Rat High Density Lipoprotein Subfraction (HDL₃) Uptake and Catabolism by Isolated Rat Liver Parenchymal Cells*

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Rat liver parenchymal cell binding, uptake, and proteolytic degradation of rat ¹²⁵I-labeled high density lipoprotein (HDL) subfraction, HDL₃ (1.110 < d < 1.210 g/ml), in which apo-A-I is the major polypeptide, were investigated. Structural and metabolic integrity of the isolated cells was verified by trypan blue exclusion, low lactic dehydrogenase leakage, expected morphology, and gluconeogenesis from lactate and pyruvate. ¹²⁵I-labeled HDL₃ was incubated with 10 x 10⁶ cells at 37°C and 4°C in albumin and Krebs-Henseleit bicarbonate buffer, pH 7.4. Binding and uptake were determined by radioactivity in washed cells. Proteolytic degradation was determined by trichloroacetic acid-soluble radioactivity in the incubation medium. At 37°C, maximum HDL₃ binding (B_max) and uptake occurred at 30 min with a B_max of 31 ng/mg dry weight of cells. The apparent dissociation constant of the HDL₃ receptor system (K_d) was 60 x 10⁻⁸ M, based on Mᵦ = 28,000 of apo-A-I, the predominant rat HDL₃ protein. Proteolytic degradation showed a 15-min lag and then constant proteolysis. After 2 hours 5.8% of incubated ¹²⁵I-labeled HDL₃ protein was degraded. Sixty per cent of cell radioactivity at 37°C was trypsin releasable. At 37°C, ¹²⁵I-labeled HDL₃ was incubated with cells in the presence of varying concentrations of native (cold) HDL₃, very low density lipoproteins, and low density lipoproteins. Incubation with native HDL₃ resulted in greatest inhibition of ¹²⁵I-labeled HDL₃ binding, uptake, and proteolytic degradation. When ¹²⁵I-labeled HDL₃ was preincubated with increasing amounts of HDL₃ antisera, binding and uptake by cells were decreased to complete inhibition. Cell binding, uptake, and proteolytic degradation of ¹²⁵I-labeled HDL₃ were markedly diminished at 4°C. Less than 1 mM chloroquine enhanced ¹²⁵I-labeled HDL₃ proteolysis but at 5 mM or greater, chloroquine inhibited proteolysis with ¹²⁵I-labeled HDL₃ accumulation in cells. L-[¹⁴C]lysine-labeled HDL₃ was bound, taken up, and degraded by cells as effectively as ¹²⁵I-labeled HDL₃. These data suggest that liver cell binding, uptake, and proteolytic degradation of rat HDL₃ are actively performed and linked in the sequence: binding, then uptake, and finally proteolytic degradation. Furthermore, there may be a specific HDL₃ (lipoprotein A) receptor or recognition site(s) on the plasma membrane. Finally, our data further support our previous reports of the important role of liver lysosomes in proteolytic degradation of HDL₃.

The definite cellular and subcellular site(s) and basic mechanisms involved in lipoprotein and apolipoprotein catabolism have not been established. Langer et al. reported data indicating that the pathologic elevation of plasma low density lipoprotein (LDL) concentration in individuals with familial type IIa hyperlipoproteinemia is due to decreased LDL degra-

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The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; B₄cAMP, dibutyryl adenosine 3'5'-monophosphoric acid.
isolated liver parenchymal cells and (b) the normal function of lysosomes. Furthermore, cultured human fibroblasts appear to regulate their intracellular cholesterol content by feedback control of the activity of the LDL receptor on the cell surface. However, the role of liver parenchymal cells in the uptake and degradation of lipoproteins is not known. Recently the method for isolation of liver parenchymal cells with satisfactory structural and metabolic integrity has been developed (12, 13) and applied in investigations of carbohydrate (14, 15), lipid (16, 17), and protein (18) metabolism and in the study of various hormonal effects on this metabolism. The purpose of the present study was to investigate binding, uptake, and proteolytic degradation of rat high density lipoprotein (HDL) subfraction, HDL₄, by isolated rat liver parenchymal cells prepared by liver perfusion with collagenase.

