Cholesteryl Ester Released	% Cell Protein Released
20% LDS	77.7 ± 4.0		50.3 ± 2.0	51.9 ± 4.2	77.4 ± 4.6
20% LDS + LDL	75.2 ± 1.5	71.4 ± 2.5	53.5 ± 2.2	50.6 ± 3.1	67.8 ± 6.3
20% LDS + HDL_2	80.9 ± 4.8	66.7 ± 3.0	48.9 ± 2.4	40.0 ± 1.8	78.7 ± 4.7

Monolayers of rabbit hepatocytes were labeled with endogenously synthesized [^{3}H]cholesterol by an incubation with [2-^{3}H]mevalonic acid lactone. The cells were then transferred to medium containing [4-^{14}C]cholesterol-labeled LDL or HDL_2. At the end of 3 hr, the cells were incubated with trypsin for 5 min. The values given above are the means ± S.E. for five dishes.

TABLE 3. Comparison of ^{3}H- and ^{125}I-labeled apoprotein catabolism by monolayers of hepatocytes over a 24-hr incubation

Medium Lipoprotein Concentration		Degradation			Cell-associated Lipoprotein	
		^{3}H-Leucine	A ^{125}I-Tyrosine plus ^{125}I	B ^{125}I-Tyrosine	^{3}H-Leucine	^{125}I-Tyrosine
pmoles/ml	*μg protein/ml*	*ng/mg cell protein*			*ng/mg*	
LDL						
20	15	200	9800	430	799	1833
40	30	770	15400	950	1129	2794
120	90	1620	31100	1360	2015	5841
200	150	2070	68500	3140	2171	7417
400	300	2900		2870	3244	11210
HDL						
2000	300	14000	55200	9720	7843	8692

Values are the averages of duplicate tissue culture dishes expressed as ng apoprotein catabolized/mg cell protein. Degraded [^{3}H]leucine-labeled apoprotein was calculated from the appearance of ^{3}H dpm in the acid-soluble fraction of the medium. Degraded ^{125}I-labeled apoprotein was calculated by two methods: method A used the increase in ^{125}I cpm in the acid-soluble fraction of the medium (this measures free ^{125}I plus ^{125}I-labeled tyrosine); method B used the increase in ^{125}I cpm in the acid-soluble fraction of the medium after extraction of free ^{125}I by the method of Bierman et al. (22) (this measures only ^{125}I-labeled tyrosine).

in this study. The amount of ^{125}I-labeled HDL degradation calculated from the total acid-soluble radioactivity was almost four times greater than the degradation measured by the release of [^{3}H]leucine; however, the HDL degradation calculated from ^{125}I-labeled tyrosine formed was only 70% of the HDL degradation calculated from the TCA-soluble [^{3}H]-leucine formed. It is possible that some of the ^{125}I-labeled tyrosine produced by degradation of ^{125}I-labeled HDL was deiodinated by hepatocytes, and that degradation calculated from the increase in medium ^{125}I-labeled tyrosine alone would result in an underestimation of the HDL apoprotein catabolism. However, this slight underestimation was deemed preferable, for subsequent calculations of lipoprotein degradation, to the large overestimation of catabolism that would result from including the increase in free ^{125}I in the incubation medium.

Comparison of free cholesterol influx from LDL and HDL to hepatocytes

The free [4-^{14}C]cholesterol influx from LDL and HDL to hepatocytes was examined more closely by considering four different periods of incubation in **Fig. 2.** The results were derived by normalizing values from two hepatocyte preparations and are graphed as a function of the concentration of free cholesterol in the medium. There is a significantly higher influx of free cholesterol from HDL than from LDL to hepatocytes after 30 min or 3 hr of incubation. The differences after 24 hr were no longer statistically significant.

