Metabolism of apoE-free high density lipoproteins in rat hepatoma cells: evidence for a retroendocytic pathway

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Abstract The cellular metabolism of apoE-free HDL (HDL) was stuided in rat hepatoma cells (FU5AH). Cells incubated with HDL showed a dose-dependent decreased incorporation of [^{14}C]acetate into cell sterol, indicating a net cholesterol delivery to the cells. HDL was localized both at the cell surface and inside the cell. This conclusion was drawn from both the association of ^{125}I-labeled HDL with the cells under different experimental conditions and morphological evidence based on the association of colloidal gold-labeled HDL with the cells. Up to 63% of the ^{125}I-labeled HDL protein initially inside the cell was subsequently recovered in the media as trichloroacetic acid precipitable (TCA-ppt) protein after a 30-min, 37°C chase with a 100-fold concentration of unlabeled HDL. About 27% of the TCA-ppt apoprotein originally inside the cell was recovered as TCA-soluble material. Thus, we conclude that of the HDL apoprotein taken up by the cells, the majority is resecreted by a retroendocytosis pathway. The quantity of HDL apoprotein reappearing in the media was stimulated by the presence of unlabeled HDL in the media, while the amount of TCA-soluble material produced was not. Retroendocytosis of HDL was inhibited at 0°C and by the presence of 10 mM NaCN, 20 mM 2-deoxy-D-glucose in the media. Thus, the pathway appears to be both temperature- and energy-sensitive. HDL resecreted by the cell were depleted of cholesteryl ester and showed an altered size distribution, indicative of lipoprotein catabolism and remodeling. This study provides evidence for the existence of an endocytosis-retroendocytosis pathway for HDL apoproteins in a rat hepatoma cell and for the possibility that the endocytosis- retroendocytosis pathway may be involved in lipid delivery to the cell. —**DeLamatre, J. G., T. G. Sarphie, R. C. Archibold, and C. A. Hornick.** Metabolism of apoE-free high density lipoproteins in rat hepatoma cells: evidence for a retroendocytic pathway. *J. Lipid Res.* 1990. **31:** 191–202.

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High density lipoproteins are thought to act as a vehicle for the transport of cholesterol from extrahepatic tissue to the liver, a process termed reverse cholesterol transport (1). Removal of cholesterol from membranes of extrahepatic tissue cells is thought to occur mainly by efflux of cholesterol from the plasma membrane, through an aqueous phase, and then to an HDL acceptor (2, 3). Work on macrophages has suggested a role for an HDL retroendocytic pathway in the clearance of cholesterol from the cell (4, 5). Three processes have been characterized with respect to delivery of HDL cholesterol to hepatic cells: *1*) lysosomal degradation; *2*) surface transfer of free cholesterol; and *3*) selective delivery of cholesteryl ester (6, 7). In the present study, we sought to determine whether an apoE-free HDL retroendocytic pathway is present in a hepatoma cell (Fu5AH). Since this cell removes cholesterol from HDL, if the pathway is present, it could potentially play a role in the delivery of HDL cholesterol to the cell or otherwise be important in HDL metbolism.

These experiments were basically designed to answer two questions. *1*) What proportion of the HDL apoprotein associated with the cell after an initial incubation is present in the cell interior? *2*) What proportion of the HDL apoprotein from the cell interior is resecreted as undegraded protein? In addition, we have examined the consequences of the retroendocytic pathway on the size distribution of apoE-free HDL. Preliminary reports of these findings have appeared (8, 9).

MATERIALS AND METHODS

Lipoprotein preparation and labeling

Three hundred-gram male Sprague-Dawley rats were bled. The blood was kept on ice, allowed to clot, and se-

Abbreviations: TCA, trichloroacetic acid; HDL, high density lipoproteins; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LCAT, lecithin:cholesterol acyltransferase; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); CE, cholesteryl ester; LDL, low density lipoprotein; CN, DG, 10 mM NaCN–20 mM 2-deoxy-D-glucose; PBS, phosphate-buffered saline.

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rum was separated by centrifugation at 2500 rpm for 20 min. The serum was collected and 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) was added from a 75 mM DTNB, 100 mM Na_2HPO_4, pH 7.4 stock solution to a final concentration of 1.5 mM. High density lipoproteins were isolated by standard ultracentrifugation techniques at d 1.08–1.21 g/ml (10) and dialyzed against 2 mM Na_2HPO_4, 50 mM NaCl, 1 mM EDTA, pH 7.4, buffer. ApoE was removed from HDL by heparin affinity column chromatography (Pharmacia) (11, 12). Analysis of this preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (13) showed that no apoE could be visualized with up to 150 μg total HDL protein in a lane (see Fig. 1). On the same gel it was possible to visualize 0.5 μg albumin. Throughout this report this apoE-free HDL will simply be referred to as HDL and, unless otherwise indicated, HDL mass is reported as protein mass. The HDL was iodinated by the iodine monochloride method of McFarlane (14). This resulted in a specific activity of 200–400 cpm/ng protein. When HDL was analyzed by SDS-PAGE, 90% of the label was present in regions of the gel where HDL apoproteins are found. The remaining 10% of the radioactivity was in the lipid fraction, consistent with values reported for ^{125}I-labeled HDL_3 (15).

Cell culture system

Cells of the rat hepatoma cell line Fu5AH (16, 17) were grown in 20 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS). The media contained 3.7 mg/ml bicarbonate, 50 μU/ml penicillin, and 50 μg/ml streptomycin. The cells were incubated at 37°C in an atmosphere of 95% air–5% CO_2. Cultures were maintained in 75-cm^2 flasks and were split 1/60 every 5 days at which time they were confluent.

Cells were plated in 60-mm tissue culture plates (Corning) 2 days prior to the experiment. On the day before the experiment, the DMEM–5% FBS was removed from the plates, the cells were washed once with DMEM, and reincubated with DMEM-5% FMS. On the day of the experiment and immediately before any of the experimental protocols, the cells were washed 2 × with DMEM-buffered with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). All lipoprotein preparations used contained 2.5 mg albumin/ml (human albumin, fraction V fatty acid-free, United States Biochemical). ApoA-I could not be detected by an ELISA with a lower detection limit of 0.5 ng. These preparations were dialyzed exhaustively against DMEM–HEPES.

Experimental protocol

In general, the experimental protocol consisted of three procedures that were altered according to the question being considered: 1) initial incubation: 1 h, 37°C incubation with 7.5 μg ^{125}I-labeled HDL/ml. For one experiment, a 2-h, 0°C incubation was used. 2) Displacement: 0°C for 2 h in all experiments except one in which the incubation was at 37°C for 30 min. Initially, a series of unlabeled HDL media concentrations was used in the experiment; 150 μg/ml (20 ×), 375 μg/ml (50 ×), 750 μg/ml (100 ×), and 1500 μg/ml (200 ×). After establishing its effectiveness, only the 100 × concentration was used for the competition procedure inmost experiments. 3) Chase: the 1-h 37°C initial incubation was followed by a 2-h, 0°C displacement with 100 × HDL and then a chase incubation for 30 min at 37°C. The chase incubation media contained either 2.5 mg/ml albumin with no HDL or albumin with a series of HDL concentrations.

