

# Binding, internalization, and degradation of high density lipoprotein by cultured normal human fibroblasts

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**Abstract** Comparative studies were made of the metabolism of plasma high density lipoprotein (HDL) and low density lipoprotein (LDL) by cultured normal human fibroblasts. On a molar basis, the surface binding of <sup>125</sup>I-HDL was only slightly less than that of <sup>125</sup>I-LDL, whereas the rates of internalization and degradation of <sup>125</sup>I-HDL were very low relative to those of <sup>125</sup>I-LDL. The relationships of internalization and degradation to binding suggested the presence of a saturable uptake mechanism for LDL functionally related to high-affinity binding. This was confirmed by the finding that the total uptake of <sup>125</sup>I-LDL (internalized plus degraded) at 5 μg LDL protein/ml was 100-fold greater than that attributable to fluid or bulk pinocytosis, quantified with [<sup>14</sup>C]sucrose, and 10-fold greater than that attributable to the sum of fluid endocytosis and adsorptive endocytosis. In contrast, <sup>125</sup>I-HDL uptake could be almost completely accounted for by the uptake of medium during pinocytosis and by invagination of surface membrane (bearing bound lipoprotein) during pinocytosis. These findings imply that, at most, only a small fraction of bound HDL binds to the high-affinity LDL receptor and/or that HDL binding there is internalized very slowly. The rate of <sup>125</sup>I-HDL degradation by cultured fibroblasts (per unit cell mass) exceeded an estimate of the turnover rate of HDL in vivo, suggesting that peripheral tissues may contribute to HDL catabolism. In accordance with their differing rates of uptake and cholesterol content, LDL increased the cholesterol content of fibroblasts and selectively inhibited sterol biosynthesis, whereas HDL had neither effect.

**Supplementary key words** low density lipoprotein · cholesterol metabolism · pinocytosis · endocytosis

In recent years considerable attention has been directed towards the metabolism of low density lipoprotein (LDL) by cultured cells. This has been stimulated by evidence from in vivo studies that the removal of LDL from plasma may occur mainly in extrahepatic tissues (1,2) and that this process is impaired in certain forms of familial hypercholesterolemia (3). Fibroblasts from normal human subjects have been shown to metabolize LDL in tissue culture.

This involves the prior binding of LDL to the cell membrane, followed by its internalization and incorporation into lysosomes, where the protein component is degraded to trichloroacetic acid-soluble fragments and the cholesteryl ester is hydrolyzed (4–7). The unesterified cholesterol released in this process inhibits endogenous cholesterol synthesis through suppression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) by mechanisms still to be elucidated (7–9).

Thus, the interactions that occur between LDL and peripheral tissues appear to be important both for the catabolism of LDL and for the regulation of cell cholesterol metabolism. Other studies have suggested that high density lipoprotein may be directly or indirectly involved in regulation of cholesterol metabolism through interactions with peripheral tissues. Thus, the body pool of exchangeable cholesterol in man has been reported to be inversely related to the plasma HDL cholesterol concentrations (10), and there is epidemiologic evidence that plasma HDL levels are inversely correlated with susceptibility to clinically manifest atherosclerosis, i.e., HDL level is a “negative” risk factor (11–13). The possibility that peripheral tissues may play a significant role in HDL catabolism has been raised by studies in this laboratory in which portacaval shunt failed to reduce the fractional catabolic rate of HDL in swine (14).

The nature of the interactions that occur between HDL and cultured human fibroblasts have not been

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; TCA, trichloroacetic acid; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; DME, Dulbecco's modification of Eagle's minimal essential medium; PBS, Dulbecco's phosphate buffered saline; FCS, fetal calf serum; LDS, lipoprotein-deficient fetal calf serum; <sup>125</sup>I-HDL and <sup>125</sup>I-LDL, <sup>125</sup>I-labeled HDL and LDL, respectively.

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systematically characterized. In the present study we have examined the surface binding, internalization, and degradation of HDL by normal human fibroblasts, and have investigated also the associated changes in cell cholesterol metabolism. Preliminary reports of some of this work have already appeared (15,16).

## METHODS

### Materials

Sodium [ $^{125}\text{I}$ ]iodide (carrier free in 0.05N NaOH) was obtained from Schwarz-Mann, Orangeburg, NY, [ $1\text{-}^{14}\text{C}$ ]acetate (sodium salt; 58 mCi/mmol) from Dhom Products Limited, North Hollywood, CA, and  $\text{D-}[^{14}\text{C}]$ sucrose (453 mCi/mmol) from New England Nuclear Corp., Boston, MA. Cholesterol (recrystallized twice from ethyl acetate) and  $5\text{-}\alpha$ -cholestane were obtained from Applied Science Laboratories, State College, PA, and pure sucrose from Schwarz-Mann. Fetal calf serum (FCS) was purchased from Irvine Scientific Sales Company. Dulbecco's modification of Eagle's minimal essential medium (DME) (17) and Dulbecco's phosphate buffered saline (PBS) (18) were obtained from GIBCO, Grand Island, N.Y. Pronase (B grade; 45,000 P.U.K./g) was purchased from Calbiochem., San Diego, CA.

### Cell cultures

Skin fibroblasts were grown in monolayer from a preputial biopsy of a normal infant. Studies of the metabolism of LDL by this cell line (B.B.) have already been reported (7). Cultures were maintained in a humidified incubator (95% air, 5%  $\text{CO}_2$ ) at  $37^\circ\text{C}$  in DME containing 24 mM  $\text{NaHCO}_3$ , 0.3 mg/ml glutamine and 10% (v/v) fetal calf serum (final protein concentration, 5 mg/ml). Cells were studied between the 9th and 20th passages; no systematic differences were noted between results in cells of early and late passage numbers. Polystyrene tissue culture dishes ( $60 \times 15$  mm; Corning) were seeded with  $1\text{--}2 \times 10^5$  cells and used 4–6 days later. At this time 50–95% of the surface of the dish was covered, and total cell protein per dish was 300–550  $\mu\text{g}$ . Growth curves had previously shown that this cell line reaches a plateau at  $1\text{--}2 \times 10^6$  cells in 6–8 days (7).

### Lipoproteins and lipoprotein-deficient serum

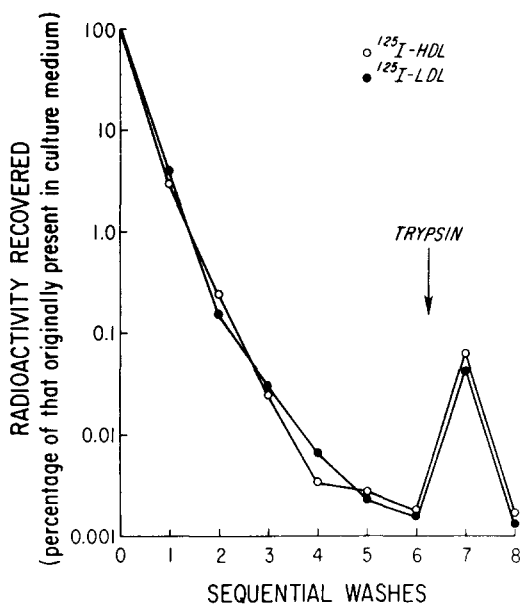
Lipoproteins in the density ranges 1.019–1.063 g/ml (LDL) and 1.090–1.21 (HDL) were isolated from pooled fasting normal human plasma by preparative ultracentrifugation (19). Isolated fractions were

dialyzed against buffer containing 20 mM Tris HCl (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA, sterilized by passage through a Millipore filter (0.22 or 0.45  $\mu\text{m}$ ) and stored at  $4^\circ\text{C}$ . Lipoproteins were used generally within 2 weeks and never after more than 4 weeks. Protein content was determined by the method of Lowry et al. (20). The Ouchterlony immunodiffusion method (21) was used to test for LDL contamination in the HDL fraction and vice versa. Using rabbit antisera against human LDL and HDL, respectively, no cross-contamination was detected. The HDL preparation was analyzed for us by Dr. John J. Albers using a radioimmunoassay method for LDL; it contained less than 0.5% apoB-reactive material.