In order to determine whether the difference between the influx of free cholesterol from HDL and LDL to hepatocytes was due to the HDL_2 ($1.063 < d < 1.125$ g/ml) subfraction, we incubated hepatocytes with [4-^{14}C]cholesterol-labeled HDL_2 and LDL. Since we had determined from the experiments described above that trypsin-treatment causes a loss of cholesterol beyond that which can be attributed to release of bound lipoprotein particles, the hepatocytes used in these experiments were harvested with a rubber policeman rather than by incubation with trypsin. The influx of [4-^{14}C]cholesterol from HDL_2 or LDL carrier to five preparations of hepatocytes is summarized in **Fig. 3.** The influx of free cholesterol to hepatocytes from HDL_2 at 25.2 μg free cholesterol/ml medium, is significantly greater ($P < 0.005$) than the influx from LDL at 24.7 μg free cholesterol/ml medium. (This concentration is equivalent to 22% of the HDL_2 present in normal rabbit serum and 69% of the LDL present in normal rabbit serum.) The smaller differences seen at lower concentrations of free cholesterol are not statistically significant. Preliminary experiments (values not shown) indicate that the influx of free cholesterol to hepatocytes from HDL_3 is also greater than the influx from LDL.

Comparison of the efflux of free cholesterol from hepatocytes to lipoproteins

We compared the efflux of free cholesterol from hepatocytes to lipoproteins using two different methods of labeling cellular cholesterol. In one experiment, hepatocytes were labeled with [4-^{14}C]cholesterol

by a 3-hr incubation with [4-^{14}C]cholesterol-labeled HDL_2. The efflux of the lipoprotein-derived [4-^{14}C]-cholesterol from hepatocytes to medium LDL or HDL_2 was then measured in a subsequent incubation. In a second experiment, we labeled hepatocytes with endogenously synthesized [^{3}H]cholesterol by incubating them for 20 hr with [2-^{3}H]mevalonic acid lactone, after which we measured the efflux of endogenously synthesized [^{3}H]cholesterol to LDL and HDL_2. The results of both experiments are summarized in **Table 4.** The percentage of cellular cholesterol recovered in the medium after a 3-hr incubation is greater if the medium contains HDL_2 than if it contains LDL, and both LDL and HDL_2 promote a greater efflux of cellular cholesterol than does incubation with medium alone. The mass of the efflux of cellular cholesterol to lipoproteins is calculated by assuming a uniform specific activity of cellular cholesterol. This is an estimate of the maximum efflux of lipoprotein-derived cellular cholesterol (Experiment A), as the specific activity of cholesterol participating in exchange may be higher than that in the interior of the cell. It is an estimate of the minimum efflux of endogenously synthesized cholesterol (Experiment B), where the specific activity of the cholesterol in the interior of the cell may exceed the specific activity of cholesterol on the cell surface. We can see from Table 4 that both endogenously synthesized free cholesterol and exogenously derived free cholesterol participate in exchange with LDL and HDL_2. The rates of efflux of endogenous free cholesterol from hepatocytes to LDL and HDL_2 are similar to the rates of influx in Experiment B. In Experiment A the rate of efflux of lipoprotein-derived cholesterol to HDL_2 is lower than the rate of influx, whereas for LDL the two rates are nearly equal.

Effects of incubation of hepatocytes on their composition

Despite the observed differences in the rates of free cholesterol influx and efflux from HDL_2 to rabbit hepatocytes in Experiment B, we have not seen a change in the free cholesterol/protein composition of hepatocytes as a result of incubation with LDL or HDL_2.

Hepatocytes incubated for 20 hr in medium supplemented with 20% lipoprotein-deficient serum plus lipoproteins at levels equivalent to 20% of normal rabbit serum levels had free cholesterol/protein ratios of 37.3 ± 3.3 μg/mg (total lipoproteins), 34.3 ± 2.1 μg/mg (LDL only), 37.9 ± 2.2 μg/mg (HDL_2 only), or 38.6 ± 3.8 μg/mg (no lipoproteins). When the dishes incubated for 20 hr without lipoproteins were transferred to new medium for 3 hr, the free cholesterol/protein ratios were 33.8 ± 3.2 μg/mg (total lipoprotein), 33.5 ± 2.1 μg/mg (LDL), 28.7 ± 5.2 μg/mg (HDL_2) or 34.6 ± 3.1 μg/mg (no lipoproteins). These values are the mean ± S.E. of ten tissue culture dishes