At the conclusion of any of the procedures outlined above the cells were washed. The washing procedure consisted of: *1*) cooling the cells to 0°C by placing them on ice for 20 min; *2*) collecting the media for analysis; *3*) two 2-ml changes of 2.5 mg albumin/ml in 0°C Dulbecco's phosphate-buffered saline (DPBS); and *4*) two 2-ml changes of 0°C DPBS. For experiments using a 37°C competition, the above wash procedure was done at 37°C. Washing the cells further (5 × and 6 ×) did not significantly decrease the amount of ^{125}I-labeled HDL associated with the cells.

In one set of experiments, the cells were digested with trypsin in order to remove ^{125}I-labeled HDL from the cell surface. After an initial incubation of 7.5 μg ^{125}I-labeled HDL/ml at 37°C for 30 min, the cells were cooled to 0°C, washed at 0°C, and incubated at 0°C with 1 ml of a solution containing 0.5 mg trypsin/ml, 4 mg EDTA/ml in a balanced salt solution without Ca^{2+} or Mg^{2+} (Gibco). The incubation was continued until the cells lifted off the tissue culture dish and were dispersed. At the time an appropriate concentration of soybean tyrpsin inhibitor (Sigma) was added in 0.5 ml of solution to stop proteolytic degradation. The cells were collected and a 200 μl suspension was placed on top of 200 μl dibutyl phthalate in a 500-μl microfuge tube. The cells were then spun in an Eppendorf microcentrifuge for 10 min. The radioactivity of the cell plates was determined by cutting the tips and counting. A 200-μl aliquot of the cell suspension was counted for total radioactivity.

In order to assess the effect of HDL on cell sterol synthesis, cells were incubated overnight in media containing 2.5 albumin and then with a series of HDL concentrations for 18 h at 37°C in DMEM-2.5 mg/ml albumin. At the conclusion of the incubation, the media was removed and the cells were washed. The cells were then incubated in media containing 4 μCi [^{14}C]acetate and 2.5 mg/ml albumin for 2 h at 37°C (18, 19). Cells lipids were extracted by the chloroform–methanol procedure of Bligh and Dyer (20) and were separated by thin-layer chromatography.

Analytical procedures

Cells were harvested using a Teflon cell scraper with three 1-ml volumes of 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 (PBS). Cell protein was determined by the method of Lowry et al. (21).

Total ^{125}I radioactivity was determined in samples that were precipitated with an equal volume (0.5 ml) of 20% trichloroacetic acid (TCA) on ice. The samples were centrifuged at 3000 rpm and 4°C. A 0.5-ml aliquot of the supernatant was removed to determine TCA-soluble radioactivity. Lipid radioactivity was measured in parallel samples that were precipitated by TCA at 0°C, centrifuged, washed with an equal volume of 10% TCA, and recentrifuged. The precipitate was then extracted by the chloroform–methanol procedure as outlined by Bligh and Dyer (20). The chloroform layer was collected, dried in a glass test tube, and counted. Radioactivity in ^{125}I-labeled HDL protein was then calculated by subtracting the lipid radioactivity (cpm) from the TCA-precipitable radioactivity.

Lipoprotein size distribution

Lipoprotein size distributions were determined by electrophoresis of lipoproteins on a 4–30% nondenaturing gradient gel (Pharmacia) using a 90 mM Tris, 80 mM borate, 3 mM EDTA, pH 8.35, buffer. Molecular weight standards were run simultaneously using a Pharmacia high molecular weight electrophoresis calibration kit. The standards included: thyroglobulin (17 nm), ferritin (12 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm).

Gold preparation and lipoprotein conjugation

Monodispersed colloidal gold particles (8–10 nm in diameter) were prepared from a 0.1% aqueous stock of chloroauric acid (J. T. Baker Chemicals) as reported previously (22). Particle sizes were inspected via transmission electron microscopy. Lipoprotein conjugation was accomplished by rapidly mixing 100 μg of freshly prepared apoE-free HDL (pH 5.5–5.7) in 0.05 M EDTA with 5 ml of the colloidal gold suspension followed by the addition of 1% polyethylene glycol (PEG, mol wt 20,000) for stabilization (23). The conjugates were then isolated from unbound lipoproteins by ultracentrifugation (28,000 rpm, 30 min) and suspended in 0.05 M EDTA–NaN_3 (pH 7.4) containing 0.2% PEG and 4% polyvinylpyrrolidone (PVP). Conjugation was then determined ultrastructurally by negative staining with 3% phosphotungstic acid (pH 7.0) on parlodion/carbon-coated, 300-mesh copper grids. Subsequently, the HDL-gold was resuspended in Dulbecco's PBS containing 14 mM glucose, 5% MEM amino acids (Gibco), PEG, and PVP (ph 7.4) and used within 24 h.

Electron microscopy

Cells to be incubated with colloidal gold-labeled HDL were grown on 25-cm^2 tissue culture flasks. The cells were incubated with 10 μg/ml colloidal gold-labeled HDL in media containing 2.5 mg/ml albumin for 1 h at 37°C. As a control, cells were incubated with the same colloidal gold concentration in DMEM with 2.5 mg/ml albumin for 1 h at 37°C. After this incubation cells were chilled on ice and washed as described above.

Subsequent to incubation with the HDL-gold conjugate, the cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose (pH 7.3–7.4) for 1 h (25°C). Monolayers were next washed several times over a 1-h period with the 0.1 M cacodylate buffer containing 0.1 M sucrose (pH 7.4), postfixed for an additional hour with 2% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through a graded series of ethyl alcohol, and transferred into plastic (Embed 812, E M Sciences), according to the method of Delcarpio, Underwood, and Moses (24). Prior to embedding, cell cultures were incubated in Gomori medium containing cytidine-5'-monophosphate and 0.1% lead nitrate according to previously reported methods (25, 26). Ultrathin sections (50 nm) were cut on an MT6000 ultramicrotome (DuPont/Sorvall) and examined in a Phillips EM 302 electron microscope.

RESULTS

Fig. 1 demonstrates the apoprotein profile of the apoE-free HDL ligand used in these studies. It is important to note that lecithin:cholesterol acyltransferase (LCAT) was immediately inhibited by DTNB when the blood was separated. If LCAT was not inhibited before HDL isolation, apoE was not able to be completely removed, even with repeated passes through the heparin affinity column. The effect of HDL on cell sterol synthesis was assessed to determine the net movement of cholesterol between HDL and Fu5AH hepatoma cells. Cells were incubated with increasing concentrations of apoE-free HDL for 18 h at 37°C. At the end of the incubation period relative rates of cell sterol synthesis were determined by pulsing the cells for 2 h with [^{14}C]acetate. The amount of radioactivity incorporated into the cell sterols decreased when the cells were incubated with increasing concentrations of HDL. The effect reached a plateau at an HDL concentration of 60 μg/ml (**Fig. 2**). This result indicated that there was a net delivery of cholesterol from HDL to the cell during the incubation period.