Lipoprotein-deficient fetal calf serum (LDS) was prepared by preparative ultracentrifugation of FCS at density 1.25 g/ml and dialysis against 0.15 M NaCl and PBS as previously described (7). The protein concentration was adjusted to 50 mg/ml by addition of PBS as required and the LDS was sterilized by filtration. The final cholesterol content, determined by gas–liquid chromatography as described below, was less than 2.5  $\mu\text{g}/\text{ml}$ .

$^{125}\text{I}$ -labeled HDL and LDL were prepared by a modification of the iodine monochloride method of McFarlane (22) as previously described (23). The  $^{125}\text{I}$ -labeled lipoproteins were dialyzed extensively against 20 mM Tris-HCL–0.15 M NaCl–0.3 mM EDTA, sterilized by filtration, and aliquots were taken for protein (20) and radioactivity determinations. Specific activities were 198–561 cpm/ng protein for  $^{125}\text{I}$ -HDL and 135–456 cpm/ng protein for  $^{125}\text{I}$ -LDL. Less than 3% of the total  $^{125}\text{I}$  in the final preparations was TCA-soluble and less than 2% was extractable into chloroform–methanol 2:1 (v/v). More than 93% of the radioactivity in  $^{125}\text{I}$ -HDL was precipitable with rabbit antiserum to human HDL; none was precipitated by rabbit antiserum to human albumin. Column chromatography of delipidated, lyophilized  $^{125}\text{I}$ -HDL on Sephadex G-100 (24) showed that approximately 53% of the radioactivity was in apoprotein A-I, 42% was in apoprotein A-II, and 5% was in C peptides.

The integrity of  $^{125}\text{I}$ -HDL was assessed by diluting it up to 20-fold with unlabeled HDL, maintaining a constant total concentration of 23  $\mu\text{g}/\text{ml}$ , and testing whether the reductions in binding, internalization, and degradation of the  $^{125}\text{I}$ -HDL by fibroblasts were theoretical or not. All three metabolic parameters were reduced in direct proportion to the extent of isotope dilution. In other words, the calculated values for total HDL binding, internalization, and degradation (in ng protein/mg cell protein) at each specific activity were unchanged, suggesting that the



**Fig. 1.** Recovery of radioactivity from cultured fibroblasts, following incubation with  $^{125}\text{I}$ -lipoproteins, by sequential washing and subsequent exposure to trypsin. Cells were incubated for 18 hr at  $37^\circ\text{C}$  in medium containing 5% LDS. This was then replaced with 2 ml of fresh medium containing 5% LDS and either  $91 \mu\text{g/ml}$  of  $^{125}\text{I}$ -LDL ( $\bullet$ ) or  $16.4 \mu\text{g/ml}$  of  $^{125}\text{I}$ -HDL ( $\circ$ ). After further incubation for 3 hr at  $0^\circ\text{C}$  the medium was removed and an aliquot taken for determination of total radioactivity. Each monolayer was then washed sequentially as follows: washes 1–6: 2 ml of ice-cold PBS were added and removed within 30 sec; wash 7: 2 ml of 0.05% trypsin in Versene buffer was added and the dishes incubated at  $37^\circ\text{C}$  for 3 min; wash 8: 2 ml of medium containing 10% FCS was added and removed within 30 sec. An aliquot of each was counted for  $^{125}\text{I}$  and the amount of radioactivity recovered in each wash expressed as a percentage of that originally present in the culture medium. Specific activities:  $^{125}\text{I}$ -LDL, 355 cpm/ng;  $^{125}\text{I}$ -HDL, 561 cpm/ng.

cells did not distinguish between labeled and unlabeled lipoprotein. Similar results have been reported for  $^{125}\text{I}$ -LDL (7). In other studies, recentrifugation of  $^{125}\text{I}$ -HDL and  $^{125}\text{I}$ -LDL at densities of 1.21 and 1.063 g/ml, respectively, was found to have no appreciable effect on the subsequent binding, internalization, or degradation of either lipoprotein.

### Lipoprotein uptake and degradation

Eighteen hours before an experiment, the medium was removed from each dish and replaced with 3 ml of fresh medium containing 5% (v/v) of LDS. Immediately before the experiment this was replaced with 2 ml fresh medium of the same composition. Cells studied at  $0^\circ\text{C}$  were placed on crushed ice for 15 min before the start of the experiment, and then held on ice in a  $4^\circ\text{C}$  cold room. Other incubations were performed at  $37^\circ\text{C}$  in a humidified incubator (95% air, 5%  $\text{CO}_2$ ).

At the end of an incubation with  $^{125}\text{I}$ -labeled lipoprotein, the medium was removed and an aliquot was assayed for total  $^{125}\text{I}$ . Lipoprotein degradation was determined on the remaining medium. Trichloroacetic acid was added to a final concentration of 10% and the mixture was placed in a boiling water bath for 5 min. After centrifugation (3000 g for 10 min) an aliquot of the supernatant was treated to remove free iodide by oxidation with  $\text{H}_2\text{O}_2$  and extraction of  $\text{I}_2$  into chloroform (25). All degradation data refer to noniodide, TCA-soluble radioactivity. In a medium containing 5% LDS, the rate of release of free iodide from  $^{125}\text{I}$ -HDL in the absence of cells was one-fifth of that from  $^{125}\text{I}$ -LDL; 2.5–3% of the total medium radioactivity was in the iodide fraction. The presence of fibroblasts did not increase the rate of deiodination of either lipoprotein. Net degradation by fibroblasts was calculated as the difference between values obtained from identical incubations in the presence and absence of cells. It was demonstrated that there was no intracellular accumulation of degradation products, i.e., TCA-soluble, noniodide  $^{125}\text{I}$  accounted for less than 10% of total radioactivity within the cells after an 18 hr incubation.

After removal of the medium, the cells were washed six times with 2 ml PBS at  $0^\circ\text{C}$ . Two ml of 0.05% trypsin in 0.54 mM Versene buffer (pH 7.4) was added to each plate and incubated at  $37^\circ\text{C}$  for 3–4 min. The cells were collected and the plates scraped with two 1-ml aliquots of DME containing 10% FCS to limit trypsin degradation of lipoprotein. The cells were separated by centrifugation (3000 g for 10 min) at  $4^\circ\text{C}$ , and an aliquot of the supernatant fluid was assayed for  $^{125}\text{I}$  radioactivity. The  $^{125}\text{I}$  released by trypsin after incubations with  $^{125}\text{I}$ -LDL is >95% precipitable with TCA and includes the same small percentage of lipid radioactivity found in the LDL preparations used (7). After incubations with  $^{125}\text{I}$ -HDL, as much as 30% of the  $^{125}\text{I}$  released by trypsin treatment was soluble in TCA. Control experiments in which  $^{125}\text{I}$ -HDL was incubated with trypsin in the absence of cells showed that 50–60% of this TCA-soluble component was not due to direct digestion of HDL by trypsin. Thus some fraction of the bound  $^{125}\text{I}$  may represent binding of TCA-soluble material in the  $^{125}\text{I}$ -HDL preparations. The release of radioactivity from dishes that had been incubated with  $^{125}\text{I}$ -LDL or  $^{125}\text{I}$ -HDL in the absence of cells was only 4–8% of that removed from identical dishes containing cells. The amount of radioactivity in the final PBS wash was shown to be less than 4% of that subsequently released by trypsin (Fig. 1). Values for  $^{125}\text{I}$ -HDL and  $^{125}\text{I}$ -LDL binding were reduced by less than



10–15% when the 4th and 5th PBS washes included albumin (2 mg/ml) and the plates were shaken for 2 min per wash.

The cells were washed by suspension in 4 ml PBS and centrifugation at 3000 *g* for 20 min. The pellet was dissolved in 0.2 ml 1 N KOH (20°C, 24 hr) and assayed for <sup>125</sup>I. The cell digest was then diluted with water to 1 ml, and aliquots were removed for protein assay (20) and measurement of total <sup>125</sup>I, lipid-soluble <sup>125</sup>I (chloroform–methanol 2:1 v/v) and TCA-soluble <sup>125</sup>I (final concentration, 10%). The TCA-precipitable, nonlipid radioactivity remaining associated with the cells after trypsinization was considered to be a measure of internalized lipoprotein (7, 25, 26).