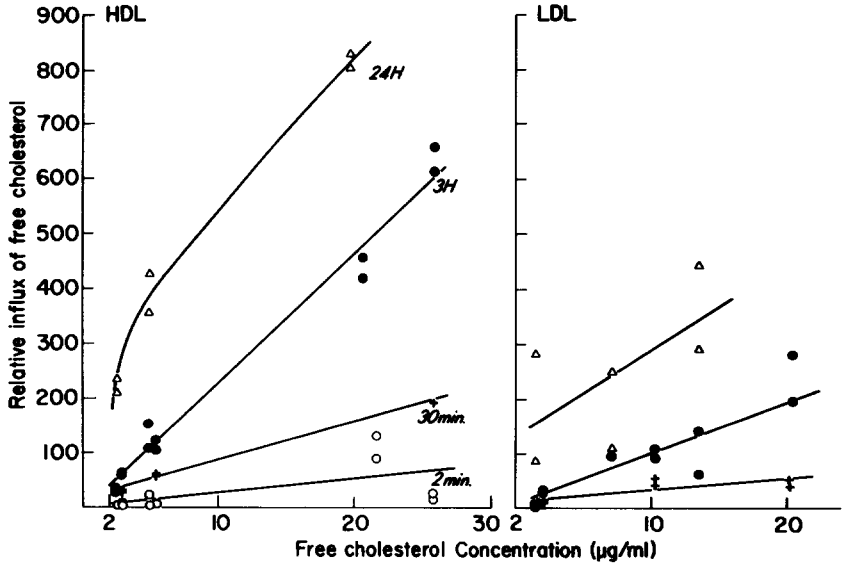

Fig. 2. Comparison of total influx of cholesterol from LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) to hepatocytes at 2 min (○), 30 min (+), 3 hr (●), and 24 hr (△). Values were derived by normalizing the results obtained with two hepatocyte preparations. The percentage of free cholesterol influx from LDL at a free cholesterol content of 10 μg/ml at 3 hr was set equal to 100. Values for LDL influx at 2 min were similar to those for LDL influx at 30 min and are not indicated on the graph. The following differences between the slopes of the HDL lines and the LDL lines are significant: 30-min incubation ($P < 0.001$) and 3-hr incubation ($P < 0.001$).

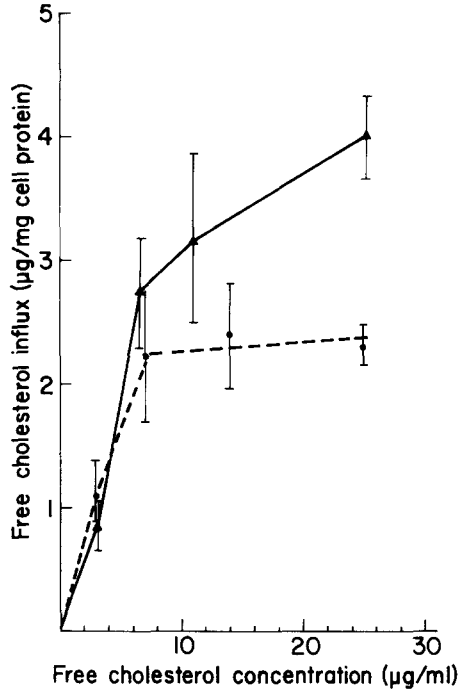

Fig. 3. Comparison of the influx of free cholesterol (to a monolayer of hepatocytes after a 3-hr incubation) from LDL (1.019–1.063 g/ml) and HDL_2 (1.063–1.125) as a function of concentration of free cholesterol in the medium. Points shown are the mean ± standard error of five to nine tissue culture plates from five hepatocyte preparations. Symbols: ●----●, LDL; ▲——▲, HDL_2. The differences in influx from LDL and HDL_2 were not statistically significant except at the 24.7 and 25.2 μg/ml concentrations ($P < 0.005$).

from one hepatocyte preparation and differences between cells incubated in different media are not statistically significant. The lack of statistically significant differences in free cholesterol content of hepatocytes incubated with LDL, HDL_2, total lipoproteins or without lipoproteins was confirmed in another similar experiment.