### MATERIALS AND METHODS

**Isolation of Rat Liver Parenchymal Cells**—Male Holtzman rats weighing 250 to 300 g were given water and standard Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum. To fast rats for the isolation of liver parenchymal cells by modifications of the method of Berry and Friend (13), using collagenase in situ in situ recirculating liver perfusion system. Each rat used for this experiment was anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, III.), 5 mg/100 g body weight. The liver was perfused by cannulating the portal vein and the thoracic portion of the inferior vena cava. A closed circuit with the perfusion system was achieved by ligation of the inferior vena cava just above the level of the renal vein and hepatic artery. The initial perfusion medium was Krebs-Henseleit bicarbonate buffer (24.9 mM NaHCO₃, pH 7.4, 19), and contained no calcium or collagenase. This buffer was gassed with 95% O₂/5% CO₂ at 37°C for 1 hour. The liver was perfused at a rate of approximately 40 ml/min by the use of a varistaltic pump (Manostat, VWR Scientific, Denver, Colo.). The first 50 to 60 ml of medium was allowed to pass through the liver directly to waste, thereby flushing all blood from the liver. Subsequently, 60 mg of collagenase (type II, Worthington Biochemical Corp., Freehold, N. J.) (30) was added to a recirculating perfusion volume of 120 ml (0.05% collagenase) in the reservoir. Hyaluronidase was not used in our method. Five minutes after adding collagenase, a calcium concentration of 2.5 mM was made by adding 1.2 ml of 0.25% CaCl₂ to 120 ml of perfusion volume. When this began to ooze from the hepatic surface and accumulate in the thoracic and abdominal cavities, it was collected by syringe and returned to the reservoir. Fifteen minutes after adding CaCl₂ (total perfusion time with medium containing collagenase was 20 min), the liver became markedly soft. The liver was then removed and transferred to a beaker containing 20 to 30 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, 19, and blood serum albumin (Fatty acid-free, Sigma Chemical Co., St. Louis, Mo.), or both, as a function of time with medium containing collagenase was 20 min), the liver became markedly soft. The liver was then removed and transferred to a beaker containing 20 to 30 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, 19, and blood serum albumin (Fatty acid-free, Sigma Chemical Co., St. Louis, Mo.), or both, as a function of time. Liver parenchymal cells, (6.3 ± 1.9) × 10⁶ cells (mean ± S.D., n = 12) per 25-ml Erlenmeyer flask in a total volume of 2 ml isolated from rats fasted 24 hours, were incubated for 2 hours in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 1.5% fatty acid-free bovine serum albumin, 2.5 mM CaCl₂, and 10 mM lactate/l mM pyruvate or 10% lactate alone. The flask was gassed with 95% O₂/5% CO₂ for 30 s, capped and incubated at 37°C with 90 to 100 oscillations per min in a Dubnoff metabolic shaker bath (Precision Scientific Co., Chicago, Ill.). The incubations were terminated by pouring the contents of the flasks into test tubes containing 0.3% of 30% perichloric acid. The perichloric acid supernatant was neutralized by 3 M K₂CO₃. The glucose produced during the incubation was measured by an enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (Boehringer Mannheim Biochemicals, New York, N. Y.) (95). Glutamine was calculated as nmol of glucose produced per mg dry weight of cells per min.

To assess further the integrity of the plasma membrane and the metabolic integrity of the isolated liver parenchymal cells, the effects of glucagon and cAMP on gluconeogenesis from lactate and pyruvate were investigated. Aliquots of the cell suspension containing (6.3 ± 1.9) × 10⁶ cells (mean ± S.D., n = 12) in 1.0 ml were added to 25-ml Erlenmeyer flasks containing 1.0 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, 1.5% fatty acid-free bovine serum albumin, 10 mM lactate, 1 mM pyruvate and 2.5 mM CaCl₂. Glucagon (crystalline porcine glucagon, Lilly Research Laboratories, Indianapolis, Ind.), 1 mg, was dissolved in 1.0 ml of diluted HCl, pH 2.5 to 3.0, to form glucagon stock solution. Glucagon and N⁵'-dibutyryl adenosine 3',5'-monophosphoric acid (Bt&AMP, monosodium salt, Sigma Chemical Co.) were added to the incubation mixture in final concentrations of 2.6 and 10 μM, respectively. The flasks were gassed with 95% O₂/5% CO₂ for 30 s, capped, and incubated at 37°C. At the end of the incubation, the reaction was terminated by pouring the contents of the flasks into test tubes containing 0.5 ml of 30% perichloric acid. Rates of gluconeogenesis were compared for the control group and for the glucagon and cAMP groups.

