Characterization and catabolism of rat very high density lipoproteins

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Abstract A previously unrecognized lipoprotein of very high density was isolated from rat serum. During zonal ultracentrifugation of whole serum or of fractions from Sepharose 4B chromatography, a peak comigrating with a peak of cholesterol was found between the typical high density lipoproteins and the residual serum proteins. Centrifugation of chylomicrons, very low density lipoproteins, and high density lipoproteins, radioiodinated in their lipid and protein moieties and mixed with serum, did not yield this peak. The pooled fractions contained about 85% protein. The remainder was lipid comprising cholesteryl esters, free cholesterol, triglycerides, phosphatidylcholine, and sphingomyelin. Polyacrylamide gel electrophoresis revealed bands in the region of apolipoproteins E and C as the major components. The composition suggested a lipoprotein, and this was substantiated by electron microscopy which showed particles with a mean diameter of 150 Å. Their average hydrated density was 1.23 g/ml and the apparent molecular weight was 1.35×10^6. These very high density lipoproteins are characterized by a rapid catabolism as compared to high density lipoproteins. Within 10 min, 84% and 70% of intravenously injected ^{125}I-labeled very high density lipoproteins were removed from plasma of male and female rats, respectively, and did not appear to be converted to lipoproteins of a different density class. Ninety-five percent of the removed ^{125}I was recovered in the liver and the radioactivity per gram of tissue was also highest for the liver. Accordingly, the rate of clearance of ^{125}I-labeled very high density lipoproteins was markedly reduced in functionally eviscerated rats. Radioautography revealed that most of the silver grains representing very high density lipoproteins were associated with hepatocytes and only about 1% was found over v. Kupffer cells. Uptake and degradation by freshly isolated rat hepatocytes were mediated by a saturable and specific binding site. Composition and metabolic pathway are compatible with a function of very high density lipoproteins in the transport of protein and lipids to the liver. **Därr, W. H., E. E. T. Windler, K. U. Stephan, A. K. Walli, and H. Greten.** Characterization and catabolism of rat very high density lipoproteins. *J. Lipid Res.* 1985. **26:** 672–683.

Supplementary key words rate zonal ultracentrifugation • LDL • HDL • VLDL • apolipoproteins • electron microscopy

Cholesterol homeostasis of peripheral tissues appears to be modulated by the antagonistic action of LDL and HDL. LDL provides cholesterol as it is endocytosed by cells, a process mediated by receptors and regulated according to demand (1). HDL on the other hand is thought to stimulate cholesterol efflux, and to mediate transport of cholesterol to the liver as the organ of catabolism and excretion (2). Whether, under physiological conditions, cholesterol homeostasis depends on this transport is not known. However, the accumulation of cholesterol leading to the formation of foam cells in atherosclerosis clearly illustrates the possibility of defective regulation of cellular cholesterol metabolism (3). Under these circumstances, reverse cholesterol transport may become critical for the elimination of cholesterol. In contrast to the well characterized metabolic pathway of LDL, the mechanisms of action of HDL are less clear. Structural differences of HDL subfractions, which in turn may entail different physiological functions, complicate the investigation of the metabolism of HDL.

Conventional equilibrium ultracentrifugation has allowed fractionation of human HDL into two populations, HDL_2 and HDL_3 (4). By employing different techniques, evidence for heterogeneity within these subspecies has been obtained. For instance, column chromatography yielded up to eight particle populations with different apolipoprotein patterns (5); gradient gel electrophoresis separated lipoproteins of the ultracentrifugal fraction $d \leq 1.2$ g/ml of human serum into four to five subpopulations (6). Taking advantage of differences in component proteins, electrophoretic procedures were successfully used to isolate an apolipoprotein E-rich particle from the bulk of HDL. Though this lipoprotein is rapidly taken up by the liver, its physiological function is uncertain (7). By means of immunoabsorption again, a functionally distinct HDL

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; VHDL, very high density lipoproteins; EDTA, ethylenediaminetetraacetic acid, disodium salt; SDS, sodium dodecylsulfate; DME, Dulbecco's minimal essential medium.