Therefore, the Fu5AH hepatoma cell represents a model system for the delivery of HDL cholesterol to a cell. Since an HDL endocytosis–retroendocytosis pathway has

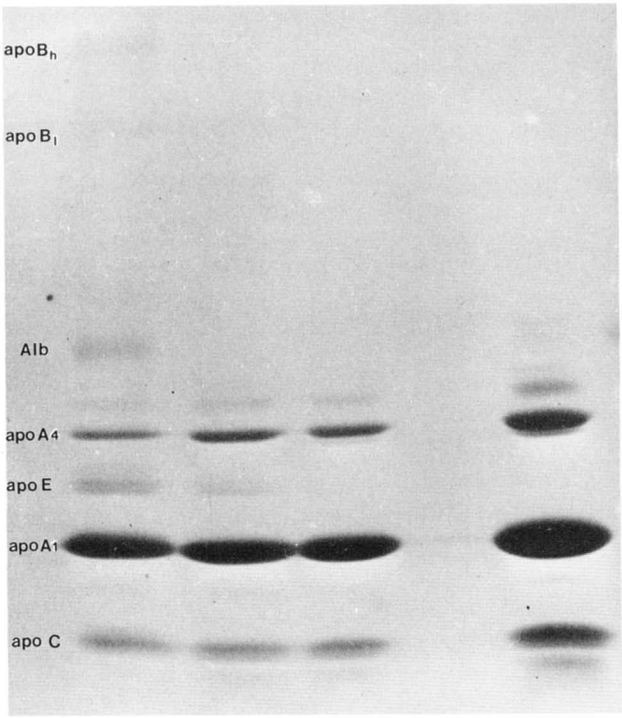

Fig. 1. SDS-PAGE of final apoE-free HDL ligand used in the study. Lane 1: d<1.21 g/ml rat lipoprotein standard (50 μg protein). Lane 2: 1.08–1.21 g/ml rat HDL preparation before heparin affinity column chromatography (50 μg protein). Lane 3: fraction of HDL preparation from lane 2 not retained on heparin affinity column (50 μg protein). Lane 4: apoE-free HDL using 150 μg protein.

never been reported in a cell in which HDL is delivering cholesterol, we sought to determine whether one is present in this cell. **Fig. 3** shows that incubating the cells with 7.5 μg ^{125}I-labeled HDL/ml for 1 h resulted in the association of 276 ng HDL protein/mg cell protein. Displacing ^{125}I-labeled HDL at 0°C for 2 h with a 100-fold excess of unlabeled HDL reduced the quantity of ^{125}I-labeled HDL associated with the cell to 101 ng HDL protein/mg cell protein. By contrast, when the incubation with a 100-fold excess of unlabeled HDL was carried out at 37°C for 30 min, the amount of ^{125}I-labeled HDL associated with the cell was reduced by 89% to 31 ng HDL protein/mg cell protein.

To investigate whether the difference between these two groups was due to material being held inside the cell at 0°C that is released at 37°C, cells were: *1*) incubated initially at 37°C for 1 h with 7.5 μg ^{125}I-labeled HDL/ml (initial incubation); *2*) cooled to 0°C and the surface-bound ^{125}I-labeled was displaced from the cells in the presence of a 100-fold excess of unlabeled HDL at 0°C for 2 h (displacement); *3*) incubated in the presence of a 100-fold excess of unlabeled HDL at 37°C for 30 min (chase). Radioactivity was measured in cells and the media after the displacement and chase procedures. The data in **Table 1** show the radio-

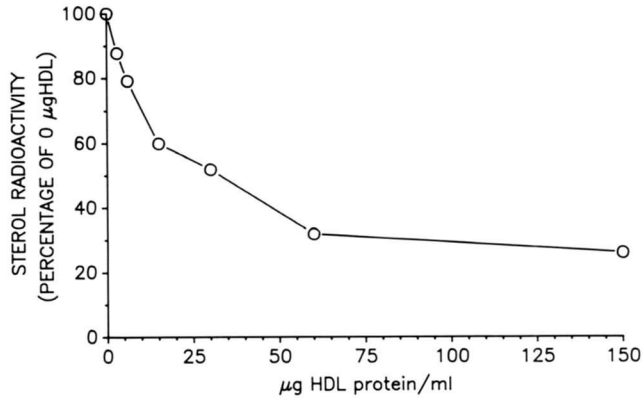

Fig. 2. Effect of HDL on cell sterol synthesis. Cells were incubated with the indicated concentrations of HDL for 1 h at 37°C. Cells were then washed and pulsed with [^{14}C]acetate for 2 h at 37°C.

activity in four fractions; total cpm/dish, TCA-soluble cpm/dish, lipid cpm/dish, and protein cpm/dish. In this experiment 47% of the protein counts associated with cells after the initial incubation remained associated with the cells after displacement and the displaced radioactivity was recovered in the media. When the cells were rewarmed to 37°C after displacement and chased with a 100-fold excess of unlabeled HDL for 30 min, more than 50% of the ^{125}I-labeled HDL protein associated with the cell after the displacement procedure was recovered in the media. Only 11.6% of the ^{125}I-labeled HDL protein associated with the cells after the initial incubation remained associated with the cells after the chase.

This result was consistent with the possibility that the cell internalized a significant quantity of HDL and resecreted the HDL as TCA-precipitable material if the cells

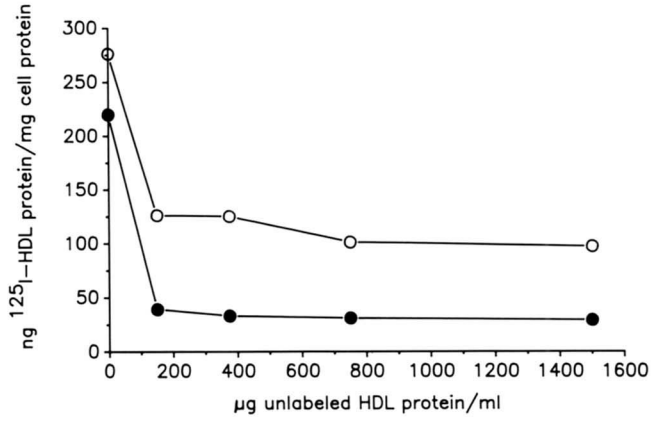

Fig. 3. Amount of HDL protein associated with the rat hepatoma cells before (0 μg HDL) and after displacement at 0°C or 37°C. The graph represents the following experiment. *1*) Initial incubation: both groups of cells were initially incubated with 7.5 μg ^{125}I-labeled HDL protein/ml for 1 h at 37°C. *2*) Displacement: the cells were incubated with various concentrations of unlabeled HDL either at 0°C for 2 h (○-○) or at 37°C for 30 min (●-●). The amount of ^{125}I-labeled HDL associated with the cell after the initial incubation and wash, but before displacement, is represented by the value at 0 μg HDL/ml. All values are the average of duplicate determinations.

TABLE 1. Distribution of ^{125}I in media and cell fractions after incubation of cells

Experimental Group	Total[a] cpm/dish × 10^{-3}	TCA-Soluble cpm/dish × 10^{-3}	Lipid cpm/dish × 10^{-3}	Protein[b] cpm/dish × 10^{-3}
Initial incubation[c]				
Cells	142 ± 11.5[d]	4.8 ± 0.4	43 ± 2.7	95 ± 9.5
Displacement[e]				
Cells	84 ± 2.6	3.6 ± 0.1	34 ± 0.6	45 ± 2.9
Media	71 ± 0.4	1.2 ± 0.1	10 ± 0.7	59 ± 1.0
Chase[f]				
Cells	39 ± 4.4	1.4 ± 0.3	29 ± 2.4	11 ± 1.8
Media	39 ± 0.7	9.1 ± 0.8	8 ± 0.6	23 ± 1.1

[a]Average protein per dish, 1.43 mg.
[b]Two hundred and two cpm/ng HDL protein.
[c]An initial incubation with 7.5 μg ^{125}I-labeled HDL protein/ml at 37°C for 1 h.
[d]Counts per minute ± SD (n = 3).
[e]An initial incubation followed by a displacement with a 100-fold excess of HDL at 0°C for 2 h.
[f]An initial incubation followed by a displacement and then a chase with a 100-fold excess of HDL at 37°C for 30 min.