DISCUSSION

Previous studies in our laboratory (11) demonstrated the induction of high affinity LDL receptors after rabbit hepatocytes were incubated in lipoprotein-deficient medium. This resulted in increased binding, internalization, and degradation of LDL protein by hepatocytes. The total rate of catabolism of LDL protein was lower than that observed in other cell types, and the induction of high-affinity LDL receptors was completely inhibited at concentrations of LDL reported for liver lymph (23). Hepatocytes had a lower affinity but a higher capacity for binding HDL than for LDL, but ^{125}I-labeled HDL binding was only partly inhibited by physiological levels of unlabeled HDL and not at all by LDL (24). The selective influx of free cholesterol compared to the uptake of apoprotein from medium lipoproteins seen in this study (Fig. 1) indicates that in hepatocytes, even under conditions that induce high-affinity LDL and HDL receptors, the primary mechanism for cellular influx of cholesterol from either LDL or HDL is not the uptake of whole lipoprotein particles. Further, this study shows that free cholesterol exchange with hepatocytes is more rapid with HDL_2 than with LDL (Fig. 2). Wu and Bailey (10) have observed that there is a similar selective influx of HDL free cholesterol over protein uptake in fibroblasts, while free cholesterol influx from LDL parallels protein uptake. Goldstein, Helgeson, and Brown (4) found that free cholesterol influx from LDL does exceed receptor-mediated uptake in Chinese hamster ovary cells, but that receptor-mediated uptake of LDL is required to supply cholesterol required for growth in compactin-treated cells. They did not examine the rate of free cholesterol exchange in compactin-treated cells (4). HDL has also been shown to stimulate the efflux of cellular cholesterol from endothelial cells while LDL had only a slight effect on cellular cholesterol efflux (25).

The rate of labeled free cholesterol flux into cells from lipoproteins occurs partly via an exchange in which a molecule transferred from a lipoprotein to the cellular plasma membrane is balanced by the transfer of another molecule in the opposite direction (2, 3, 7). If this transfer is dependent on contact between lipoproteins and cell membranes, then HDL may be more efficient than LDL in exchanging free cholesterol with cell membranes because of their much smaller size. HDL particles are approximately one-tenth the mass of LDL particles and they carry approximately one-twentieth as much free cholesterol per particle as LDL particles do. Thus when HDL is compared with LDL at equal concentrations of lipoprotein-bound free cholesterol, the free cholesterol carried by HDL is distributed into twenty times as many lipoprotein particles as the free cholesterol carried by LDL. Free cholesterol carried by HDL is thus more likely to come into contact with cell membranes and to be exchanged with them.

There are situations in which the net transfer of free cholesterol occurs, probably as a result of a shift in the equilibrium of the exchange reaction so that influx exceeds efflux or vice versa. For example, Stein, Vanderhoek, and Stein (26) have recently characterized certain plasma factors that promote a net removal of cholesterol from skin fibroblasts. Rothblatt, Arbogast, and Ray (7) and Ray et al. (8) have induced changes in the free cholesterol content of hepatomas by changes in the free cholesterol/esterified cholesterol ratio in HDL in the medium. Although we observed no changes in free cholesterol content of hepatocytes in these experiments, it is possible that a net transfer

TABLE 4. Efflux of cellular cholesterol from hepatocytes to LDL and HDL_2 and the simultaneous influx from lipoproteins to cells during a 3-hr incubation