Data on lipoprotein concentrations and on binding, internalization, and degradation are expressed in terms of lipoprotein protein unless otherwise stated. In experiments with replicate dishes, the coefficients of variation for binding, internalization, and degradation averaged 10.3, 5.4, and 10.9%, respectively.

#### Sterol synthesis and cell cholesterol content

Eighteen hours before an experiment the medium was removed from each dish and replaced with 3 ml of medium containing 5% LDS. At the beginning of the experiment this was replaced with 2 ml of fresh medium containing 5% LDS with or without added unlabeled lipoproteins. After further incubation for 17 hr, 2  $\mu$ Ci of [1-<sup>14</sup>C]acetate and unlabeled carrier acetate (final concentration, 0.1  $\mu$ mol/ml) were added to the dish and incubation was continued for 1 hr. The medium was then removed and the cells were washed with 3  $\times$  2 ml PBS before being harvested with a rubber policeman. The cells were pelleted by centrifugation (3000 *g*, 10 min, 4°C) and dissolved by incubating overnight at room temperature in 0.2 ml 1 N KOH. The dissolved pellet was diluted to 1.0 ml with water and aliquots were removed for protein assay (20). After addition of 5  $\mu$ g of 5- $\alpha$ -cholestane to serve as internal standard, the remainder was saponified (1 N KOH in 70% ethanol for 2–3 hr at 60°C), diluted with 1.0 ml water, and the nonsaponifiable lipids were extracted with 4.2 ml of hexane. The hexane layer was washed with 0.1 M sodium acetate, and an aliquot was taken for radioactivity determination (scintillation solution: 40 mg of 2,5-diphenyloxazole and 0.5 mg of [1,4-bis-2-(5-phenyloxazolyl)-benzene] in 10 ml of toluene). It has been shown that the incorporation of acetate into sterols remains linear for 3 hr under these conditions, and that increasing the total acetate concentration by addition of further unlabeled acetate does not in-

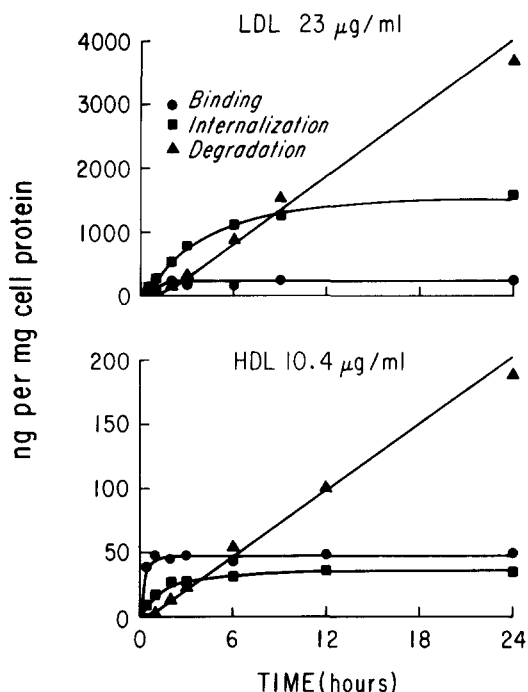
crease the rate of incorporation (7). Data for sterol synthesis refer to the incorporation of <sup>14</sup>C radioactivity into nonsaponifiable lipids. The remainder of the hexane phase was concentrated under N<sub>2</sub>, and the cholesterol content determined by gas–liquid chromatography (injection volume, 2–5  $\mu$ l; column: 91 cm  $\times$  2 mm, 3% SP-2250 on 80/100 mesh Supelcoport, 200°C) (27).

The aqueous phase of the original saponification mixture was acidified with 1 ml of 12 M HCl and extracted with 4 ml of hexane. The fatty acid fraction (hexane) was washed with 0.1 M sodium acetate and an aliquot removed for scintillation counting.

The transfer of newly-synthesized sterol to the medium was monitored by measuring the radioactivity in the nonsaponifiable lipid fraction of the medium.

#### [<sup>14</sup>C]Sucrose uptake (fluid endocytosis)

The uptake of radiolabeled sucrose by cultured fibroblasts has been shown to be a suitable marker for the quantitation of pinocytosis (28, 29). Eighteen hours before an experiment the medium was removed from each dish and replaced with medium containing 5% LDS. At the beginning of the experiment this was replaced with fresh medium containing 5% LDS and D-[U-<sup>14</sup>C]sucrose at a concentration of 5.5  $\mu$ mol/l (2.5  $\mu$ Ci/ml). After further incubation at 37°C for 18 hr, the medium was removed and an aliquot added to 15 ml of scintillation solution (10.5 ml of toluene, 4.5 ml of Triton, 60 mg of 2,5-diphenyloxazole, 0.8 mg of [1,4-bis-2-(5-phenyloxazolyl)-benzene]) for determination of <sup>14</sup>C radioactivity. The cells were washed 10 times with 3 ml of PBS, harvested with a rubber policeman, and dissolved in 0.2 ml of 1 N NaOH. Aliquots were taken for protein determination (20) and liquid scintillation counting. Counting efficiency, measured by internal standardization, averaged 84%. Clearance of [<sup>14</sup>C]sucrose ( $\mu$ l medium/mg cell protein) was calculated by dividing the uptake of radioactivity by the cells (cpm/mg cell protein) by the concentration of radioactivity in the medium (cpm/ $\mu$ l). The coefficient of variation was 8.6%. [<sup>14</sup>C]sucrose uptake was shown to be linear for at least 27 hr, and to be unaffected by the addition of unlabeled sucrose to the medium up to a concentration of 45  $\mu$ mol/l. The total radioactivity in the final PBS wash was less than 3% of that subsequently associated with the cells. There was no significant release of radioactivity from cells that had been pre-incubated for 18 hr with [<sup>14</sup>C]sucrose during a second incubation of 3 hr in sucrose-free medium.



**Fig. 2.** Time courses for binding (●), internalization (■), and degradation (▲) of human  $^{125}\text{I}$ -LDL (upper panel) and  $^{125}\text{I}$ -HDL (lower panel) by cultured normal human fibroblasts at  $37^\circ\text{C}$ . Cell monolayers were first incubated for 18 hr in medium containing 5% LDS. This was then replaced by fresh medium containing 5% LDS and either  $23\ \mu\text{g/ml}$  of  $^{125}\text{I}$ -LDL (specific activity, 355 cpm/ng) or  $10.4\ \mu\text{g/ml}$  of  $^{125}\text{I}$ -HDL (239 cpm/ng). After the indicated times of incubation at  $37^\circ\text{C}$ , the cells were harvested and the extent of binding, internalization, and degradation of the lipoproteins determined as described under Methods. Results are given as the means of replicate determinations (2–4 dishes).

## RESULTS

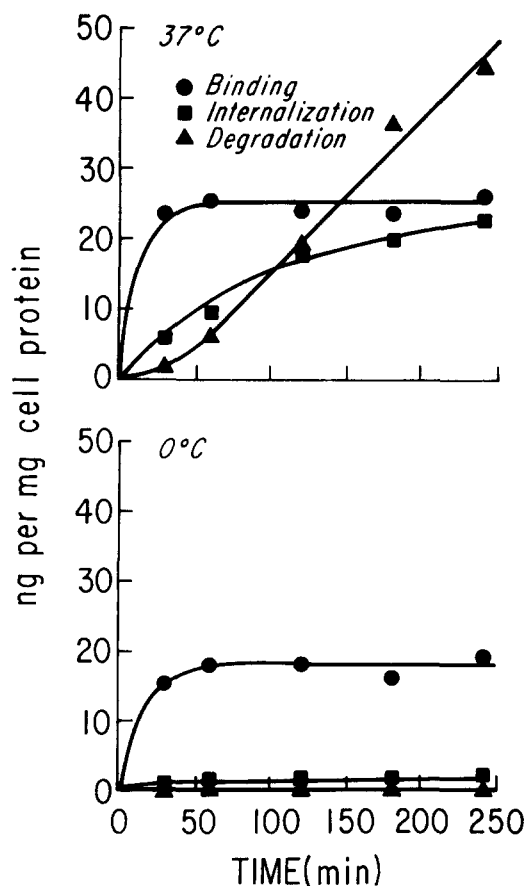
### Binding, internalization, and degradation

The time course for surface binding, internalization, and degradation of  $^{125}\text{I}$ -labeled HDL incubated with normal human skin fibroblasts at  $37^\circ\text{C}$  is shown in **Fig. 2**. Data for labeled LDL are shown for direct comparison. Binding of each lipoprotein was relatively rapid, showing little increase beyond 1–2 hr; the amount internalized continued to increase for several hours but reached a plateau by 6–12 hr. In contrast, degradation proceeded linearly for at least 24 hr, after an initial lag period of 30 min. As discussed below in greater detail, the internalization and degradation of HDL relative to surface binding was much less than the internalization and degradation of LDL.