**Isolation of Rat High Density Lipoprotein (HDL) Subfraction, HDL₄**—Blood was obtained from healthy male Holtzman rats fasted for 16 hours. They had previously been fed a commercial rat food which provided approximately 27% of calories as protein, 11% as fat, and 62% as carbohydrate (Purina Laboratory Chow). Blood was placed in test
labeled HDL, by native HDL, very low density lipoproteins (VLDL)
terminated by adding ovomucoid trypsin inhibitor (Worthington
incubation in the standard assay and the radioactivity remaining in
One milligram of this trypsin inhibitor inhibits 1.2 to 1.8 mg of trypsin.
lyophilized) in a concentration of 1 mg/ml was added after a 64 min
HDL,. Nonspecific binding, uptake, and proteolytic degradation were defined
representing 2,000 times as much protein as from 'z5-labeled HDL,).
Specific binding, uptake, and proteolytic degradation were defined as
for 20 min on an International centrifuge, 1.5 ml of the aqueous phase
trichloroacetic acid supernatant, 20 ~1 of 40% KI and 100 ~1 of 30%
precipitated by 1.0 ml of 30% trichloroacetic acid and 0.2 ml aliquots
radioactivity in the chloroform phase and the water phase were
counted using aliquots of both.
Incubation Study of '131-labeled HDL, with Isolated Rat Liver
Parenchymal Cells—Our standard incubation of '131-labeled HDL,
binding, uptake, and proteolytic degradation by rat liver parenchymal
cells consisted of (0.5 ~ 2.5) x 10^6 cells (mean ± S.D., n = 13) of HDL
protein. The 26-ml Erlenmeyer flasks were gassed with 98% O_2/2% CO_2 for 30 s, capped,
and incubated at 90 to 100 oscillations per min in a Dubnoff metabolic
shaker-bath. After the incubation, flasks were placed on ice and their
contents were poured into chilled test tubes. Medium and cells were
separated immediately by centrifugation (2,000 rpm (720 g), 15 min)
on an International centrifuge at 4°C. Radioactivity in cells was counted
using aliquots of both.
Specific binding, uptake, and proteolytic degradation were defined as
the radioactivity in the presence of an excess of native (cold) HDL
(200 µg of native HDL protein/ml, representing 2,000 times as much protein as from
'131-labeled HDL,). Nonspecific binding, uptake, and proteolytic degradation were defined as
the radioactivity obtained in the presence of an excess of native HDLs.
In order to determine the per cent of radioactivity bound to the plasma membrane (binding) plus the '131-labeled HDL, inside of cells (uptake) were determined. Aliquots of medium (1.5 ml) were
precipitated by 1.0 ml of 30% trichloroacetic acid and 0.2 ml aliquots of the supernatant were counted on the Auto-Gamma spectrometer to measure the rate of proteolytic degradation of HDLs. Using aliquots of trichloroacetic acid supernatant of medium, free '131, and '131-labeled monooiodotyrosine were separated according to the method of Goldstein (4) and Rierman et al. (6) as follows. Two-milliliter portions of trichloroacetic acid supernatant, 20 µl of 40% KI and 100 µl of 30%
H_2O_2 were added, mixed, and allowed to stand for 5 min at room
temperature. Four milliliters of chloroform were added to those mixtures
and shaken for 5 min. After centrifugation at 2,000 rpm (720 x g) for 20 min on an International centrifuge, 1.5 ml of the aqueous phase
(containing 131-labeled monooiodotyrosine) and 1.0 ml of the chloro-
form phase (containing oxidized free 131I) were counted on the
Auto-Gamma spectrometer. All samples were assayed in duplicate.
Specific binding, uptake, and proteolytic degradation were defined as
the difference between the radioactivity in the absence and presence of an
excess of native (cold) HDL (200 µg of native HDL protein/ml, representing 2,000 times as much protein as from
'131-labeled HDL,). Nonspecific binding, uptake, and proteolytic degradation were defined as
the radioactivity obtained in the presence of an excess of native HDLs.
In order to determine the per cent of radioactivity bound to the surface of the plasma membrane, trypsin (Worthington Biochemical Corp., Freehold, N.J., twice crystallized, dialyzed salt-free, and lyophilized) in a concentration of 1 mg/ml was added after a 60 min
incubation in the standard assay and the radioactivity remaining in the cells was measured for a 2-hour period. Trypsin activity was terminated by adding eomozzoid trypsin inhibitor (Worthington Biochemical Corp.) in a concentration of 3 mg/ml at each time period. One milligram of this trypsin inhibitor inhibits 1.2 to 1.8 mg of trypsin.
To investigate the specificity of lipoprotein binding and uptake by cells and to investigate the functional linkage between lipoprotein binding and uptake, and proteolytic degradation, competition with '131-
labeled HDL, by native HDL, very low density lipoproteins (VLDL)
(d < 1.006 g/ml) and LDL (1.019 < d < 1.050 g/ml) for binding.
uptake, and proteolytic degradation by cells was examined. Using our
standard assay, 131-labeled HDL, was incubated with isolated liver parenchymal cells in a medium containing different concentrations of VLDL, LDL, and HDL, Lipoprotein protein concentrations tested were 0 to 500 µg/ml.
The effects of rabbit anti-rat HDL, serum on binding and uptake of 131-labeled HDL, by isolated cells were investigated. Aliquots of 131-labeled HDL, were preincubated with increasing quantities of 0 to 500 µl of rabbit anti-rat HDL, serum for 30 min at 37°C
followed by the addition of isolated rat liver parenchymal cells and incubation for 30 min.
Effect of Chloroquine on Binding, Uptake, and Proteolytic Degradation of 131-labeled HDL, by Isolated Rat Liver Parenchymal Cells—Isolated rat liver parenchymal cells were preincubated with chloro-
quione (diphosphate salt) in a concentration range of 0 to 50 µm, for 30 min. Chloroquine was purchased from Sigma Chemical Co., St. Louis, Mo. After preincubation, 131-labeled HDL, alone or 131-labeled HDL, and native HDL, were added and incubation was continued for another 2 hours. Chloroquine was dissolved in medium immediately before use and the pH was adjusted to 7.4. Binding, uptake, and proteolytic degradation of 131-labeled HDL, by cells treated with chloroquine were investigated using our standard assay.
Incubation Study of L-[U-14C]Lysine-labeled HDL, with Isolated
Liver Parenchymal Cells—In order to obtain L-[U-14C]Lysine-labeled HDL, 100 µCi of L-[U-14C]lysine per rat was injected intravenously into eight rats fasted for 16 hours. One hour after injection, blood was obtained in tubes containing 1 mg of KFI/A/tube. HDL, was fractionated as previously described and dialyzed against 0.15 M NaCl containing 0.3 mm EDTA, pH 7.4, at 4°C. The L-[U-14C]lysine-labeled HDL, (62.9 µg of protein with specific radioactivity 9586 dpm/mg of protein) was incubated with isolated rat liver parenchymal cells in our
2-ml standard assay. Binding, uptake, and proteolytic degradation
were investigated in the same way as with 131-labeled HDL, Radio-
activity of all samples in universal liquid scintillation cocktail, Insta-Gel
(Packard), was measured using a Packard model 2409 liquid scintilla-
tion spectrophorometer equipped with automatic external standardization
to measure quenching (Packard Instrument Co., Downers Grove, Ill.). All samples were counted up to 10,000 cpm.