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subfraction has been identified exhibiting activity for cholesterol esterification and transfer (8).

Rate zonal ultracentrifugation has proved to be a powerful method for the separation of lipoproteins according to flotation rate on a preparative scale (9). We have employed zonal centrifugation in an attempt to isolate HDL-subfractions of possible metabolic significance. In the present research we provide evidence for the existence of a distinct rat lipoprotein of very high density with particular structural and metabolic features.

EXPERIMENTAL PROCEDURES

Animals

Sprague-Dawley rats (Zentrale Versuchstieranstalt, Hanover, FRG), males weighing 300–350 g and females weighing 200–250 g, were maintained on standard rat chow and tap water. Rats were bled under light ether anesthesia and, after clotting at room temperature, serum was separated from blood cells by low speed centrifugation. For functional hepatectomy the mesenteric arteries, the hepatic artery, and the portal vein were ligated and the liver was freed from all its ligaments. The abdominal wall was closed with wound clips.

Preparation of lipoproteins

Fractionation of lipoproteins was carried out by rate zonal ultracentrifugation in a Kontron TZT 48.65 rotor using the discontinuous NaBr-gradient (density range 1.0–1.4 g/ml, 0.013% EDTA, pH 7.4) as originally described by Patsch et al. (10). Thirty ml of a solution of d 1.4 g/ml containing 15–30 ml of female rat serum was layered under the gradient followed by a cushion of 20 ml of the 1.4 g/ml solution, while the rotor was spinning at 2,500 rpm at room temperature. After centrifugation at 40,000 rpm for the indicated times at 14°C, the rotor was unloaded at 2,500 rpm from the center by pumping 1.4 g/ml solution into the periphery of the rotor. The effluent was continuously monitored at 280 nm and fractions of 20 ml were collected.

For isolation of VLDL and HDL, after 8 hr of centrifugation, the first two fractions or the two peak fractions of HDL, respectively, were dialyzed against several changes of 0.15 M NaCl, 0.01% EDTA, pH 7.4, and concentrated by ultrafiltration (Amicon-Diaflow UM 30, Lexington, MA). For isolation of VHDL, indicated fractions after 16 hr of centrifugation of up to five preparations were pooled, concentrated by ultrafiltration to 30 ml, adjusted to a density of 1.4 g/ml, and recentrifuged for 24 hr as above. The peak, which now was separated from the residual serum proteins, was dialyzed and concentrated as described. Small chylomicrons were obtained from mesenteric lymph of male rats during intraduodenal infusion of 10% glucose in 0.15 M NaCl as described previously (11). Isolation was achieved by two centrifugations for 3×10^7 g_{av}-min (12).

Radioiodination of lipoproteins

Lipoproteins were labeled with ^{125}I (Na[^{125}I]iodide, carrier free, Amersham-Buchler, Braunschweig, FRG) according to the method of McFarlane as modified for lipoproteins (13), and dialyzed against five changes of 0.15 M NaCl, 0.01% EDTA, pH 7.4. Lipoproteins in density solution were labeled without prior dialysis and dilution with 2 N NaCl was omitted. More than 95% of the radioactivity was precipitable in 10% trichloroacetic acid. About 10% of the radioactivity in VHDL and about 50% of the radioactivity in chylomicrons, VLDL and HDL were extractable into ether–ethanol 1:3 (v/v).

Column chromatography

Twenty ml of female rat serum was fractionated by gel permeation chromatography on Sepharose 4B (2.5 × 100 cm) at 4°C mixed with a trace of ^{125}I-labeled HDL or ^{125}I-labeled VHDL, using a buffer of 0.9% NaCl, 0.01% EDTA, pH 7.4. The flow rate was 25 ml/hr and fractions of 6.0 ml were collected. Indicated fractions of up to five column runs were pooled, concentrated by ultrafiltration (Amicon UM 30) to 30 ml, and centrifuged as described for serum.