remained at 37°C. However, in order to conclude that the material was inside the cell, it was necessary to validate that displacement at 0°C with unlabeled HDL could completely remove all the ^{125}I-labeled HDL from the cell surface. Since endocytosis is inhibited at 0°C, HDL associated with cells after a 0°C incubation is only bound to the cell surface. The displacement procedure was evaluated by following a 0°C initial incubation with a 0°C displacement. In the experiment by depicted in **Fig. 4**, an initial 1 h, 37°C incubation with ^{125}I-labeled HDL followed by a 0°C displacement with a 100-fold excess of unlabeled HDL (750 μg HDL protein/ml) reduced the quantity of HDL associated with the cell by 49%. In contrast, when the cells were initially incubated with ^{125}I-labeled HDL at 0°C for 2 h and HDL was displaced at 0°C with a 100-fold excess of unlabeled HDL, 88% of the ^{125}I-labeled HDL originally associated with the cell was removed. An important issue addressed by this experiment was what proportion of the ^{125}I-labeled HDL associated with the cell after a 1 h, 37°C initial incubation was due to nonspecific binding. The amount of ^{125}I-labeled HDL remaining with the cell after a 0°C initial incubation and a 0°C displacement represented 8% of ^{125}I-labeled HDL originally associated with the cell after the 37°C initial incubation, a value that is consistent with nonspecific binding found for HDL at this concentration in a number of cell systems, including Fu5AH hepatoma cells (27-29) and fibroblasts (19). This result indicated that nonspecific binding did not account for the 49% of ^{125}I-labeled HDL remaining with the cell when the ^{125}I-labeled HDL was displaced from the cell surface at 0°C after a 1 h, 37°C initial incubation.

To verify whether a 2-h incubation with unlabeled HDL was an adequate time period to allow for complete removal of surface-bound ^{125}I-labeled HDL, cells were incubated with 7.5 μg ^{125}I-labeled HDL protein/ml at 37°C for 1 h, chilled to 0°C, and ^{125}I-labeled HDL was displaced at 0°C with a 100-fold excess of unlabeled HDL for 1 h, 2 h, and 4 h. The results shown in **Fig. 5** demonstrate that by 2 h the amount of ^{125}I-labeled HDL associated with the cell reached a minimum value.

In all of the above experiments ^{125}I-labeled HDL was removed from the cell surface by displacement with excess unlabeled HDL. Others have shown that proteolytic en-

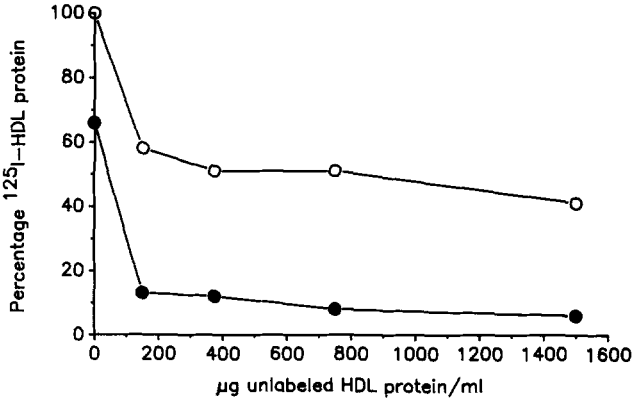

Fig. 4. Percentage of HDL protein associated with rat hepatoma cells that were initially incubated with HDL at either 37°C or 0°C and then displced at 0°C. The graph represents the following experiment. *1*) Initial incubation: 7.5 μg ^{125}I-labeled HDL protein/ml was initially incubated with the cells for either 1 h at 37°C (○-○) or 2 h at 0°C (●-●). *2*) Displacement: both groups were incubated with various concentrations of unlabeled HDL for 2 h at 0°C. The amount of ^{125}I-labeled HDL associated with the cell after the initial incubation and wash, but before the displacement, is represented by the value at 0 μg HDL/ml; 100% represents the amount of ^{125}I-labeled HDL associated with the cell after the 37°C incubation (220 ng HDL/mg cell protein). All values are the average of duplicate determinations.

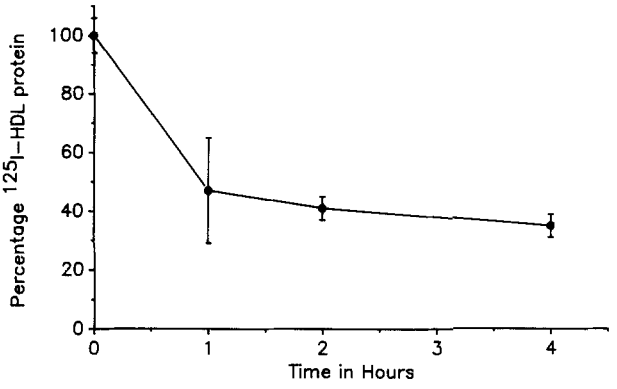

Fig. 5. Percentage of ^{125}I-labeled HDL protein associated with the rat hepatoma cells immediately after an initial incubation of 1 h at 37°C and after displacement with a 100-fold excess of unlabeled HDL at 0°C for 1, 2, and 4 h. The 100% value represents 299 ng HDL/mg cell protein. Values are mean ± SD for triplicate determinations.

zymes remove material bound to cell surfaces (HDL, LDL, and peptide hormones) while leaving internalized material undigested due to the inaccessibility of the internalized material to the proteolytic enzyme (30). Therefore, we subjected the cells to tryptic digestion and compared the amount of ^{125}I-labeled HDL associated with the cell after tryptic digestion to the amount of ^{125}I-labeled HDL associated with the cell after displacement with a 100-fold excess of unlabeled HDL. The data showed that both procedures produced the same result (i.e., the same amount of ^{125}I-labeled HDL protein remained associated with the cell). This is consistent with the concept that incubating cells with a 100-fold excess of unlabeled HDL was effective in displacing ^{125}I-labeled HDL off the cell surface and also that ^{125}I-labeled HDL associated with cells after displacement was located inside the cell.

The displacement procedures and the tryptic digestion procedure used above were all carried out at 0°C. Because the binding at 0°C may be different than at 37°C, the displacement procedure was carried out at 37°C. To do this we needed to use a treatment that would prevent ^{125}I-labeled HDL that was potentially inside the cell from being resecreted. We predicted that inhibiting ATP production with this treatment would stop endocytosis and retroendocytosis of HDL. Therefore, cells were inhibited with 10 mM NaCN and 20 mM 2-deoxyglucose-D-glucose (CN, DG) after the initial incubation and prior to displacement. After an initial incubation, cells were: *1*) chilled to 0°C, washed, and ^{125}I-labeled HDL was displaced with unlabeled HDL at 0°C for 2 h; *2*) treated with CN, DG (added to the media) for 30 min, washed with CN, DG wash buffer at 37°C, and displaced for 30 min at 37°C with CN, DG media and a 100-fold excess of unlabeled HDL; or *3*) incubated an additional 30 min at 37°C, washed at 37°C, and ^{125}I-labeled HDL was displaced with unlabeled HDL for 30 min at 37°C. The results in **Fig. 6** show that when cells were inhibited by incubation with CN, DG and ^{125}I-HDL was displaced with 750 μg unlabeled HDL protein/ml at 37°C, the amount of ^{125}I-labeled HDL that remained associated with the cells was close to that associated with cells when incubated with the same amount of unlabeled HDL at 0°C (82 ng HDL/mg cell protein vs 101 ng HDL/mg cell protein). The amount of ^{125}I-labeled HDL associated with the cells after displacement with 750 μg unlabeled HDL in the presence of CN, DG at 37°C was 2.6-times that associated with cells displaced with the same amount of HDL at 37°C with no inhibitors. The difference in ^{125}I-labeled HDL remaining in cells displaced in the presence of CN, DG versus displacement at 0°C could be due to: *1*) the 37°C temperature in the CN, DG group allowing a greater quantity of the ^{125}I-labeled HDL to be displaced from the cell surface than at 0°C; or, *2*) less inhibition of the retroendocytosis of ^{125}I-labeled HDL by CN, DG than that at 0°C. Nevertheless, the observation is consistent with the conclusion that 0°C displacement adequately removes surface-bound ^{125}I-labeled HDL and that CN, DG is able to block retroendocytosis of HDL.