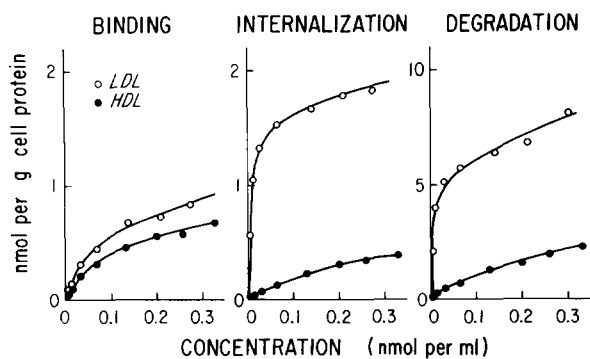
As previously reported for LDL (7), the rates of internalization and degradation of HDL were strongly temperature dependent, whereas surface binding was relatively independent of temperature. In **Fig. 3**

are shown the binding, internalization, and degradation of HDL as functions of time at  $37^\circ\text{C}$  and at  $0^\circ\text{C}$ . At  $0^\circ\text{C}$  little internalization or degradation was detectable whereas both processes were very active at  $37^\circ\text{C}$ . In contrast, surface binding at  $0^\circ\text{C}$  was only slightly less than that at  $37^\circ\text{C}$ .

Because the rates of internalization and degradation of HDL were so low relative to those of LDL, comparisons of the two lipoproteins were made using incubations of 6–18 hr duration in order to obtain values less influenced by background corrections (no-cell controls). The results of a representative experiment (18-hr incubations) are presented in **Fig. 4**. The values for concentration of lipoprotein in the medium are here expressed in molar terms (30–33) to permit a direct comparison of the two



**Fig. 3.** Time courses for binding (●), internalization (■), and degradation (▲) of  $^{125}\text{I}$ -HDL by cultured human fibroblasts at  $37^\circ\text{C}$  (upper panel) and at  $0^\circ\text{C}$  (lower panel). Cells were first incubated for 18 hr in medium containing 5% LDS. This was then replaced by fresh medium containing 5% LDS and  $7.7\ \mu\text{g/ml}$   $^{125}\text{I}$ -HDL (specific activity: 239 cpm/ng) and the dishes were incubated at  $37^\circ\text{C}$  or  $0^\circ\text{C}$ . After the indicated periods of time, the cells were harvested and the extent of binding, internalization, and degradation determined as described under Methods.



**Fig. 4.** Binding (left panel), internalization (middle panel), and degradation (right panel) of  $^{125}\text{I}$ -LDL (○) and  $^{125}\text{I}$ -HDL (●) by normal human fibroblasts at  $37^\circ\text{C}$  as a function of lipoprotein concentration. After an 18 hr incubation in medium containing 5% LDS, fresh medium was added containing 5% LDS and the indicated concentrations of  $^{125}\text{I}$ -HDL (specific activity: 561 cpm/ng) or  $^{125}\text{I}$ -LDL (355 cpm/ng). After a further 18 hr incubation at  $37^\circ\text{C}$ , the cells were harvested and the extent of binding, internalization, and degradation determined as described under Methods. Molar values were calculated from the relationships: 1 mg LDL protein  $\sim$  1.8 nmol LDL; 1 mg HDL protein  $\sim$  9.0 nmol HDL (see Results).

lipoproteins on a particle basis<sup>3</sup>. The binding, internalization, and degradation of each lipoprotein increased as functions of concentration, but the relationships were not linear. The binding of HDL was only slightly less than that of LDL at equimolar medium concentrations. In contrast, the rates of internalization and degradation of HDL were much less than the corresponding rates for LDL. This difference was most marked at concentrations below 0.02 nmol/ml (1.8  $\mu\text{g}$  protein/ml for HDL; 11  $\mu\text{g}$ /ml for LDL). At these lower concentrations the rates of internalization and degradation of HDL were less than 5% of those of LDL.

While only 5–6% of the  $^{125}\text{I}$  in the HDL preparation resided in the C apoprotein fraction, we considered the possibility that this fraction, which exchanges readily among lipoproteins, might contribute disproportionately to the observed binding and degradation results. Two sterilized  $^{125}\text{I}$ -HDL preparations were incubated 48 hr at  $37^\circ\text{C}$  with a large excess of unlabeled VLDL (25 mg of VLDL protein plus 2 mg of  $^{125}\text{I}$ -HDL protein) and reisolated. The fraction of  $^{125}\text{I}$  in apo C was reduced in one case to 1.1% and in the other to 0.3% of the total. Comparison of these labeled HDL preparations with

<sup>3</sup> Molar lipoprotein concentrations were calculated assuming a molecular weight of 550,000 for LDL protein (30, 31) and a molecular weight of 110,000 for HDL protein (32). Thus a 5:1 molar ratio of HDL to LDL corresponds to a 1:1 protein concentration ratio. It was assumed that HDL in the density range used (1.09–1.21 g/ml) consisted predominantly of HDL<sub>3</sub> (refs. 32, 33).

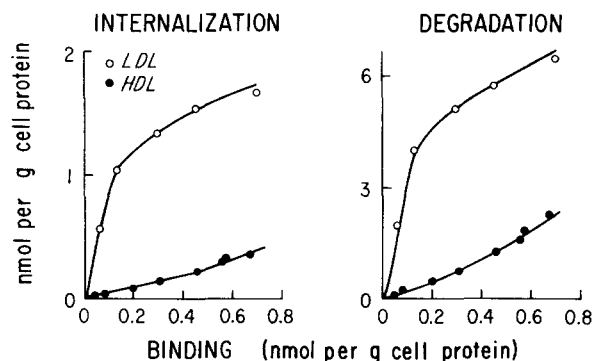
the original, untreated labeled HDL showed that binding, internalization, and degradation were altered only slightly (23% reduction in binding, 7% reduction in internalization, and 16% reduction in degradation).

The relationships of internalization and degradation to binding for both lipoproteins are shown in Fig. 5. At any given degree of binding, internalization of HDL was much less than that of LDL. The relationship of internalization to binding for LDL was curvilinear, the slope decreasing very obviously beyond a binding figure of 0.15–0.3 nmol/g cell protein (75–150 ng protein/mg cell protein; medium LDL concentration, 10–25  $\mu\text{g}$  protein/ml). In contrast, the same relationship for HDL was, with different preparations, either linear or slightly concave upwards. Similar results were obtained when degradation was related to binding (Fig. 5).

The nature of the HDL binding sites was explored by examining the effect on binding of prior exposure of the cell surface to mild proteolytic digestion with pronase. Pronase treatment (2  $\mu\text{g}/\text{ml}$  in DME without LDS;  $37^\circ\text{C}$ ; 25 min) only slightly reduced the subsequent binding of  $^{125}\text{I}$ -HDL ( $0^\circ\text{C}$ , 1 hr) at concentrations of 1–100  $\mu\text{g}/\text{ml}$  (mean decrease, 11%). In contrast, prior treatment of the cells with pronase markedly reduced the binding of  $^{125}\text{I}$ -LDL at LDL concentrations of 5, 20, and 100  $\mu\text{g}/\text{ml}$  (by 76, 64, and 50%, respectively).