Mixing experiments and catabolism of lipoproteins

Seventy-five μg of apolipoprotein in ^{125}I-labeled VHDL or ^{125}I-labeled HDL and 1.0 mg of triglyceride in ^{125}I-labeled chylomicrons or ^{125}I-labeled VLDL were incubated with 5 and 8 ml of female rat serum for 60 min at room temperature and centrifuged for 16 hr as described. ^{125}I-labeled VHDL or ^{125}I-labeled HDL (150 μg of protein per rat) were injected into two female rats each. After 60 min the rats were bled and the serum was centrifuged for 16 hr as above. In fractions of 20 ml the radioactivity was measured and the distribution was compared to that after incubation in vitro.

For determination of the plasma clearance, ^{125}I-labeled VHDL (about 5 μg protein per rat) or ^{125}I-labeled HDL (about 30 μg per rat) were injected intravenously under light ether anesthesia. At indicated times, about 200 μl or 50 μl blood was taken from the tail veins of intact or functionally eviscerated rats, respectively. Radioactivity was counted in serum samples. The injected dose of ^{125}I per estimated plasma volume was taken as 100%. The calculation was based on a volume of 4.5% of body weight in intact rats and 2.65% in functionally hepatectomized rats. The plasma volume of hepatectomized rats was determined with ^{125}I-labeled human albumin. Fifteen min after injection into five male rats, the space of distribution was 2.65 ± 0.18% of body weight. The sites of

catabolism of VHDL were determined 10 min after intravenous injection of 30 or 300 μg of ^{125}I-labeled VHDL into two female rats each. The rats were exhaustively bled and the indicated organs and specimens of the psoas muscles and retroperitoneal adipose tissue were removed and thoroughly rinsed with normal saline. Radioactivity per gram wet weight was measured.

Determination of hydrated density and molecular weight

The hydrated density of VHDL was determined by equilibrium centrifugation in a zonal rotor (Kontron TZT 48.65) using a linear gradient with respect to rotor volume of NaBr with a range of 1.0–1.3 g/ml (14). VHDL (1.5 mg of protein) in 30 ml of d 1.3 g/ml solution and a cushion of 20 ml of the same solution were added into the rotor and centrifuged for 48 hr and 40,000 rpm as above. The hydrated density of the peak fraction of absorption and the density range of VHDL were determined by gravimetry.

For an approximation of the molecular weight, lipoproteins were chromatographed on a column (1.6 × 100 cm) of Sepharose 4B in 2 M NaBr, 0.1 mM EDTA, pH 7.6, at a flow rate of 5 ml/hr at room temperature (15). The absorption at 280 nm of fractions of 1.5 ml was measured. A semilogarithmic plot of the peak elution volume / void volume versus molecular weight of the following reference molecules gave a straight line: cytochrome C (12,500 daltons), human HDL_3 (170,000 daltons), and glutamate dehydrogenase (1×10^6 daltons).

Radioautography

Ten minutes after injection of 2.5 μg ^{125}I-labeled VHDL into a female rat, the liver was flushed from the portal vein with normal saline and subsequently fixed with 500 ml of 2.2% glutaraldehyde in phosphate buffer, pH 7.4 (flow rate 12 ml/min). Thick sections were coated with Kodak NTB 3 photoemulsion at 45°C and incubated at 4°C for 7 days. The exposed slides were developed in Kodak D-19 and stained in toluidine–pyronine–water 4:1:5 (v/v/v). Association of 2400 silver grains with hepatic cells was evaluated on light microscopic radioautographs.