RESULTS
Characterization of Isolated Rat Liver Parenchymal Cells—The technique utilized consistently resulted in a cell yield of (6.6 ± 1.5) x 10^6 cells (mean ± S.D., n = 30) per liver. Approximately 60 min elapsed from the initial intraarterial injection of Nembutal into a rat to the final cell preparation. Light microscopic study of isolated liver parenchymal cells showed that cells were completely dissociated from each other and demonstrated intact cell membranes with a bright halo as well as intact nuclear and cytoplasmic components. The liver cells were completely free of broken connective tissue and blood cells. After staining by 0.2% trypan blue, the intact round cells with a bright halo did not take up trypan blue in contrast to the damaged cells, which were stained darkly with more diffuse margins. The initial liver parenchymal cell viability, as measured by the ability of the cells to exclude trypan blue, was usually greater than 95% (96.9 ± 1.5%, mean ± S.D., n = 28) and in our standard assay usually remained greater than 85% for at least 2 hours. An electron micrograph of isolated rat liver parenchymal cells immediately after isolation demonstrated that most of the cells had intact cell membranes, on the surface of which were present large numbers of microvilli. No vacuoles were present in the cell cytoplasm. There were normal-shaped mitochondria which showed no swelling or contraction and no change in configuration of their cristae. The cells also contained parallel strands of rough endoplasmic reticulum. The percentage of intact cells was approximately 90% and the percentage of contamination by Kupffer cells was less than 3% on electron microscopic study. If isolated liver parenchymal cells remain structurally and...
functionally intact during incubation, they should retain soluble cytoplasmic enzymes. The percentage of leakage of lactic dehydrogenase from cell to medium in Krebs-Henseleit bicarbonate buffer/1.5% bovine serum albumin, pH 7.4, was 0.49, 0.56, 9.31, 3.18, 3.33, 3.65, 9.88, and 3.58% at 0, 5, 15, 30, 45, 60, 90, and 120 min, respectively, after incubation at 37°C (average values of two different experiments). In experiments without added bovine serum albumin, the percentage of loss of lactic dehydrogenase from cell to medium was 9.73, 9.25, 14.42, 12.68, 14.48, 12.57, 11.3, and 14.9% at 0, 5, 15, 30, 45, 60, 90, and 120 min, respectively, after incubation at 37°C. Total lactic dehydrogenase activity (the sum of activities in cells and medium) remained relatively constant. Total lactic dehydrogenase activities at 30, 60, and 120 min after incubation were 98.6, 93.5, and 91.1% of initial total lactic dehydrogenase produced/mg dry weight of cells/2 hours. Therefore, to investigate gluconeogenesis and the combination of 10 mM glucagon to glucose produced without glucagon were 1.36 and 12.68, 14.48, 12.57, 11.3, and 14.9% at 0, 5, 15, 30, 45, 60, 90, and 120 min, respectively, after incubation at 37°C. Total lactic dehydrogenase activity (the sum of activities in cells and medium) remained relatively constant. Total lactic dehydrogenase activities at 30, 60, and 120 min after incubation were 98.6, 93.5, and 91.1% of initial total lactic dehydrogenase activity. The differences in lactic dehydrogenase leakage between cells with and without added bovine serum albumin indicated a protective effect of albumin on the plasma membrane. Mean value of the percentage of leakage of lactic dehydrogenase from cell to medium in Krebs-Henseleit bicarbonate buffer/1.5% bovine serum albumin, pH 7.4, after approximately 30 min at 4°C in 23 separate isolated liver parenchymal cell preparations was 3.0 ± 0.9 (mean ± S.D.) %.

The highest glucose production rate (790 nmol of glucose produced/mg dry weight of cells/2 hours) was observed with the combination of 10 mM lactate/1 mM pyruvate as substrate. Other glucose production rates were as follows. 10 mM lactate alone, 580 nmol of glucose/mg dry weight of cells/2 hours; 10 mM pyruvate alone, 550 nmol of glucose/mg dry weight of cells/2 hours. Therefore, to investigate gluconeogenesis and the effect of glucagon, cAMP, and bovine serum albumin on gluconeogenesis, the combination of 10 mM lactate/1 mM pyruvate was used as substrate. Liver parenchymal cells isolated from rats fasted 24 hours produced approximately 4.05 nmol of glucose/mg dry weight of cells/min when incubated with 10 mM lactate/1 mM pyruvate at 37°C for 2 hours. After approximately a 15-min lag phase, the rate of gluconeogenesis was constant throughout the 2-hour incubation. In Fig. 1, the effects of 1.5% bovine serum albumin and 2.6 µM glucagon on gluconeogenesis are shown. By preparing and incubating the cells in Krebs-Henseleit bicarbonate buffer/1.5% bovine serum albumin, pH 7.4, the rate of gluconeogenesis was increased. Even though glucagon exhibited its effect on gluconeogenesis both in the cells with and without bovine serum albumin, the glucagon effect was much greater in the presence of albumin. At 120 min of incubation, the ratios of glucose produced with glucagon to glucose produced without glucagon were 1.36 and 1.92, with and without added serum albumin, respectively. This indicates that albumin has a protective effect on the structural and metabolic integrity of liver parenchymal cells, especially on the plasma membranes. B4cAMP, 10 µM, showed an effect on gluconeogenesis comparable to 2.6 µM glucagon. Glucose production through gluconeogenesis was always less than 10% of total glucose production during incubation.