#### [ $^{14}\text{C}$ ]Sucrose uptake

The more rapid internalization of LDL than HDL might reflect a stimulatory effect of the former on pinocytosis. Therefore, [ $^{14}\text{C}$ ]sucrose uptake was measured in the absence and in the presence of the lipo-



**Fig. 5.** Relationships of internalization (left panel) and degradation (right panel) to surface binding for  $^{125}\text{I}$ -LDL (○) and  $^{125}\text{I}$ -HDL (●) after incubation at different concentrations ( $^{125}\text{I}$ -LDL: 3.5–69  $\mu\text{g}$  protein/ml;  $^{125}\text{I}$ -HDL: 0.7–33  $\mu\text{g}$  protein/ml) with normal fibroblast monolayers for 18 hr at  $37^\circ\text{C}$ . Same experiment as Figure 4. 1 mg LDL protein  $\sim$  1.8 nmol LDL; 1 mg HDL protein  $\sim$  9.0 nmol HDL (see Results).

TABLE 1. Volume of medium cleared of [<sup>14</sup>C]sucrose, <sup>125</sup>I-LDL, or <sup>125</sup>I-HDL during incubation with normal human fibroblasts

Addition to Medium (concentration)	Clearance		
	[ <sup>14</sup> C]Sucrose	<sup>125</sup> I-LDL	<sup>125</sup> I-HDL
	<i>μl medium/mg cell protein</i>		
[ <sup>14</sup> C]Sucrose (5.5 μmol/l)	3.40		
[ <sup>14</sup> C]Sucrose (5.5 μmol/l) plus:			
LDL (5 μg/ml)	3.35		
LDL (250 μg/ml)	3.03		
HDL (1 μg/ml)	3.43		
HDL (50 μg/ml)	3.79		
HDL (250 μg/ml)	3.38		
<sup>125</sup> I-LDL (5 μg/ml)		595	
<sup>125</sup> I-LDL (250 μg/ml)		25.6	
<sup>125</sup> I-HDL (1 μg/ml)			24.9
<sup>125</sup> I-HDL (50 μg/ml)			10.6
<sup>125</sup> I-HDL (250 μg/ml)			5.9

Cell monolayers were incubated in medium containing 5% LDS for 18 hr. The medium was then replaced with fresh medium containing 5% LDS and the indicated concentrations of D-[U-<sup>14</sup>C]sucrose (453 mCi/mmol) and/or lipoproteins. After 18 hr further incubation at 37°C, the medium was removed and the cells were harvested for measurement of [<sup>14</sup>C]sucrose uptake or <sup>125</sup>I-labeled lipoprotein internalization and degradation, as described under Methods. The volume of medium cleared of sucrose was calculated by dividing the uptake of radioactivity (cpm/mg cell protein) by the concentration of radioactivity in the medium (cpm/μl). Clearance of <sup>125</sup>I-labeled lipoproteins was calculated by dividing internalized plus degraded lipoprotein (ng/mg cell protein) by the concentration of lipoprotein in the medium (ng/μl). Each result is the mean of duplicate determinations. Specific activities: <sup>125</sup>I-LDL, 221 cpm/ng; <sup>125</sup>I-HDL, 128 cpm/ng.

proteins. The results of a representative experiment are summarized in **Table 1**. Neither LDL nor HDL had any effect on the rate of removal of sucrose from the culture medium during an 18 hr incubation at 37°C. The clearance of <sup>125</sup>I-LDL at 5 μg/ml in the medium, measured in the same experiment, was more than 100-fold greater than that of sucrose. The clearance of <sup>125</sup>I-HDL, on the other hand, was only 7-fold greater than that of sucrose even at the lowest concentration (1 μg/ml) and less than 2-fold greater at higher HDL levels (250 μg/ml). A closer analysis of the uptake of lipoproteins in relation to pinocytosis is presented in the Discussion.

#### Cell cholesterol and sterol synthesis

As reported from previous studies (7, 34–36), incubation of fibroblasts at 37°C with LDL (102 μg protein/ml; 243 μg cholesterol/ml) for 18 hr was associated with a net increase in cell cholesterol content and a marked inhibition of [1-<sup>14</sup>C]acetate incorporation into cell sterols relative to values obtained with cells incubated in the absence of lipoproteins. These findings were confirmed as shown in **Table 2**. In contrast, incubation with a similar molar concentration of unlabeled HDL (protein: 20 μg/ml; cholesterol: 4.9 μg/ml) had no measurable effect on cell cholesterol content or on sterol synthesis. Incubation with HDL even at a much higher molar concentration (protein: 978 μg/ml; cholesterol: 241 μg/ml) also

failed to measurably alter cell cholesterol content. At these higher concentrations of HDL, sterol synthesis was inhibited but the inhibition was not selective, i.e., there were proportionate reductions of [1-<sup>14</sup>C]acetate incorporation into cell sterols and fatty acids.

The recovery of radioactivity in the sterol fraction of the culture medium, relative to the total radioactivity in cell sterols (or cell plus medium sterols), was greater (*P* < 0.01) following incubations with HDL than following incubations with LDL at the same cholesterol concentration.

#### DISCUSSION

In the present experiments the metabolism of HDL and LDL by fibroblasts was examined using lipoproteins labeled with <sup>125</sup>I in the protein moiety. Validation of the methods used for studying LDL has been previously reported (7, 25, 36). The validity of using <sup>125</sup>I-HDL as a tracer for HDL was established by dilution analysis, which showed that fibroblasts do not differentiate between labeled and unlabeled HDL molecules. Surface binding of lipoprotein was quantified from the release of radioactivity upon brief exposure of the cells to trypsin. Radioactivity that was not released by trypsin, and that was neither lipid- nor TCA-soluble, was considered to represent internalized lipoprotein. Bierman, Stein,



TABLE 2. Effects of high density and low density lipoproteins on cell cholesterol content and the synthesis of sterols and fatty acids from [ $^{14}\text{C}$ ]acetate in normal human fibroblasts

	Lipoprotein Additions to the Medium			
	None	LDL	HDL	HDL
$\mu\text{g}$ protein/ml		102	20	978
approx. nmol/ml		0.18	0.22	10.7
$\mu\text{g}$ cholesterol/ml		243	4.9	241
Cell cholesterol content ( $\mu\text{g}/\text{mg}$ cell protein)	$27.9 \pm 0.85$	$38.6 \pm 1.9$ (4) $P < 0.001$	$26.4 \pm 2.6$ (5) NS	$29.9 \pm 1.1$ (4) NS
Sterol synthesis (cpm/mg cell protein)	$14,197 \pm 878$ (5)	$3,791 \pm 124$ (4) $P < 0.001$	$14,095 \pm 1,004$ (5) NS	$7,779 \pm 543$ (4) $P < 0.001$
Fatty acid synthesis (cpm/mg cell protein)	$51,068 \pm 2,762$ (5)	$42,095 \pm 2,076$ NS	$50,831 \pm 1,824$ (5) NS	$35,419 \pm 1,962$ (4) $P < 0.005$
Radioactivity in medium sterols (% of total in- corporation into sterols)	$1.25 \pm 0.19$ (5)	$3.94 \pm 0.35$ (4)	$1.40 \pm 0.10$ (5)	$6.08 \pm 0.76$ (4)

Cell monolayers were incubated in medium containing 5% LDS for 18 hr. The medium was then replaced with 2 ml of fresh medium containing 5% LDS and the indicated concentration of HDL or LDL. After further incubation for 17 hr at  $37^\circ\text{C}$ ,  $2 \mu\text{Ci}/\text{ml}$  of [ $^{14}\text{C}$ ]acetate ( $58 \text{ mCi}/\text{mmol}$ ) and unlabeled carrier acetate were added (final concentration,  $0.1 \mu\text{mol}/\text{ml}$ ) and the incubation continued for 60 min. The cells were then harvested for measurement of cell cholesterol content and incorporation of radioactivity into cell sterols, cell fatty acids, and medium sterols as described under Methods. Results are expressed as: mean  $\pm$  SEM (number of replicates). Probability values were obtained by  $t$  test analysis against those results obtained in the absence of lipoproteins. NS, not statistically significant ( $P > 0.05$ ).

and Stein (25) have confirmed by radioautography that the protein radioactivity associated with rat aortic smooth muscle cells is mostly intracellular following incubation with  $^{125}\text{I}$ -HDL and subsequent trypsinization. Evidence for the validity of the method as applied in the present studies is provided by the demonstration that the amount of radioactivity released by trypsin after incubation with  $^{125}\text{I}$ -HDL at  $0^\circ\text{C}$  is comparable to that released after incubation at  $37^\circ\text{C}$ , whereas that not released by trypsin is an order of magnitude lower at  $0^\circ\text{C}$  than at  $37^\circ\text{C}$  (Fig. 3). Furthermore, during incubation at  $37^\circ\text{C}$  the amount of trypsin-releasable  $^{125}\text{I}$ -HDL was near maximum after 1 hr, whereas that remaining after trypsinization continued to rise for 6–12 hr (Fig. 2).