Cell binding and degradation assays

Hepatocytes were isolated by standard methods (16). Binding assays were carried out as previously described (17). Fibroblasts derived from skin biopsies of normal subjects or rats were cultured in a humidified incubator (95% air, 5% CO_2) at 37°C in DME which contained 10% fetal calf serum, 25 mM $NaHCO_3$, 20 mM HEPES buffer (pH 7.4) with 100 U penicillin and 100 μg streptomycin/ml (17). After 3 days the medium was changed to DME containing 10% human lipoprotein-deficient serum. After 24 hr the medium was replaced by 2 ml of DME containing lipoprotein-deficient serum. For binding or degradation assays, ^{125}I-labeled VHDL was added with or without excess of unlabeled lipoproteins, and cell cultures were incubated for 2 hr at 4°C or 6 hr at 37°C, respectively. The medium was removed, and cells were placed on ice and washed five times with ice-cold DME medium containing 2 mg/ml bovine serum albumin and twice with DME. Cell pellets were dissolved in 0.1 M NaOH and radioactivity and protein content were measured. Non-iodide radioactivity soluble in trichloroacetic acid served as a measure of lipoprotein degradation (18). Dishes without fibroblasts served as controls.

Chemical analysis

Determination of protein and lipids in lipoproteins and thin-layer chromatography of phospholipids were performed by standard procedures as previously described (19). Total cholesterol in zonal rotor effluent fractions was determined by gas–liquid chromatography after saponification at pH 11 and extraction of the lipids according to Bligh and Dyer (20). A Varian-chromatograph 3700 with codein as internal standard was used. For analytical isoelectric focusing, lipoproteins were delipidated with ethanol–ether 3:1 (v/v) and ether (21). Electrophoresis was performed in polyacrylamide gels in the absence of reducing agents (22). SDS gel electrophoresis was performed as described by Kane, Hardman, and Paulus (23). Electrophoretic mobility of lipoproteins was determined in agarose gels (24).

Electron microscopy

VHDL and HDL were negatively stained with 2% phosphotungstic acid and their size was assessed from electron micrographs (EM Philips 300).

RESULTS

Fig. 1 shows the fractionation of normal female rat serum by rate zonal ultracentrifugation. After 8 hr of centrifugation, the major part of HDL was separated from VLDL and LDL and the residual plasma proteins, respectively, in a single peak. Two minor fractions between the lipoproteins of lower density and HDL were originally described by Danielsson et al. (25) and further characterized by Oschry and Eisenberg (26) (Fig. 1A). After 16 hr, at the edge of the residual proteins a shoulder appeared (Fig. 1B), which emerged as a distinct plasma fraction from the residual plasma proteins (Fig. 1C). Complete separation of this fraction from the residual plasma proteins (Fig. 1D) could be achieved by recentrifugation for 24 hr of the indicated fraction in Fig. 1B.

The peak of absorption of 280 nm coincided with a peak of cholesterol, indicating the presence of a lipopro-