Characterization of Rat HDL—Column chromatography of totally delipidated HDL₃ on Sephadex G-100 showed three peaks comparable with the column chromatographic pattern of canine apo-HDL₃ reported previously (2). Polyacrylamide gel electrophoresis of HDL₃, apo-HDL₃, and the column fractions indicated the presence of apo A-I as the major protein moiety and suggested apo-A-II, apo-C-II, apo-C-III, and apo-D as minor protein moieties. Immunodiffusion and immunoelectrophoresis confirmed this and ruled out contamination by apo-B. After iodination of HDL₃, approximately 97 to 98% of ¹²⁵I-labeled HDL₃ was precipitable by 15% trichloroacetic acid and less than 5% radioactivity could be extracted with chloroform/methanol (2/1, v/v) solution.

Binding and Uptake of ¹²⁵I-Labeled HDL₃ by Isolated Rat Liver Parenchymal Cells—Radioactivity in cells reached a maximum at 30 min of incubation and this plateau or steady state persisted for at least 2 hours (Fig. 2). The amount of specific binding and uptake of ¹²⁵I-labeled HDL₃ at the plateau was 1.8% (mean of three experiments) of incubated ¹²⁵I-labeled HDL₃. The binding and uptake of ¹²⁵I-labeled HDL₃ by isolated liver parenchymal cells was decreased with increasing native HDL₃, as shown in Fig. 3. The apparent maximum HDL₃, binding and uptake capacity of rat liver parenchymal cells (B₅₇₃₃) and the apparent dissociation constant of the liver cell system (K₅₇₃₃) were estimated with one of the Woolf plots (30) commonly known as a Scatchard plot (31, 32). B₅₇₃₃ was 31 ng of HDL₃ protein/mg dry weight of cells, and K₅₇₃₃ was 17 µg of HDL₃ protein/ml liver cell system or K₅₇₃₃ = 60 × 10⁻⁷ M based on M, 28,000 for apo-A-I as the predominant protein in HDL₃ (33). These data indicate a saturable or specific process for binding and uptake of rat HDL₃ by rat liver cells. Only a minimal amount of specific binding and uptake of ¹²⁵I-labeled HDL₃ occurred at 4°C. There were slight but constant increments in nonspecific binding and uptake of ¹²⁵I-labeled HDL₃ by cells, which were of similar magnitude at both 4°C and 37°C. Therefore, specific binding and uptake of ¹²⁵I-labeled HDL₃ by liver cells is temperature-dependent and is saturable in contrast to nonspecific processes. To measure the percentage of radioactivity on the surface of the plasma membrane, the cells were treated with trypsin at 60 min of incubation, which was after radioactivity in liver cells had reached a steady state plateau.
Approximately 60% of radioactivity in the cells was trypsin-releasable and 40% remained inside the cells after trypsin treatment. Thus, 60% of radioactivity was \(^{125}\text{I}-\)labeled HDL bound on the plasma membrane surface (binding) and 40% of radioactivity was internalized \(^{125}\text{I}-\)labeled HDL (uptake). To investigate further the specificity of the lipoprotein binding and uptake by cells, competition of native HDL, VLDL, and LDL with \(^{125}\text{I}-\)labeled HDL, for binding and uptake was studied. Native HDL, inhibited \(^{125}\text{I}-\)labeled HDL, binding and uptake much more effectively than did VLDL and LDL (Fig. 4). When \(^{125}\text{I}-\)labeled HDL, was preincubated with varying amounts of rabbit antiserum to rat HDL, binding and uptake by cells were decreased by increasing amounts of antiserum, and complete inhibition was observed with 500 \(\mu\text{l}\) of antiserum (Fig. 5). Both the antibody binding site and the receptor attachment site on the HDL, molecule may be in close proximity so that there can be an effect of steric hindrance.

**Fig. 2.** Specific binding, uptake, and proteolytic degradation of \(^{125}\text{I}-\)labeled HDL, by isolated rat liver parenchymal cells at 37°. Specific binding, uptake, and proteolytic degradation were defined as the difference between the radioactivity in the absence and presence of an excess of native HDL, (200 \(\mu\text{g}\) of native HDL, protein/ml). Specific binding and uptake were determined by the appearance of radioactivity in cells, expressed as dpm/mg dry weight of cells accumulated over a 120-min incubation. Specific proteolytic degradation of \(^{125}\text{I}-\)labeled HDL, by isolated rat liver parenchymal cells is also shown. Specific proteolytic degradation was determined by the release of trichloroacetic acid-soluble radioactivity into the incubation medium as dpm/mg dry weight of cells over a 120-min incubation. \(^{125}\text{I}-\)labeled HDL, protein concentration was 100 \(\mu\text{g}\)/ml and specific radioactivity was 420 dpm/ng of HDL, protein. In each flask, there were 10.2 \(\times\) 10\(^6\) cells with a total dry weight of 8.9 mg.