The surface binding of HDL by normal human fibroblasts, expressed in molar terms, was only slightly less than that of LDL at equimolar concentrations of lipoprotein in the medium. Except at very low HDL concentrations, binding was a linear function of HDL concentration; the same was true for internalization and degradation. In contrast, the internalization and degradation of LDL was relatively much greater at low than at high LDL concentrations. Thus, as shown by Goldstein and co-workers (4, 35), LDL appears to bind with higher affinity at low concentrations but there is little if any comparable high-affinity binding in the case of HDL. In the case of LDL, Goldstein and Brown (4) have reported that the high-affinity, but not low-

affinity, uptake of LDL is reduced by prior treatment of the cells with pronase (4). This effect was confirmed in the present studies. No evidence was obtained, however, for a similar selective effect of pronase on the binding sites for HDL. This marked difference in the effects of proteolytic enzymes on LDL and HDL binding has been examined in detail by Koschinsky, Carew, and Steinberg (38).

Despite the comparable density of surface binding of HDL and LDL, HDL was internalized and degraded much more slowly than LDL at equimolar concentrations (Fig. 4). Stein and Stein (39) and Carew et al. (40) have reported similar findings with regard to the binding and internalization of homologous LDL and HDL by rat and swine aortic smooth muscle cells in culture. In cultured human arterial smooth muscle cells Bierman and Albers (41) have observed a much lower rate of internalization of HDL than of LDL at equal lipoprotein protein concentrations. However, these investigators compared only the intracellular accumulation of the two lipoproteins without reference to possible differences in surface binding and degradation.

When internalization and degradation were related to the extent of surface binding, a further notable difference between LDL and HDL emerged. The slopes of both relationships for LDL showed a decrease beyond a binding figure of 75–150 ng/mg cell protein (Fig. 5). Thus, the rate of internalization of LDL relative to its binding was greatest at



TABLE 3. Comparison of observed lipoprotein uptake with the calculated sum of uptake by fluid endocytosis and adsorptive endocytosis

Labeled Lipoprotein in Medium	Observed Surface Binding <sup>a</sup> (ng/mg)	Calculated Uptake (ng/mg)			Observed Lipoprotein Uptake (ng/mg)
		By Fluid Endocytosis <sup>b</sup>	By Adsorptive Endocytosis <sup>c</sup>	Sum	
HDL, 1 $\mu\text{g/ml}$	6	3.4	26	29	24
HDL, 50 $\mu\text{g/ml}$	120	170	530	700	530
HDL, 250 $\mu\text{g/ml}$	325	850	1,436	2,286	1,475
LDL, 5 $\mu\text{g/ml}$	48	17	212	229	2,975
LDL, 250 $\mu\text{g/ml}$	505	850	2,236	3,086	6,400

<sup>a</sup> Mean value for binding at 37°C from studies like that shown in Figure 5.

<sup>b</sup> Mean value for sucrose clearance (3.4  $\mu\text{l/mg}$  cell protein per 18 hr)  $\times$  concentration of lipoprotein in medium (ng/ $\mu\text{l}$ ).

<sup>c</sup> Density of lipoproteins binding to cell surface (ng/ $\mu\text{m}^2$ )  $\times$  total area of cell surface internalized in 18 hr due to pinocytosis ( $\mu\text{m}^2/\text{mg}$  cell protein per 18 hr). Cell surface area was calculated assuming a mean cell diameter of 25  $\mu\text{m}$  and using the formula proposed by Steinman, Brodie, and Cohn (28) to estimate true surface area (3PIID<sup>2</sup>). From cell counts and cell protein measurements, 1 mg cell protein represents about  $2.5 \times 10^6$  cells. Mean surface area and volume of pinocytotic vessels were assumed to be 0.162  $\mu\text{m}^2$  and 0.00847  $\mu\text{m}^3$ , respectively, as reported by Steinman, Brodie, and Cohn (28) for the mouse fibroblast L cell line.

<sup>d</sup> Sum of observed internalization and degradation over 18 hr.

low concentrations, suggesting that internalization was occurring more readily when binding was to high-affinity, rather than to low-affinity, sites. In contrast, the relationships of internalization and degradation to surface binding for HDL showed no tendency to flatten over a similar range of surface binding densities. In fact, the tendency in most experiments (Fig. 5) was for the slopes of the curves to increase slightly as a function of increasing surface binding. These results indicate that the entry of HDL into the cell interior is equally probable no matter which sites it occupies.

The precise mechanisms by which cells internalize plasma lipoproteins have not been elucidated. One theoretical possibility is that both LDL and HDL are taken up by pinocytosis occurring generally over the entire cell surface. The low rate of internalization of HDL relative to that of LDL, despite a similar density of surface binding, might then reflect differing effects of the two lipoproteins on the overall rate of pinocytosis—either an inhibition by HDL or a stimulation by LDL. However, neither HDL nor LDL had any measurable effect on the rate of uptake of [<sup>14</sup>C]sucrose by fibroblasts (Table 1); the latter has been shown by others to be a valid measure of bulk fluid uptake by pinocytosis (28, 29). In the same experiments, the rates of clearance of <sup>125</sup>I-LDL and <sup>125</sup>I-HDL from the culture medium were compared with that of sucrose. The volume of medium cleared of LDL during the incubation was substantially greater than that of sucrose; at low concentrations there was more than a 100-fold difference. This is consistent with the report by Goldstein and Brown (4)

that the rate of clearance of LDL by normal human fibroblasts is an order of magnitude greater than that of albumin. On the other hand, the present studies show that the volume of medium cleared of HDL was only slightly greater than that of sucrose, the difference again being greater at low than at high concentrations.

From the sucrose uptake data it is possible to calculate the expected rates of uptake of LDL and HDL referable to the bulk uptake of medium during pinocytosis. Those values are shown in Table 3, column 3. Observed uptake (the sum of lipoprotein internalized and that degraded) was considerably greater, as shown in the last column of Table 3. An additional component of uptake could reflect internalization of lipoprotein bound to those areas of the cell membrane that invaginate during pinocytosis and thus become the walls of the forming vesicles (adsorptive endocytosis, ref. 29). The latter can be calculated from the measured surface binding of lipoproteins and the sucrose clearance if it is assumed that binding and pinocytosis both occur randomly over the cell surface. Values for the mean volume and surface area of the pinocytotic vesicles were taken from those determined for mouse fibroblasts (L cells) by Steinman, Brodie, and Cohn (29) (see legend to Table 3). The sum of the calculated uptakes by fluid and adsorptive endocytosis are shown in Table 3, column 5, and the observed uptake in column 6. In the case of HDL, the calculated and observed values were reasonably close, especially at low HDL concentrations. In contrast, the observed uptake of LDL at 5  $\mu\text{g/ml}$  in the medium was an order of

magnitude greater than that attributable to fluid and adsorptive endocytosis using the same assumptions. At 250  $\mu\text{g/ml}$  LDL, observed uptake was twice that calculated for ordinary endocytosis. These data support the operation of an efficient internalization mechanism for LDL that is somehow related to the high-affinity binding of LDL to the cell membrane. Since the presence of LDL did not measurably increase fluid endocytosis as measured by [ $^{14}\text{C}$ ]-sucrose uptake, it becomes necessary to postulate either: (a) a selective stimulation of endocytosis at LDL binding sites, these representing so small a fraction of the cell surface that the increment in accompanying fluid uptake is too small to measure; or (b) that LDL is internalized by some mechanism that does not involve the uptake of extracellular fluid. The selective binding of ferritin-labeled LDL to "coated pits"—specialized areas of the cell membrane believed to play a special role in protein uptake (42–44)—recently reported by Anderson, Goldstein, and Brown (45) to correlate with high-affinity binding could very well represent the specific, nonrandom site for LDL internalization. On the other hand, the internalization of HDL by fibroblasts can be explained by the internalization of medium (with its HDL content) plus invagination of membrane (with its complement of surface-bound HDL) during pinocytosis without the need to postulate specialized sites of binding.