	LP Concentration $\frac{\mu g \text{ Free Cholesterol}}{ml \text{ Medium}}$	% Efflux of Cellular Free Cholesterol	Efflux of Free Cholesterol to LP	Influx of LP Cholesterol to Cells
			ng/dish	*ng/dish*
Experiment A				
No LP	0	35.9		
LDL	1.95	38.6	136	135
	2.87	37.2	101	178
	6.03	40.8	226	262
	9.85	41.2	242	286
HDL_2	2.18	40.6	217	389
	3.73	40.6	217	612
	7.96	45.2	428	860
	12.45	42.6	306	1370
Experiment B				
No LP	0	17.7 ± 2.3		
LDL	6.8	27.1 ± 2.3	508	697 ± 80
HDL_2	8.9	39.0 ± 1.6	937	926 ± 36

Monolayers of rabbit hepatocytes were labeled with [4-^{14}C]cholesterol by exchange with labeled HDL_2 (Experiment A) or with endogenously synthesized [^{3}H]cholesterol (Experiment B). The cells were then transferred to medium containing LDL, HDL_2, or no added lipoprotein. Values given below for the efflux and influx of free cholesterol during the second incubation are the average of duplicate tissue culture dishes (Experiment A) or the mean ± S.E. of five tissue cultures dishes (Experiment B). The efflux of free cholesterol to lipoproteins (ng/dish) has been corrected by subtracting the value for efflux to medium alone.

of cholesterol in these cells could be accommodated by cholesterol metabolism, for example by increased production of bile or by secretion of nascent lipoproteins. We therefore chose to evaluate the balance between the rates of influx and efflux of free cholesterol in hepatocytes by prelabeling the cells with cholesterol containing one isotope and then incubating the cells with lipoproteins containing cholesterol with a second isotope. It is difficult to calculate the precise output of hepatocyte cholesterol because the distribution of labeled free cholesterol in subcellular cholesterol pools is not known and it is possible that the specific activity of the freely exchangeable pool of free cholesterol is different from that of free or total cholesterol in the whole cell. To avoid this problem, we measured the efflux of cellular cholesterol labeled both through exogenous exchange or endogenous synthesis, which would not be likely to sequester label in the same intracellular pools. (Table 4). In both cases, rates of cholesterol influx and efflux were higher with HDL than with LDL, confirming that the rate of free exchange was higher. The influx of free cholesterol from LDL equalled efflux in both cases. While efflux of endogenously labeled cholesterol to HDL_2 equalled influx, efflux of cholesterol labeled by previous exchange with HDL_2 was less than influx. This result may be explained by the findings of Schwartz and coworkers (27), who found that plasma-derived cholesterol is preferred to new synthesized cholesterol as a substrate for bile acids in man and that HDL free cholesterol is preferred to LDL free cholesterol (28). Results from our laboratory have confirmed the preferential utilization of HDL free cholesterol for bile acid synthesis in squirrel monkeys (29).

The results obtained by studying hepatocytes in vitro may have been affected by the unique anatomical positioning of cells in culture. We have shown that maintenance of hepatocytes in culture for 20 hr allows attachment of cells, and that much of the cell surface exposed to the medium is covered by microvilli (11).[4] Although we could not identify the different areas of the surface of the hepatocyte in culture by scanning electron microscopy, we assume that the sinusoidal, canalicular, and intervening portions of the plasma membrane may all be exposed to the medium. This is not representative of the interaction between blood and hepatocytes in vivo (30). Care must also be taken in interpreting data on lipoprotein metabolism obtained from isolated parenchymal cells since, as van Berkel et al. (31) have shown, non-parenchymal liver cells appear to metabolize more lipoproteins per unit of cell protein than do hepatocytes.

The work described in this article, publication no. 1203 from the Oregon Regional Primate Research Center, was supported by grants-in-aid from the National Institutes of Health, Animal Resources Branch, RR-00163 and General Research Support Grant RR-05694 (Oregon Regional

[4] Soltys, P. A., J. P. O'Malley, and O. W. Portman. Unpublished observations.

Primate Research Center) and by grant HL-09744 (University of Oregon Health Sciences Center).

Manuscript received 31 October 1980 and in revised form 14 May 1981.

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