**Fig. 3.** Estimation of the maximum rat HDL, binding capacity \((B_{\text{max}})\) and the dissociation constant \((K_d)\) of the isolated rat liver cell system. Aliquots of rat liver cells were incubated with a fixed amount of \(^{125}\text{I}-\)labeled HDL, (100 \(\mu\text{g}\)/ml, specific radioactivity of 512 dpm/ng of HDL, protein). Increasing amounts of native HDL, were added to a maximum concentration of 200 \(\mu\text{g}\)/ml. In each flask, there were 5.4 \(\times\) 10\(^6\) cells with a total dry weight of 7.0 mg. The binding and uptake of \(^{125}\text{I}-\)labeled HDL, by isolated rat liver parenchymal cells was decreased with increasing native HDL, and a straight line \((r = -0.98)\) was obtained by a Scatchard plot. \(B_{\text{max}}\) was 31 ng/mg dry weight of cells and \(K_d\) was 16.8 \(\mu\text{g}\)/ml or 60 \(\times\) 10\(^{-8}\) \(M\), based on \(M_1\), 28,000 of apo-A-I, the predominant protein moiety of rat HDL,.

**Fig. 4 (left).** Effects of VLDL, LDL, and HDL, on \(^{125}\text{I}-\)labeled HDL, binding and uptake by isolated rat liver parenchymal cells. \(^{125}\text{I}-\)labeled HDL, was incubated with isolated rat liver parenchymal cells in a medium containing increasing concentrations of VLDL, LDL, and native HDL, at 37°. Lipoprotein protein concentrations ranged from 0 to 500 \(\mu\text{g}\)/ml. Incubation time was 60 min. In each flask, there were 7.0 \(\times\) 10\(^6\) cells with a total dry weight of 6.1 mg.

**Fig. 5 (center).** Effect of rabbit anti-rat HDL, serum on binding and uptake of \(^{125}\text{I}-\)labeled HDL, by isolated rat liver parenchymal cells. \(^{125}\text{I}-\)labeled HDL, was preincubated with varying amounts of antibody to rat HDL, for 30 min at 37°, followed by a 30-min incubation with isolated rat liver parenchymal cells at 37°. \(^{125}\text{I}-\)labeled HDL, protein concentration was 100 ng/ml (specific radioactivity 463 dpm/ng of HDL, protein). In each flask, there were 6.4 \(\times\) 10\(^6\) cells with a total dry weight of 6.4 mg.

**Fig. 6 (right).** Effect of increasing concentrations of chloroquine on specific binding, uptake, and proteolytic degradation of \(^{125}\text{I}-\)labeled HDL, by isolated rat liver parenchymal cells at 37°. Specific binding and uptake were determined by the appearance of radioactivity in cells, expressed as dpm/mg dry weight of cells accumulated during 120-min incubation. Specific proteolytic degradation was determined by the release of trichloroacetic acid-soluble radioactivity into the incubation medium, expressed as dpm/mg dry weight of cells during 120-min incubation. \(^{125}\text{I}-\)labeled HDL, protein concentration was 100 ng/ml and specific radioactivity was 463 dpm/ng of HDL, protein. In each flask, there were 10.0 \(\times\) 10\(^6\) cells with a total dry weight of 10.4 mg.
Mechanism of the initial increase in binding and uptake at very low amounts of rabbit anti-rat HDL<sub>2</sub> serum is not clear but may be due to an unspecified effect of serum factor(s) which promote binding (34, 35). These observations on lipoprotein competition for binding and uptake and on antisera inhibition of binding and uptake further support the specificity of binding and uptake for each lipoprotein.

Proteolytic Degradation of <sup>111</sup>I-Labeled HDL<sub>2</sub> by Isolated Rat Liver Parenchymal Cells—The rate of <sup>111</sup>I-labeled HDL<sub>2</sub> proteolytic degradation was measured by counting radioactivity obtained from the incubation medium in the trichloroacetic acid-soluble fraction and in the chloroform phase and the resultant aqueous phase after extracting the trichloroacetic acid-soluble fraction with chloroform, as described under "Materials and Methods." After peroxidation, 72.6 ± 5.4% (mean ± S.D., n = 10) of radioactivity in the trichloroacetic acid-soluble fraction, aqueous phase (<sup>14</sup>Clabeled tyrosine), and chloroform phase (oxidized free <sup>125</sup>I) were comparable to each other. There was no significant radioactivity increase observed in the trichloroacetic acid-soluble fraction, aqueous phase, or chloroform phase by incubating <sup>131</sup>I-labeled HDL<sub>2</sub> without cells, indicating that both <sup>131</sup>I-labeled tyrosine and free <sup>131</sup>I were formed through active catabolism by the liver cells. These data and information on liver microsomal idotyrosine deiodinase (36-38) suggest that radioactivity released from the cells to the medium following <sup>131</sup>I-labeled HDL<sub>2</sub>, proteolytic degradation is not only in the form of <sup>131</sup>I-labeled tyrosine but also in the form of free <sup>131</sup>I. Therefore, we chose to express the rate of <sup>131</sup>I-labeled HDL<sub>2</sub> degradation by radioactivity change in the trichloroacetic acid-soluble fraction of the medium, since this represents the full liver cell catabolism of our <sup>131</sup>I-labeled HDL<sub>2</sub> used in these studies. At 37°C, there was a lag phase for 15 min and after that a constant rate of degradation was observed up to 2 hours in which 5.8% (mean of three experiments) of radioactivity bound to the external surface of the plasma membrane was degraded and released from liver cells to medium (Fig. 2). However, no significant degradation of <sup>125</sup>I-labeled HDL<sub>2</sub>, was observed at 4°C. By incubating <sup>131</sup>I-labeled HDL<sub>2</sub> with isolated rat liver parenchymal cells in a medium containing increasing concentrations of lipoproteins (VLDL, LDL, and HDL<sub>2</sub>), there was much greater inhibition of <sup>131</sup>I-labeled HDL<sub>2</sub> proteolytic degradation by HDL<sub>2</sub> than by VLDL or LDL.