These differences in the internalization of LDL and HDL by fibroblasts despite similar overall density of binding imply that the high affinity site for LDL either does not bind HDL to a significant degree, or does not internalize HDL subsequent to binding. While the present results do not permit certain distinction between these possibilities, the data available suggest at least that HDL binds mostly to sites other than the high-affinity site for LDL. Thus, the binding of HDL over a wide range of concentrations was reduced only slightly following treatment of the cells with pronase, whereas that of LDL at low concentration (5  $\mu\text{g/ml}$ ) was reduced by 76%. Secondly, Koschinsky, Carew, and Steinberg (38) have recently shown that pre-incubation of fibroblasts with cholesterol, which was shown by Brown and Goldstein (46) to reduce the number of LDL binding sites, does not reduce the number of HDL binding sites. Finally, we have found in other studies that neither the high affinity nor the low affinity binding of HDL is reduced in fibroblasts from subjects with homozygous familial hypercholesterolemia (15), which appear to lack the high affinity binding site for LDL (4, 37). Other data, however, have indicated that there is at least an interaction between HDL

(or some component in the d 1.090–1.21 lipoprotein fraction) and LDL binding sites. Thus, we have shown previously that HDL significantly inhibits the binding of LDL to human fibroblasts when the molar ratio of HDL to LDL is high, e.g., 25% inhibition at a molar ratio of 25:1 (15, 16, 47). Similar phenomena have been observed with swine aortic smooth muscle cells (40) and with human and rabbit endothelial cells (48, 49) in culture. LDL, reciprocally, reduces the binding of HDL (15, 16). It is not yet clear, however, whether this reflects a competition between LDL and HDL for common binding sites, or site–site interaction. It should be noted that when high concentrations of HDL are used to effect a significant reduction in LDL binding, only a small fraction of the HDL bound would have to be bound to LDL receptor sites.

In the experiment illustrated in Fig. 4, a 47-fold increase in  $^{125}\text{I}$ -HDL concentration (from 0.7 to 33  $\mu\text{g/ml}$ ) produced a proportionately greater increase (31-fold) in total uptake (internalized plus degraded) than in surface binding (14-fold). This is consistent with uptake by pinocytosis, since that fraction of uptake due to fluid endocytosis would be expected to remain a linear function of increasing concentration as the increments in surface binding progressively decrease. It is of interest that the increase in internalization (23-fold) was less than that in degradation (34-fold). A similar result was observed in other experiments. Internalized lipoprotein comprises an intracellular pool, the size of which will be determined by the relative rates of entry of lipoprotein into the cell and its subsequent degradation. The finding of a proportionately greater increase in  $^{125}\text{I}$ -HDL degradation than in internalization with increasing medium concentration indicates at least that the overall rate of HDL degradation is not a simple linear function of the total intracellular pool size. One possibility is that HDL molecules internalized by different processes (adsorptive or fluid endocytosis) have differing metabolic fates. Goldstein and Brown (4) have presented evidence that the metabolism of LDL by fibroblasts differs according to the mechanism of internalization.

There is evidence that the degradation of LDL by peripheral tissues may be quantitatively important *in vivo*. Thus, studies in this laboratory have shown that the fractional catabolic rate of LDL in swine and dogs is not reduced following hepatectomy (1, 2). Furthermore, fibroblasts cultured from subjects with familial hypercholesterolemia, who have defective LDL catabolism *in vivo* (3, 50), do not degrade LDL at a normal rate (4, 7). Carew et al. (14) have recently reported that the fractional catabolic rate

of neither LDL nor HDL was reduced following portacaval shunt in swine, suggesting that HDL catabolism also may occur to a large extent at the periphery. The present experiments demonstrate that at least one peripheral tissue in man is able to degrade HDL, albeit at a low rate relative to that of LDL. In the present experiments, the rate of HDL degradation by fibroblasts at a concentration of 75  $\mu\text{g}$  protein/ml (i.e., 5% of the normal plasma HDL concentration (32, 33)) averaged 30 ng protein/mg cell protein per hour. Extrapolating to a daily rate and expressing the result per kg of cell mass (assuming 15% protein by weight), a value of 105 mg HDL protein/kg per day is obtained. This is actually in excess of the estimated turnover rate for HDL in vivo (48, 49). Thus, the degradation of HDL by peripheral cells may make an important contribution to HDL catabolism in vivo.

In confirmation of previous reports (7, 34–36), the addition of LDL to fibroblasts previously incubated with lipoprotein-deficient medium increased the cholesterol content of the cells and selectively inhibited the biosynthesis of sterols from acetate, whereas incubation with HDL did not have these effects. Although a reduction of sterol synthesis was observed at high HDL concentrations, this was non-selective in that it was accompanied by a comparable reduction of acetate incorporation into fatty acids. The inhibition of sterol synthesis by LDL is due to suppression of HMG CoA reductase activity by free cholesterol released during intracellular degradation of the lipoprotein (6, 8, 9, 34). The failure of HDL to increase the cholesterol content of fibroblasts and thereby to inhibit HMG CoA reductase can now be explained, at least in part, by the low rate of internalization of HDL. Further, that HDL that is internalized would be less effective due to the low cholesterol content of HDL relative to LDL (31, 32). The possibility that HDL may have promoted an efflux of cholesterol from the cells also requires consideration, in view of the evidence for such an effect of HDL or delipidated HDL in studies with erythrocytes (51), aortic smooth muscle cells (52), ascites tumor cells (52), and L cell fibroblasts (53). It is of interest in this context that, following incubation of fibroblasts with [ $^{14}\text{C}$ ]acetate, a greater proportion of total sterol radioactivity was isolated from media containing HDL than from those containing LDL at a similar cholesterol concentration (Table 2). This could reflect either a greater efflux of cholesterol from the cells in the presence of HDL or a more rapid rate of exchange of cholesterol between HDL and the cell pool of newly synthesized cholesterol. ■

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## REFERENCES

1. Sniderman, A. D., T. E. Carew, J. G. Chandler, S. Hayes, and D. Steinberg. 1973. The role of the liver in metabolism of low density lipoproteins (LDL). *J. Clin. Invest.* **52**: 79a (Abstr).
2. Sniderman, A. D., T. E. Carew, J. G. Chandler, and D. Steinberg. 1974. Paradoxical increase in rate of catabolism of low-density lipoproteins after hepatectomy. *Science*. **183**: 526–528.
3. Langer, T., W. Strober, and R. I. Levy. 1972. The metabolism of low density lipoprotein in familial Type II hyperlipoproteinemia. *J. Clin. Invest.* **51**: 1528–1536.
4. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoprotein by cultured human fibroblasts. *J. Biol. Chem.* **249**: 5153–5162.
5. Goldstein, J. L., G. Y. Brunschede, and M. S. Brown. 1975. Inhibition of the proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A, and Triton WR 1339. *J. Biol. Chem.* **250**: 7854–7862.
6. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Nat. Acad. Sci. USA*. **72**: 2925–2929.
7. Stein, O., D. B. Weinstein, Y. Stein, and D. Steinberg. 1976. Binding, internalization, and degradation of low density lipoprotein by normal human fibroblasts and by fibroblasts from a case of homozygous familial hypercholesterolemia. *Proc. Nat. Acad. Sci. USA*. **73**: 14–18.
8. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1973. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts by lipoproteins. *Proc. Nat. Acad. Sci. USA*. **70**: 2162–2166.
9. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts. *J. Biol. Chem.* **249**: 789–796.
10. Miller, N. E., P. J. Nestel, and P. Clifton-Bligh. 1976. Relationships between plasma lipoprotein cholesterol concentrations and the pool size and metabolism of cholesterol in man. *Atherosclerosis*. **23**: 535–547.
11. Miller, G. J., and N. E. Miller. 1975. Plasma high density lipoprotein concentration and development of ischaemic heart disease. *Lancet* **I**: 16–19.