All of the experiments described above were discussed in terms of changes in ^{125}I-labeled HDL protein. In the ^{125}I-labeled HDL preparation the amount of ^{125}I associated with the lipid fraction of HDL averaged 10% of total counts. After incubation of cells with ^{125}I-labeled HDL for 1 h at 37°C, 31% to 49% of the cell-associated radioactivity was in the lipid fraction. Analysis by thin-layer chromatography showed that approximately 85% of the radioactivity was in the phospholipid fraction. The data

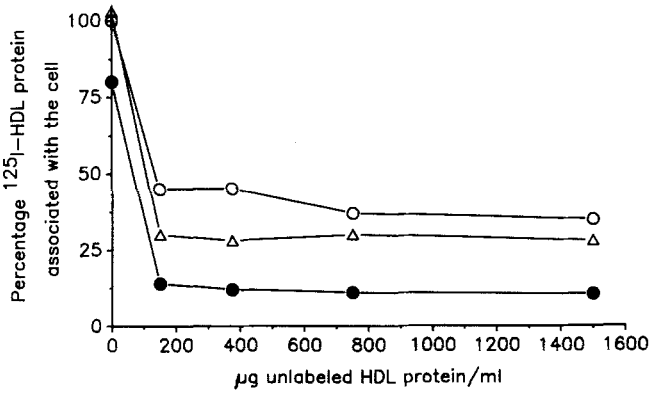

Fig. 6. Effect of metabolic inhibitors, NaCN and 2-deoxyglucose-D-glucose, on the retention of HDL protein by rat hepatoma cells. The graph represents the following experiments. *1*) Initial incubation: all groups of cells were initially incubated with 7.5 μg ^{125}I-labeled HDL protein/ml for 1 h at 37°C. *2*) Displacement: the cells were divided into three groups, cooled to 0°C, and incubated with various concentrations of unlabeled HDL (○-○), and either *a*) incubated for 20 min with 10 mM NaCN, 20 mM 2-deoxy-D-glucose that had been added to the initial incubation media and then incubated with media containing (CN, DG) and various concentrations of unlabeled HDL for 30 min at 37°C (△-△); or *b*) incubated an additional 30 min at 37°C and then incubated with various concentrations of unlabeled HDL for an additional 30 min at 37°C (●-●); (100% represents 276 ng/mg cell protein). All values are the average of duplicate determinations.

in **Fig. 7** show the cell lipid counts from the experiment described in Fig. 4. Lipid results were qualitatively similar to the protein results. *1*) Displacement with increasing concentrations of HDL decreased the amount of lipid label associated with the cell to approximately 40% of that present after the initial 37°C incubation. *2*) In the cells incubated initially at 0°C, the same HDL displacement decreased the amount of lipid associated with the cell to approximately 10% of the lipid radioactivity found in the cells incubated at 37°C for 1 h.

Although the protein and lipid results were qualitatively and quantitatively similar in the experiment shown in Figs. 4 and 7, this was not the case in the experiment reported in Fig. 1. As just described for Figs. 4 and 7, a 37°C initial incubation followed by a 0°C displacement resulted in a reduction of both protein and lipid radioactivity by 60%. In contrast, when the initial incubation and displacement procedures were both carried out at 37°C, lipid radioactivity decreased by an amount equivalent to the decrease seen when the displacement was done at 0°C (63%), while the decrease in cell protein radioactivity was significantly greater (89%).

These results can be explained if one assumes that the initial 37°C incubations with ^{125}I-labeled HDL resulted in two pools of cellular lipids: *1*) a surface pool mainly associated with the cell plasma membrane; and, *2*) an internal pool associated with intracellular structures. The observation that incubating the cells with excess unlabeled HDL had a qualitatively similar effect on lipid radioactivity at either 0°C or 37°C indicated that the unlabeled HDL is able to exchange plasma membrane-associated lipid radioactivity at 0°C or 37°C. This conclusion is supported by the experiment in which ^{125}I-labeled HDL is initially incubated at 0°C (Figs. 4 and 7). Here the labeled lipid could have only been associated with the cell plasma membrane (31) and after displacement with unlabeled HDL at 0°C, 90% of the lipid radioactivity associated with the cell was removed. In contrast, the protein results from the experiment in which both sets were initially incubated with ^{125}I-labeled HDL at 37°C for 1 h were qualitatively different from the lipid when the displacement was done at 0°C versus 37°C (much less ^{125}I-labeled HDL protein was associated with the 37°C displacement). Since the lipid associated with the plasma membrane is apparently displaced at 0°C while all of the ^{125}I-labeled HDL protein is not, this indicates that the portion of ^{125}I-labeled HDL protein that remains associated with the cell after the displacement procedure is not on the plasma membrane but inside the cell.

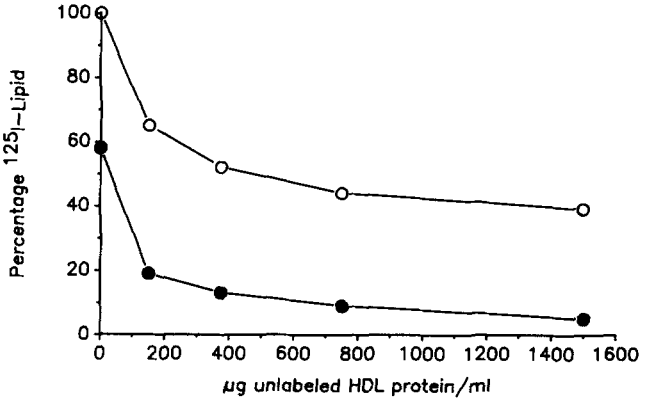

Fig. 7. Percentage of ^{125} I-labeled HDL lipid associated with rat hepatoma cells that were initially incubated with ^{125}I-labeled HDL at either 37°C or 0°C and then HDL displaced at 0°C. The graph represents the following experiment. *1*) Initial incubation: 7.5 μg ^{125}I-labeled HDL protein/ml was initially incubated with the cells for either 1 h at 37°C (O-O) or 2 h at 0°C (●-●). *2*) Displacement: both groups were incubated with various concentrations of unlabeled HDL for 2 h at 0°C. The amount of ^{125}I-labeled lipid associated with the cell after the initial incubation and wash, but before the displacement, is represented by the value at 0 μg HDL/ml. All values are the average of duplicate determinations.