By incubating isolated rat liver parenchymal cells with increasing concentrations of chloroquine, proteolytic degradation of <sup>131</sup>I-labeled HDL<sub>2</sub> was enhanced at concentrations less than 1 mM and inhibited at concentrations equal to and greater than 5 mM (Fig. 6). Radioactivity in cells increased hyperbolically with increasing chloroquine concentrations, indicating cellular accumulation of <sup>131</sup>I-labeled HDL<sub>2</sub>.

Binding, Uptake, and Proteolytic Degradation of L-[U-<sup>14</sup>C]Lysine-labeled HDL<sub>2</sub> by Isolated Rat Liver Parenchymal Cells—Time course of binding, uptake, and proteolytic degradation of L-[U-<sup>14</sup>C]labeled HDL<sub>2</sub> by isolated liver parenchymal cells was similar to that of <sup>131</sup>I-labeled HDL<sub>2</sub>. Radioactivity in cells reached a maximum at 45 min to 60 min of incubation and this plateau was maintained up to 2 hours. The rate of L-[U-<sup>14</sup>C]lysine-labeled HDL<sub>2</sub> proteolytic degradation, expressed by radioactivity change in the trichloroacetic acid-soluble fraction of medium, had a lag phase for approximately 5 to 10 min and after that a constant rate of degradation was observed for up to 2 hours. Of the total L-[U-<sup>14</sup>C]lysine-labeled HDL<sub>2</sub> protein incubated, 2.2% was degraded and released into the medium.

Discussion

Our previous studies indicate that the liver and liver lysosomes are important organ and subcellular organelle sites of apo-A-I catabolism in dogs (2, 3). We could not rule out some role of reticuloendothelial cells (Kupffer cells) in apo-A-I degradation (2, 3). To investigate the important role of liver parenchymal cells and basic mechanisms for cell binding, uptake, and proteolytic degradation of lipoprotein A, we have studied rat HDL<sub>2</sub> catabolism by isolated rat liver parenchymal cells. An incubation system with liver parenchymal cells has several advantages. Metabolic functions can be investigated without effects of other liver cell types (especially Kupffer cells) as compared to studies of perfused liver. The ability to control the cellular environment is a major advantage with isolated cell systems as compared to in vivo studies. Hormone receptors appear to be retained in isolated liver cells (14, 39, 40). In addition, numerous comparative studies on hepatocytes derived from a single mammalian liver are advantageous, due to the variations which occur with different animals.

Since Howard et al. (12) described an enzymatic method for preparation of isolated liver parenchymal cells from rat by shaking liver slices in a buffered medium containing hyaluronidase and collagenase, many modifications have been made in order to isolate large quantities of viable liver parenchymal cells that retain in vivo metabolic characteristics, maintain intact plasma membranes, and respond to hormones at physiological concentrations (41). Liver parenchymal cells isolated by this enzymatic digestion method have been used to investigate several aspects of protein synthesis and degradation (18, 42), gluconeogenesis (14, 15, 39, 40), fatty acid metabolism (15, 43), lipoprotein synthesis and secretion (44, 45), bile acid synthesis and secretion (46, 47), binding and degradation of insulin (48, 49), and chylomicron catabolism (43, 50). These reports indicate that metabolic activities by isolated liver parenchymal cells are comparable to liver function in vivo. The original method of Berry and Friend (13) subjects the liver to 0.05% collagenase and 0.10% hyaluronidase for prolonged periods (60 to 90 min). By modifying these procedures to expose the liver to 0.05% collagenase without hyaluronidase, by perfusing for a shorter period (20 min) and by adding albumin to a concentration of 1.5% at the time the cells were washed, we were able to isolate large quantities of viable liver parenchymal cells with good morphologic and metabolic properties, as shown under "Results."

These well characterized cells were used in the present study to investigate binding, uptake, and proteolytic degradation of rat <sup>131</sup>I-labeled HDL<sub>2</sub>. Our working hypothesis on the process of lipoprotein catabolism of liver parenchymal cells, shown in Fig. 7, was constructed from published data on: (a) lipoprotein binding, uptake, and proteolytic degradation by human skin fibroblasts (4, 7–11) and human and rat aortic smooth muscle cells (5, 6, 51) in culture, (b) glycoprotein uptake and catabolism by liver cells (38, 52, 53), and (c) endocytosis and catabolism of proteins (54–56). These steps may be divided into at least six major steps as described in the legend to Fig. 7. Both binding and uptake of <sup>131</sup>I-labeled HDL<sub>2</sub> by rat liver parenchymal cells were measured by counting radioactivities in cells washed and centrifuged three times at 4°C. Following this procedure, radioactivity in cells was always constant. Radioactivity bound to the external surface of the plasma...
membrane (binding) and radioactivity inside of cells (uptake) were not determined separately in each experiment. However, trypsin treatment 60 min after incubation of \( ^{125}I \)-labeled HDL, with liver parenchymal cells indicated that 60% of cell radioactivity is trypsin-releasable and is therefore bound to the external surface of the plasma membrane and is not internalized.