12. Castelli, W. P., J. T. Doyle, T. Gordon, C. Hames, S. B. Hulley, A. Kagan, D. McGee, W. J. Vicic, and W. J. Zukel. 1975. HDL cholesterol levels (HDLc) in coronary heart disease (CHD): a cooperative lipoprotein phenotyping study. *Circulation*. **51**: 11-97. (Abstr).
13. Rhoads, G. G., C. L. Gulbrandsen, and A. Kagan. 1976. Serum lipoproteins and coronary heart disease in a population study of Hawaiian Japanese men. *N. Engl. J. Med.* **294**: 293-298.
14. Carew, T. E., R. P. Saik, K. H. Johansen, C. A. Dennis, and D. Steinberg. 1976. Low density and high density lipoprotein turnover following portacaval shunting in swine. *J. Lipid Res.* **17**: 441-450.
15. Miller, N. E., D. B. Weinstein, and D. Steinberg. 1976. Metabolism of human lipoproteins, high density (HDL) and low density (LDL), by fibroblasts of normal and of homozygous familial hypercholesterolemic (FH) subjects. *Clin. Res.* **24**: 459A (Abstr.).
16. Steinberg, D., N. E. Miller, and D. B. Weinstein. 1976. Uptake of high density and low density lipoproteins by cultured human fibroblasts: relationship to endocytosis. *Circulation*. **54**: 11-210 (Abstr.).
17. Dulbecco, R., and G. Freeman. 1959. Plaque production by the Polyoma virus. *Virology*. **8**: 396-397.
18. Dulbecco, R., and M. Vogt. 1960. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**: 167-182.
19. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345-1353.
20. Lowry, O. H., H. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
21. Ouchterlony, Ö. 1964. Gel diffusion techniques. In *Immunological Methods*. J. J. Ackroyd, editor. Blackwell Scientific Publications, Oxford. 55-78.
22. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature (London)*. **182**: 53.
23. Sniderman, A. D., T. E. Carew, and D. Steinberg. 1975. Turnover and tissue distribution of low density lipoprotein apoprotein in swine and dogs. *J. Lipid Res.* **16**: 293-299.
24. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244**: 5687-5694.
25. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in culture. *Circ. Res.* **35**: 136-150.
26. Stein, O., and Y. Stein. 1975. Comparative uptake of rat and human serum low-density and high-density lipoproteins by rat aortic smooth muscle cells in culture. *Circ. Res.* **36**: 436-443.
27. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20  $\mu$ l of plasma. *J. Lipid Res.* **15**: 286-290.
28. Becker, G., and M. J. Ashwood-Smith. 1973. Endocytosis in Chinese hamster fibroblasts. *Exp. Cell Res.* **82**: 310-314.
29. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. *J. Cell Biol.* **68**: 665-687.
30. Bjorklund, R., and S. Katz. 1956. The molecular weights and dimensions of some human serum lipoproteins. *J. Amer. Chem. Soc.* **78**: 2122-2126.
31. Bragdon, J. H., R. J. Havel, and E. Boyle. 1956. Human serum lipoproteins. I. Chemical composition of four fractions. *J. Lab. Clin. Med.* **48**: 36-42.
32. Scanu, A., and J. L. Granda. 1966. Effects of ultracentrifugation on the human serum high-density ( $1.063 < \rho < 1.21$  g/ml) lipoprotein. *Biochemistry*. **5**: 446-455.
33. Gofman, J. W., and R. K. Tandy. 1967. Lipid transport in hyperlipidemia. In *Atherosclerotic Vascular Disease*. A. N. Brest, and J. H. Moyer, editors. Butterworths, London. 162-187.
34. Khachadurian, A. K., M. Lipson, and F. S. Kawahara. 1975. Diagnosis of familial hypercholesterolemia by measurement of sterol synthesis in cultured skin fibroblasts. *Atherosclerosis*. **21**: 235-244.
35. Brown, M. S., J. R. Faust, and J. L. Goldstein. 1975. Role of the low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.* **55**: 783-793.
36. Weinstein, D. B., T. E. Carew, and D. Steinberg. 1976. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* **424**: 404-421.
37. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell*. **7**: 85-95.
38. Koschinsky, T., T. E. Carew, and D. Steinberg. 1977. A comparative study of surface binding of human low density and high density lipoproteins to human fibroblasts: Regulation of sterols and susceptibility to proteolytic digestion. *J. Lipid Res.* **18**: 451-458.
39. Stein, O., and Y. Stein. 1975. Surface binding and interiorization of homologous and heterologous serum lipoproteins by rat aortic smooth muscle cells in culture. *Biochim. Biophys. Acta.* **398**: 377-384.
40. Carew, T. E., T. Koschinsky, S. B. Hayes, and D. Steinberg. 1976. A mechanism by which high-density lipoproteins may slow the atherogenic process. *Lancet*. **I**: 1315-1317.
41. Bierman, E. L., and J. J. Albers. 1975. Lipoprotein uptake by cultured human arterial smooth muscle cells. *Biochim. Biophys. Acta.* **388**: 198-202.
42. Fawcett, D. W. 1965. Surface specializations of absorbing cells. *J. Histochem. Cytochem.* **13**: 75-91.
43. Bennett, H. S. 1969. The cell surface: movements and recombinations. In *Handbook of Molecular Cytology*. A. Lima-de Faria, editor. North Holland Publishing Co., Amsterdam. 1294-1319.
44. Korn, E. D. 1975. Biochemistry of endocytosis. In *Biochemistry of Cell Walls and Membranes*. Vol. 2. C. R. Fox, editor. MTP International Review of Science, London. 1-26.
45. Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of low density lipoprotein receptors of plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc. Nat. Acad. Sci. USA.* **73**: 2434-2438.
46. Brown, M. S., and J. L. Goldstein. 1975. Regulation of



the activity of the low density lipoprotein receptor in human fibroblasts. *Cell*. **6**: 307–316.

47. Miller, N. E., D. B. Weinstein, T. E. Carew, T. Koschinsky, and D. Steinberg. 1977. Interaction between high density and low density lipoproteins during uptake and degradation by cultured human fibroblasts. *J. Clin. Invest.* In press.
48. Stein, O., and Y. Stein. 1976. High density lipoproteins reduce the uptake of low density lipoproteins by human endothelial cells in culture. *Biochim. Biophys. Acta*. **431**: 363–368.
49. Reckless, J. P. D., D. B. Weinstein, and D. Steinberg. 1976. Lipoprotein metabolism by rabbit arterial endothelial cells. *Circulation*. **54** (Suppl. II): 56.
50. Simons, L. A., D. Reichl, N. B. Myant, and M. Mancini. 1975. The metabolism of the apoprotein of plasma low density lipoproteins in familial hyperbetalipoproteinemia in the homozygous form. *Atherosclerosis*. **21**: 283–298.
51. Glomset, J. A. 1970. Physiological role of lecithin: cholesterol acyltransferase. *Amer. J. Clin. Nutr.* **23**: 1129–1136.
52. Stein, Y., M. C. Gangeaud, M. Fainaru, and O. Stein. 1975. The removal of cholesterol from aortic smooth muscle cells in culture and Landschutz ascites cells by fractions of human high-density apolipoprotein. *Biochim. Biophys. Acta*. **380**: 106–118.
53. Bates, S. R., and G. H. Rothblat. 1974. Regulation of cellular sterol flux and synthesis by human serum lipoprotein. *Biochim. Biophys. Acta*. **360**: 38–55.