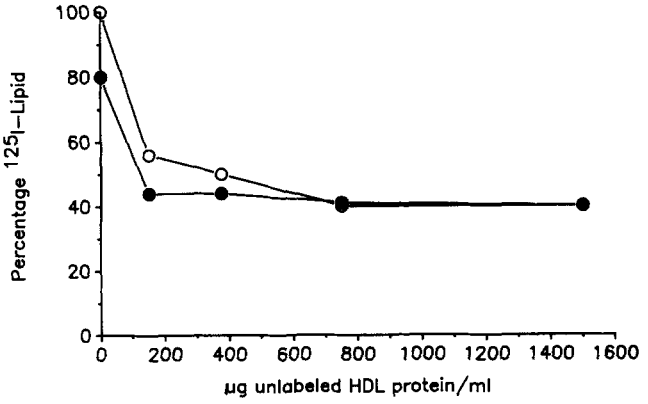

Fig. 8. Percentage of ^{125}I-labeled HDL lipid associated with rat hepatoma cells before and after competition at 0°C or 37°C. The graph represents the following experiment. *1*) Initial incubation: both groups of cells were initially incubated with 7.5 μg ^{125}I-labeled HDL protein/ml for 1 h at 37°C. *2*) Displacement: the cells were incubated with various concentrations of unlabeled HDL either for 2 h at 0°C (O-O) or for 30 min at 37°C (●-●). The amount of ^{125}I-labeled lipid associated with the cell after the initial incubation and wash, but before the displacement, is represented by the value of 0 μg HDL/ml. All values are the average of duplicate determinations.

As an attempt to understand the effect that the endocytosis–retroendocytosis pathway has on the cellular metabolism of the ^{125}I-labeled HDL lipid, we examined the ^{125}I-lipid/^{125}I-labeled protein ratios in the cells from the experiments depicted in Fig. 3 and **Fig. 8**. After an initial incubation of 1 h with ^{125}I-labeled HDL, the cell ^{125}I-labeled lipid/^{125}I-labeled protein ratio was 0.55. This ratio was 0.6 after displacemnt with a 100-fold excess of unlabeled HDL for 30 min at 37*C*, the lipid/protein ratio increased to 1.99 and was 1.12 even when the TCA-soluble counts generated during this time period were added to the protein counts. Thus, while the displacement by unlabeled HDL at 0°C was able to remove all lipid and protein radioactivity associated with the cell surface, in order to remove all protein radioactivity, the cells had to be chased at 37°C. This was most likely due to protein being held inside the cell.

TABLE 2. Distribution of ^{125}I counts in cell and media fractions

Experimental Group	Total cpm/mg Cell Protein	TCA-Soluble cpm/mg Cell Protein	Lipid cpm/mg Cell Protein	Protein cpm/mg Cell Protein
Cells				
Initial incubation[a]	77,777[b]	6,932	25,264	45,581[c]
Displacement	42,162	4,962	16,161	21,040
Chase media[d]				
2.5 mg Albumin/ml	18,019	7,944	1,912	8,164
75 μg HDL/ml + albumin	22,532	7,945	2,588	11,249
150 μg HDL/ml + albumin	22,440	7,342	3,273	11,825
375 μg HDL/ml + albumin	24,630	8,141	3,260	13,228
750 μg HDL/ml + albumin	24,814	7,843	3,913	13,059
1500 μg HDL/ml + albumin	23,926	7,246	4,028	12,652

[a]Initial incubation of 7.5 μg ^{125}I-labeled HDL protein/ml at 37°C for 1 h or after an initial incubation followed by displacement with a 100-fold excess of unlabeled HDL for 2 h at 0°C.
[b]Average of duplicate determinations.
[c]Two hundred and twelve cpm/ng HDL protein.
[d]Media fractions from cells that were initially incubated followed by displacement, then followed by a chase with 2.5 albumin/ml or albumin and increasing concentrations of HDL for 30 min at 37°C.

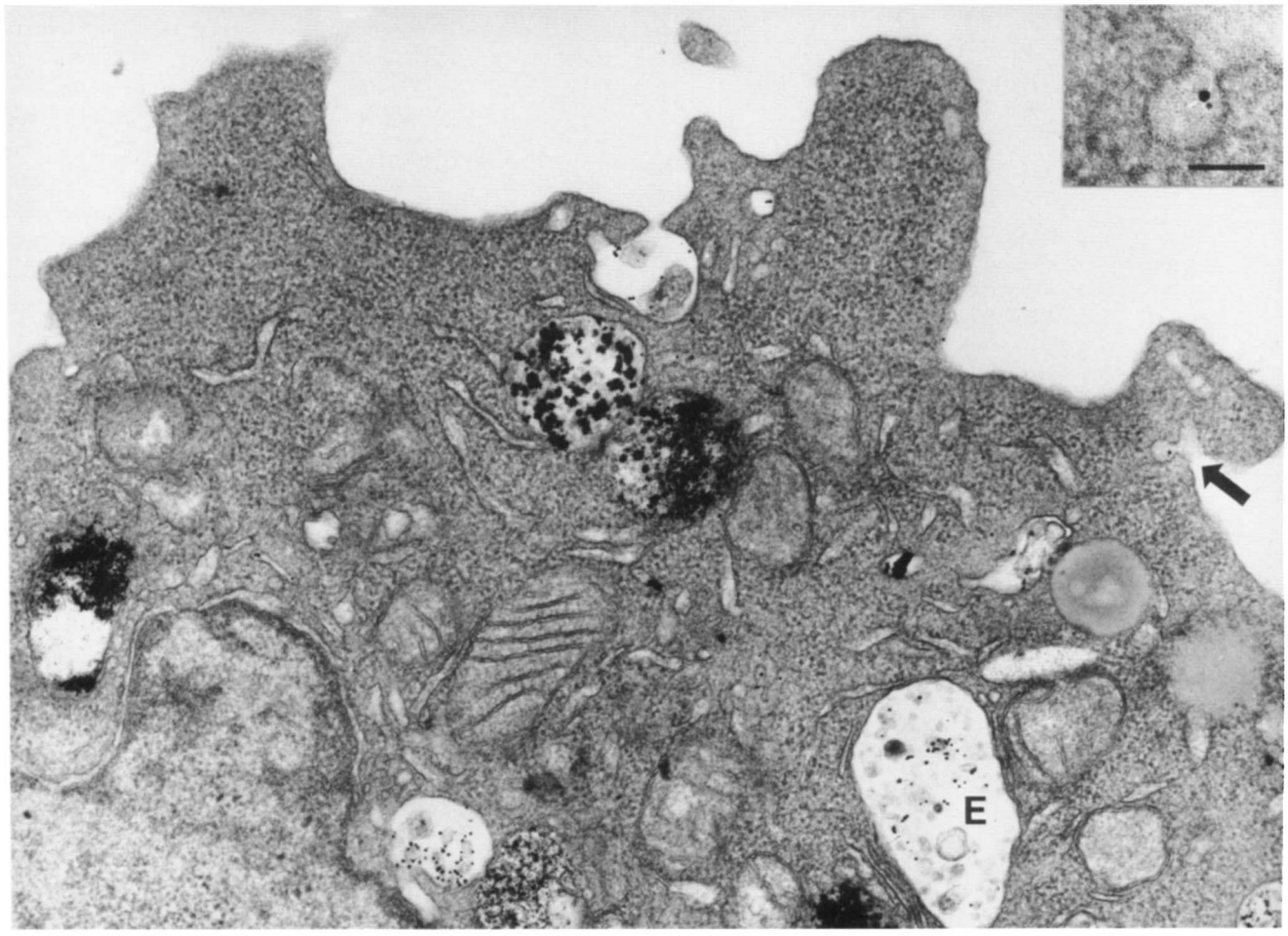

Fig. 9. Transmission electron micrographs of the Fu5AH hepatoma cells from monolayers reacted with 8-10 nm colloidal gold conjugates of apoE-free HDL (10 μg/ml) for 60 min at 37°C. Micrograph shows an acid phosphatase-positive cell demonstrating the internalization of the HDL-gold conjugates (see arrow and insert). HDL conjugates are seen in endosomes (E); x 45,334. Insert: x 150,000; bar represents 50 nm.