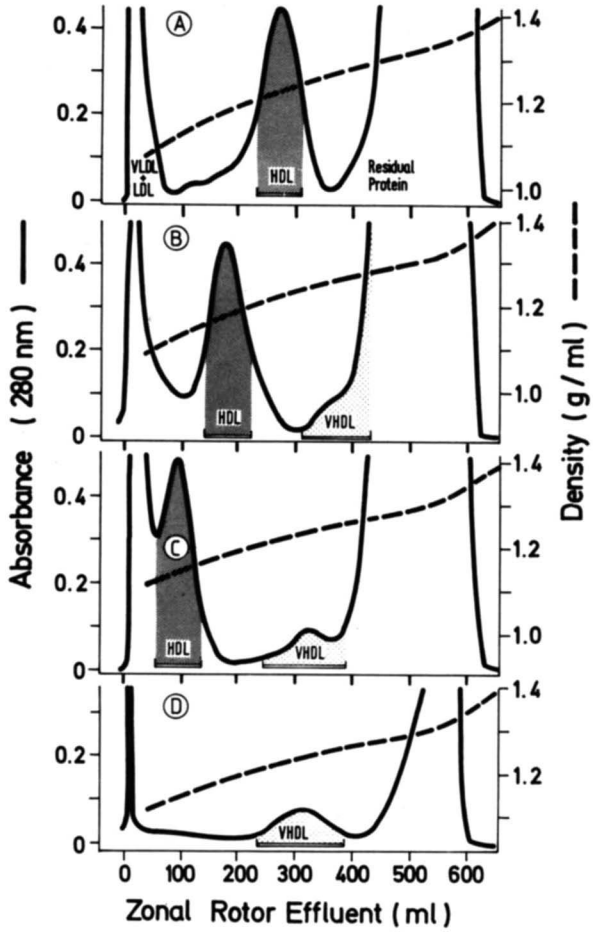

Fig. 1. Fractionation of normal female rat serum by rate zonal ultracentrifugation. Fifteen ml of serum was centrifuged for 8 hr (A), 16 hr (B), or 24 hr (C). For isolation of VHDL, fractions indicated in (B) were re-centrifuged for 24 hr (D).

tein (**Fig. 2**). Its mean hydrated density was determined to be 1.23 g/ml (range 1.20–1.27 g/ml) by equilibrium centrifugation. Therefore the isolated fraction was tentatively termed very high density lipoprotein (VHDL).

Chromatography on Sepharose 4B of serum resulted in separation into two fractions (**Fig. 3**). The minor peak coinciding with the void volume of the column represented the triglyceride-rich lipoproteins. The position of radioactive tracers suggested that the second peak contained VHDL and HDL, partially separated from each other. Accordingly, four major fractions of the column effluent were collected, which should contain no VHDL (I), VHDL (II), VHDL plus HDL (III), or HDL (IV). As expected, rate zonal ultracentrifugation (Fig. 3) revealed that fraction I contained VLDL and LDL, but no HDL or VHDL. Centrifugation of fraction II showed a peak of absorbance coinciding with ^{125}I-labeled VHDL and eluting at the position of VHDL as characterized above (Fig. 3, IIA and B). HDL could not be detected in this fraction. The major portion of VHDL was found in fraction III together with the bulk of HDL (Fig. 3, IIIA and B). Fraction IV contained HDL, but no VHDL could be detected, even after recentrifugation of the fractions in the position of the gradient characteristic of VHDL (Fig. 3, IVA and B).

The chemical composition of VHDL isolated by zonal ultracentrifugation of serum (Fig. 1) or of the column fractions II or III (Fig. 3) were very similar, but there was a higher content of unesterified cholesterol in VHDL from the column (**Table 1**). VHDL contained about 85% protein as compared to 40% in HDL. The relative distribution of the lipid components of VHDL and HDL was comparable except for a higher content of sphingomyelin and a lower content of phosphatidylcholine in VHDL.

VHDL was eluted from a permeation chromatography column ahead of HDL as one symmetrical peak. Its apparent molecular weight was estimated to be 1.35×10^6 (**Fig. 4**). On electron micrographs VHDL appeared as spherical particles with an average diameter of 150 Å as opposed to 106 Å for HDL (**Fig. 5**). Both the electron microscopic appearance and the width of the peak of VHDL in Sepharose 4B chromatography suggested greater heterogeneity within the VHDL population compared with HDL. In agarose gel electrophoresis ^{125}I-labeled VHDL constantly appeared as a single band. Mixed with HDL or whole serum, VHDL migrated with an electrophoretic mobility intermediate between alpha and prebeta lipoproteins.

Isoelectric focusing of the soluble proteins of VHDL revealed two major groups of bands in the region of apolipoprotein E and the C-apolipoproteins (C-II, C-III-0, C-III-3) as compared with the pattern of VLDL (**Fig. 6**). In contrast, HDL contained mainly apolipoprotein A-I. In some preparations an additional faint band appeared in the region of apolipoprotein E, possibly indicating the presence of apolipoprotein A-IV, which partially overlaps with apolipoprotein E in isoelectric focusing gels. SDS-electrophoresis in 5% acrylamide showed bands with a mobility comparable to apolipoprotein E and the C-apolipoproteins, but also small amounts of material co-