One of the major objectives of the present study was to investigate the existence of saturable (specific) receptor or recognition sites for HDL, or lipoprotein A on the plasma membrane of liver parenchymal cells. Although the quantitative aspects of reactant-receptor interaction appear to be complex in all cell systems studied, binding has been considered to be the sum of at least two processes: a saturable (specific) component and a nonsaturable (nonspecific) component (57). Goldstein and Brown reported that there are specific and nonspecific binding sites for human LDL on plasma membranes of human fibroblasts in culture (4, 8, 9, 11). The binding and uptake of \( ^{131}I \)-labeled HDL, by isolated rat liver parenchymal cells was decreased with increasing native HDL, binding and uptake of \( ^{125}I \)-labeled HDL, by isolated rat liver parenchymal cells (0.4 x 10\(^{-6}\) M) (58). It was observed that a small amount of \( ^{125}I \)-labeled HDL, would always bind and be taken up by isolated liver parenchymal cells, even in the presence of high concentrations of native HDL. The amount of this nonspecific binding and uptake was less than 10% of total binding and uptake. These observations indicate that isolated liver parenchymal cells have a saturable (specific) component and a nonsaturable (nonspecific) component for binding and uptake of HDL, but the role of the nonspecific component appears to be significantly less in HDL, catabolism. The time course of saturable (specific) binding and uptake of HDL, is rapid (Fig. 2) and largely dependent on temperature. Further evidence for specific binding and uptake is provided by the greater inhibition of \( ^{125}I \)-labeled HDL, binding and uptake by native HDL, than by VLDL or LDL and by the complete inhibition of \( ^{125}I \)-labeled HDL, binding and uptake in the presence of rabbit anti-rat HDL, serum.

It should be emphasized that the cell-free medium did not degrade \( ^{125}I \)-labeled HDL, so that iodinated degradation products resulted only from catabolism by cells. There was an initial lag in the appearance of iodinated degradation products which suggests that there may be an obligatory translocation of HDL, from binding site to intracellular degradation site. In contrast, there was no lag in proteolytic degradation of HDL, by acid protease in liver lysosomes (3).

Chloroquine has been shown to stabilize lysosomes against damage by ultraviolet irradiation, thermal stress, oxygen excess, vitamin A, carbon tetrachloride, endotoxins, viral infections, traumatic shock, and streptolysin O (59). In addition, chloroquine appears to retard the release of hydrolytic enzymes from rabbit liver granules induced by streptolysin S, lysocysteine, etiocholanolone, or progesterone (60). In cells in culture (61, 62), chloroquine appears to be selectively taken up and concentrated in lysosomes. \( ^{125}I \)-labeled HDL, accumulated more in chloroquine-treated liver parenchymal cells than in untreated cells. Proteolytic degradation of HDL, was enhanced at chloroquine concentrations of less than 1 mM. These observations may be explained by a stabilizing effect of chloroquine on lysosomal membranes, possibly facilitating fusion of primary lysosomes with endocytic vacuoles. We did not observe any inhibitory effect of chloroquine on proteolytic degradation of HDL, at this concentration, even though inhibition of mucopolysaccharide degradation (63), cellular protein degradation (61), and lipoprotein degradation (7) in cultured fibroblasts has been reported at concentrations less than 1 mM. However, at concentrations equal to and greater than 5 mM, chloroquine inhibited degradation of HDL, by isolated liver parenchymal cells. Wibo and Poole (61) reported that chloroquine was taken up by fibroblasts in culture and that the concentration in the cells reached several hundred times that in medium (100 \(\mu\)M) over 2 hours. Chloroquine, at concentrations attained inside the lysosomes, inhibited cathepsin B1 (EC 3.4.22.1) but not cathepsin D (EC 3.4.23.5). Inhibition of proteolytic degradation of HDL, by chloroquine at concentrations equal to and greater than 5 mM may be explained by these chloroquine effects on cathepsin B1, which we reported previously to be a key enzyme for HDL, proteolysis in lysosomes (3). These chloroquine effects on HDL, proteolysis further confirm the important role of lysosomes in this process.

Most studies of lipoprotein metabolism in vivo and in vitro...
have been made with lipoproteins or apolipoproteins labeled with \(^{125}\text{I}\) in vitro, or both. Labeling with \(^{125}\text{I}\) is probably the only practical way to obtain high specific activities in complex lipoprotein molecules (64). Nevertheless, it is difficult to prove that \(^{125}\text{I}\)-labeled lipoproteins behave exactly like native lipoproteins when studied in biological systems. In our studies, binding, uptake, and proteolytic degradation of \(^{125}\text{I}\)-labeled HDL, were effective and were competitively inhibited by native HDL,.

In conclusion, our data suggest that binding, uptake, and proteolytic degradation of HDL, by isolated rat liver parenchymal cells are as effective as binding, uptake, and proteolytic degradation of \(^{125}\text{I}\)-labeled HDL, These data suggest that HDL, behaves essentially as native HDL, in our in vitro isolated rat liver parenchymal cell system. This is fortunate because \(^{125}\text{I}\)-labeled HDL, or other \(^{14}\text{C}\)-aminoacid-label

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