The above results suggest that HDL is internalized by endocytosis and then resecreted by retroendocytosis. In order to examine the effect that media HDL concentration has on the endocytosis–retroendocytosis pathway, we used the standard 1 h, 37°C initial incubation followed by displacement at 0°C for 2 h in the presence of a 100-fold excess of unlabeled HDL. Cells were then rewarmed to 37°C for 30 min in the presence of either 2.5 mg albumin/ml or 2.5 mg albumin/ml and increasing concentrations of HDL (chase). **Table 2** shows the radioactivity that appeared in the media during the 30-min chase. The amount of TCA-soluble cpm released into the media during this time was the same in all groups, averaging 7743 cmp/mg cell protein. This indicated that the presence of unlabeled HDL in the chase media did not affect the amount of ^{125}I-labeled HDL after a lysosomal degradation pathway. However, the amount of ^{125}I-labeled protein that reappeared in the media was apparently affected by the presence of HDL in the chase media. When there was no HDL in the chase media, 8,164 cpm protein/mg cell protein appeared in the media. This was 62% greater (13,228 cpm protein/mg cell protein) when the concentration of unlabeled HDL present in the chase media was 375 μg protein/ml. However, a substantial amount of HDL reappeared in the media in the absence of HDL, indicating that there was not an absolute requirement for HDL to be present in order for retroendocytosis of HDL protein to occur.

Uptake of colloidal gold-labeled HDL was examined by electron microscopy in order to verify that HDL was internalized (**Fig. 9**). Cells were stained for acid phosphatase before embedding so that lysosomes could be identified and differentiated from endosomes. HDL could readily be found in internal endocytic structures that did not stain positively for acid phosphatase. HDL was frequently observed in appendages attached to the endocytic structures. These may either be endosomes budding to form a recycling vesicle or newly formed endosomes from the cell surface fusing with other internal endosomes. In micrographs of cells incubated with colloidal gold not bound to HDL, it was very difficult to find any gold associated with the cell.

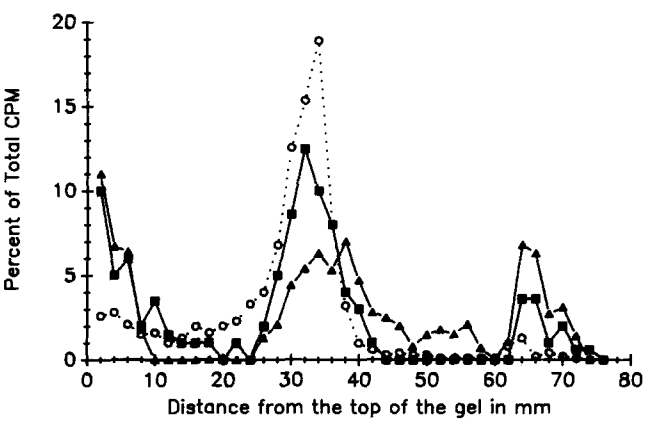

Fig. 10. Gradient gel analysis of ^{125}I-labeled HDL. The graph represents the following experiment. *1*) Initial incubation: 7.5 μg ^{125}I-labeled HDL protein/ml was initially incubated with the cells for 1 h at 37°C. *2*) Displacement: *a*) one group was incubated with 2.5 mg/ml albumin for 2 h at 0°C. The media collected during this incubation represented surface HDL; *b*) the other group was incubated with 750 μg unlabeled HDL and 2.5 mg/ml albumin for 2 h at 37°C. *3*) Chase: for group *b* the displacement was followed by a 30-min, 37°C incubation with 2.5 mg/ml albumin in DMEM. The media from this incubation represented resecreted HDL. Samples shown include: (O-O) unincubated HDL; (■-■) surface HDL; (▲-▲) resecreted HDL.

TABLE 3. Analysis of lipoprotein fractions by gradient gel electrophoresis

Fraction	Unincubated	Surface	Resecreted
		percent of total	
1–10	10.6	26.5	25.9
11–40	83.0	56.1	32.2
40–60	3.4	6.0	20.5
60–76	3.0	11.4	21.4

Data are taken from the experiment described in Fig. 10. The radioactivity in each are of the gel was summed to give the amount in different areas of the gel.

The size distribution of ^{125}I-labeled HDL resecreted from the cell surface was examined. To obtain resecreted ^{125}I-labeled HDL, cells were *1*) incubated with 7.5 μg/ml ^{125}I-labeled HDL for 1 h at 37°C; *2*) incubated at 0°C with 750 μg/ml unlabeled HDL; and *3*) incubated at 37°C for 30 min in DMEM containing 2.5 mg/ml albumin. The media from the final incubation was collected and the lipoproteins were separated by electrophoresis on a nondenaturing gradient gel (**Fig. 10**). The original HDL was basically present as a single, homogenous peak. Several new populations appeared in the resecreted HDL: a larger population and three smaller populations. Included in the smaller populations was a peak of radioactivity in a region of the gel where molecules below the size of albumin migrate. The distribution of ^{125}I in the apoproteins of resecreted HDL was the same as that of the original HDL; this indicated that the apoproteins in the resecreted HDL had not been subjected to proteolysis. Thus, it is apparent that the resecreted HDL had been remodeled.

The profile of the resecreted HDL was compared to that of HDL from the cell surface. This material was collected from the media after cells were initially incubated for 1 h at 37°C with 7.5 μg ^{125}I-labeled HDL/ml and then incubated with DMEM containing 2.5 mg/ml albumin. While the changes in the surface HDL reflected resecreted HDL, they were generally of a smaller magnitide than resecreted HDL. To illustrate this point, the gel was divided into four regions and the amount of material in each region was determined (**Table 3**). In fractions 1–10

there was more material in both the surface HDL (26%) and resecreted HDL (26%) than the original HDL (11%). Fractions 10–40 contained 83% of the original HDL. The amount of radioactivity in this peak was 56% in the surface HDL and 32% in the resecreted HDL. Fractions 40–60, i.e., those fractions between the original HDL peak and albumin, contained 3% of the total radioactivity in the original HDL, 6% of that in the surface HDL, and 20% of that in the resecreted HDL. In the region of the gel below albumin, the fractions contained 3% of the radioactivity of the original HDL, 11% of that in the surface HDL, and 21% of that in the resecreted HDL. Thus, both regions of the gel that represent smaller HDL were increased in the surface HDL, but increased to an even greater extent in the resecreted HDL. This is expected if HDL is remodeled inside the cell, since the surface HDL should consist of two populations: HDL that has not entered the cell, and HDL that is being resecreted.

DISCUSSION

The present study examines the metabolism of HDL by rat hepatoma cells (Fu5AH). HDL is a cholesterol-transporting lipid-protein complex whose physiological role with respect to cholesterol depends on the cell type and the metabolic state of the cell under consideration. Cells thought to take up HDL cholesterol for cell metabolism include, hepatocytes for bile formation (32), cells of adrenal (33), ovary (34), and testis (35) for steroid hormone production, and growth-stimulated cells for membrane synthesis (36, 37). In all other tissues HDL is thought to remove cholesterol from cell membranes for transport to the liver (reverse cholesterol transport) (1). In a rapidly dividing cell, such as the hepatoma cells used in this study, HDL can serve as a source of lipids. The observation that steroid synthesis is suppressed in cells incubated with HDL (Fig. 2) is indicative of a net cholesterol delivery to these cells.