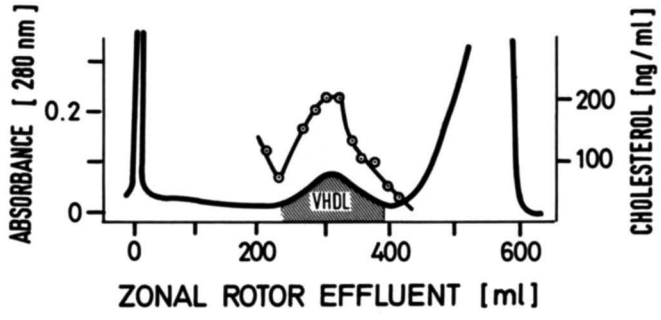

Fig. 2. Gas-liquid chromatographic determination of cholesterol (⊙-⊙) in fractions of the zonal rotor effluent after centrifugation of rat serum as in Fig. 1D. Absorbance 280 nm (—).

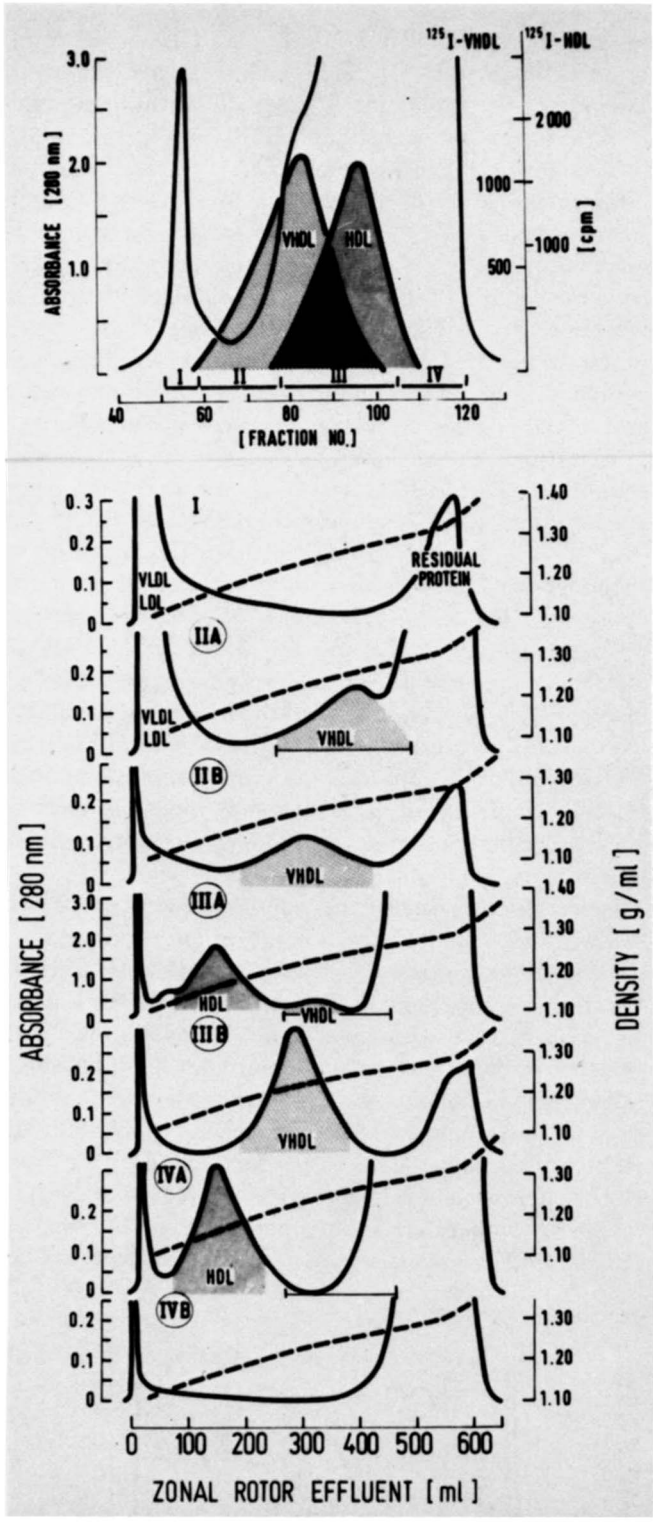

Fig. 3. Upper panel: fractionation of 20 ml of female rat serum by gel permeation chromatography on Sepharose 4B. Indicated elution volumes of VHDL and HDL were determined by the admixture of trace amounts of the respective radioiodinated lipoproteins in separate runs. Lower panel: pooled fractions as indicated in the upper panel were subjected to rate zonal ultracentrifugation for 16 hr (I, IIA, IIIA, IVA). Indicated fractions were recentrifuged for 24 hr (IIB, IIIB, IVB). Note the difference in the absorbance range in IIIA. The results of one of three experiments, each from three to five pooled fractions after gel chromatography, are shown.

TABLE 1. Composition of HDL and VHDL

	HDL n = 6 Zonal Ultra-centrifugation	VHDL n = 6 Zonal Ultra-centrifugation	VHDL n = 4 Gel Permeation Chromatography
		% ± *SD*	
Protein	40.8 ± 2.0	87.1 ± 0.8	84.1 ± 2.3
Lipids			
Cholesteryl ester	38.8 ± 2.2	41.9 ± 2.3	36.3 ± 25.0
Free cholesterol	4.4 ± 0.3	4.7 ± 3.9	15.0 ± 2.5
Triacylglycerol	8.0 ± 3.4	10.7 ± 9.3	6.9 ± 2.5
Phosphatidylcholine	38.1 ± 1.9	30.2 ± 3.1	28.7 ± 10.0
Sphingomyelin	8.5 ± 2.0	13.2 ± 1.6	13.1 ± 3.9
Lysophosphatidylcholine	2.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0

For calculation the following molecular weights were used: phosphatidylcholine, M_r 800; sphingomyelin, M_r 750; lysophosphatidylcholine, M_r 550; cholesteryl ester, M_r 650; cholesterol, M_r 386; triacylglycerol, M_r 880.