Retroendocytosis of HDL has been reported in rat aortic smooth muscle cells (38), mouse peritoneal macrophages (4), and liver macrophages (39). However, others were not able to demonstrate this pathway in human fibroblasts or mouse peritoneal macrophages (30). Thus, the presence of the pathway remains controversial for cells that presumably efflux cholesterol to HDL. Since the Fu5AH hepatoma cells used in this study remove cholesterol from HDL, it is of interest to determine whether an HDL retroendocytic pathway is present in this cell.

The evidence presented above demonstrates that when apoE-free ^{125}I-labeled HDL is incubated with rat hepatoma cells it is present both on the cell surface and in the cell interior, as would be expected. This point was supported by electron microscopy of cells incubated with gold-labeled HDL. The fact that incubating the cells with excess unlabeled HDL at 0°C sufficiently removed ^{125}I-labeled HDL from the cell surface was confirmed by: *1*) an HDL displacement time course; *2*) displacement of HDL from cells in which HDL was initially bound at 0°C; *3*) tryptic digestion of cells; *4*) displacment of ^{125}I-labeled HDL at 37°C from CN, DG-treated cells with excess unlabeled HDL; and *5*) movements of cell ^{125}I-labeled lipid during displacement and chase experiments. As much as 63% of the ^{125}I-labeled HDL protein held inside the cell was recovered in the media as TCA-precipitable protein when the cells were subsequently incubated at 37°C for 30 min (Table 2). During that 30-min time period, the ratio of TCA-soluble counts produced to protein counts appearing in the media was 0.3–0.44. This indicated that 23–30% of HDL apoprotein was being channeled into the lysosomal pathway. Further support for the HDL being held inside the cell and then resecreted was obtained by gradient gel analysis of ^{125}I-labeled HDL in that ^{125}I-labeled HDL collected from the media after a 37°C chase (resecreted HDL) was modified to a greater extent than ^{125}I-labeled HDL from the cell surface. Thus, we conclude that the majority of internalized HDL apoproteins follow a retroendocytic pathway in this rat hepatoma cell.

Considering the rate of normal membrane endocytosis and recycling in many cell types, it is logical to conclude that HDL should be internalized if one accepts that HDL binds to cell membranes. For example, L-cell fibroblasts and macrophages internalize 0.9% and 3%, respectively, of their surface membrane each minute (40). Others have indicated that human fibroblasts, HeLa cells, and baby hamster kidney cells take up their membrane surface each 50 min or less (41). Since the membrane content of the internal vacuolar system is constant, and the rate of cellular membrane degradation is slow compared to internalization, Steinman et al. (40) have concluded that membrane continually recycles through cells. The possibility that these findings may be merely a consequence of membrane recycling phenomena and have no functional relevance for the cell needs to be considered. Data from nondenaturing gradient gels (Fig. 10) showed that resecreted HDL had been structurally modified. Since the HDL from the cell surface was not modified to as great an extent, we conclude that the modification occurred while the HDL was inside the cell. Because the modification resulted in the generation of smaller lipoprotein populations and apoproteins in the lipoprotein-free fraction, it is probable that these new lipoproteins were depleted of their original lipid content.

Using parallel experiments with HDL labeled in the cholesteryl ester and protein moieties, we determined the CE/protein ratio of the resecreted HDL in a pulse-chase

experiment. The CE/protein ratio of the unincubated material was 95 × 10^{-3} and 78 × 10^{-3} after incubation with cells for 1 h at 37°C. HDL resecreted into the media during a 30-min, 37°C chase incubation had a CE/protein ratio of 34 × 10^{-3}. Thus, the resecreted HDL had been depleted of cholesteryl ester (DeLamatre, J. G., and C. A. Hornick, unpublished observations). It is tempting to speculate that the cholesteryl ester was removed from the lipoprotein while in an endocytic compartment, but without more detailed analysis of endocytic processing of lipoproteins we are unable to determine whether this occurs in an endosome or on the cell surface.

Pittman et al. (42) examined the effect of a series of metabolic inhibitors on the selective uptake of cholesteryl ether versus apoA-I by HepG2 human hepatoma cells. They concluded that endocytosis was not responsible for the selective uptake of cholesteryl ether. However, their evidence for the lack of HDL endocytosis was circumstantial in that lysosomal hydrolysis of asialofetuin was determined as an indicator of endocytosis. Without using some direct measure of the HDL retroendocytic pathway, it is not possible to completely rule out HDL endocytosis, especially since all incubations were followed by a 2-h chase incubation before harvesting the cells; this would provide ample time for endocytosed HDL to be resecreted. The conclusion drawn from this work is that the selective transfer of cholesteryl ether occurs exclusively on the cell surface. Indeed, Knecht and Pittman (43) and Israeli et al. (44) have shown selective delivery of cholesteryl ether into plasma membranes.

Studies of mouse peritoneal macrophages by electron microscopy showed that HDL accumulated in endosomes with transferrin while acetylated LDL was localized in lysosomes (4). Upon further incubation at 37°C, the macrophages resecreted the HDL, providing morphological evidence for an HDL retroendocytic pathway in macrophages. While the present report was in preparation, Takata et al. (39) reported that HDL are internalized and resecreted in rat liver macrophages. Using fluorescein isothiocyanate-labeled HDL, they also demonstrated that HDL was present in an acidic endocytic, nonlysomal compartment. The data in the present report agree very well with both these studies but, again, we observed net HDL cholesterol delivery and not cholesterol efflux in these cells.

Klausner et al. (45) demonstrated that the presence of excess unlabeled transferrin in the incubation media resulted in the release of a significantly greater quantity of transferrin from a human leukemic cell line. This same phenomenon was observed here for HDL retroendocytosis. The data in Table 2 showed that in the absence of HDL in the incubation media, 39% of the ^{125}I-labeled protein inside the cell was recovered in the media at 30 min while 63% was recovered in the media that contained 375 μg HDL/ml. This represented a 62% increase in the amount of ^{125}I-labeled HDL protein that was resecreted during the 30-min time period. The observation that the presence of HDL in the incubation media increased the retroendocytosis of ^{125}I-labeled HDL could be due to several factors, including: *1*) preventing reuptake of ^{125}I-labeled HDL by displacing the ^{125}I-labeled HDL when it returns to the surface; *2*) increased HDL mass in the retroendocytic pathway; *3*) binding of HDL to a receptor on the cell surface sending a signal to the interior of the cell that resulted in a greater return of an HDL-receptor complex to the surface; or *4*) the presence of HDL in the media having a generalized effect on the retroendocytic pathway that resulted in the accelerated movement of ligands through the pathway.

In conclusion, these data support the hypothesis that HDL follow a retroendocytic pathway in a cell that is removing cholesterol from HDL: the rat hepatoma cell, Fu5AH. We present evidence that resecreted HDL has been remodeled. It appears likely that the retroendocytic pathway has an important function in HDL lipid metabolism. To what extent the pathway affects the metabolism of HDL cholesterol, cholesteryl ester, phospholipids, free fatty acids, or triacylglycerols must still be addressed.

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