migrating with apolipoprotein A-I and A-IV. Above these bands an additional component was visible, possibly containing aggregated or higher molecular weight apolipoproteins. Electrophoresis in 3% SDS gels did not provide evidence for the presence of apolipoprotein B-100 or B-48 within VHDL, but suggested the presence of at least two unidentified proteins with a molecular weight lower than apolipoprotein B. Neither SDS, nor isoelectric focusing gels showed detectable amounts of albumin.

When ^{125}I-labeled HDL, incubated with normal female rat serum for about 60 min, was recentrifuged for 16 hr as in Fig. 1B, about 70% of the radioactivity was associated with HDL, while 15% and 12% were in the region of lighter lipoproteins or the residual proteins, respectively (**Fig. 7A**). After centrifugation of ^{125}I-labeled VHDL mixed with rat serum, more than 90% of the radioactivity was recovered as one peak in the position of VHDL (Fig. 7B). About 6% of the radioactivity co-eluted with the residual protein fraction. ^{125}I-labeled VHDL or ^{125}I-labeled HDL were injected intravenously into rats. After 1 hr the serum was subjected to rate-zonal ultracentrifugation. In the case of HDL, about 60% of the injected radioactivity was recovered in the plasma (Fig. 7A). Forty percent was found in the zone of HDL, whereas 15% and 5% were associated with the lipoproteins of lower density and the residual plasma proteins, respectively. This distribution of radioactivity was similar to that in the experiments in vitro. By contrast, in the experiment with ^{125}I-labeled VHDL in vivo, only about 10% of the injected radioactivity was recovered in the plasma, and it was widely distributed over the zone of VHDL and the adjacent zones after ultracentrifugation (Fig. 7B). Centrifugation, under the same conditions, of ^{125}I-labeled lymph chylomicrons or VLDL led to a redistribution of less than 4% of radioactivity to the position of HDL and the residual proteins (**Fig. 8**). Apparently no radioactivity was transferred from ^{125}I-labeled VHDL to other lipopro-

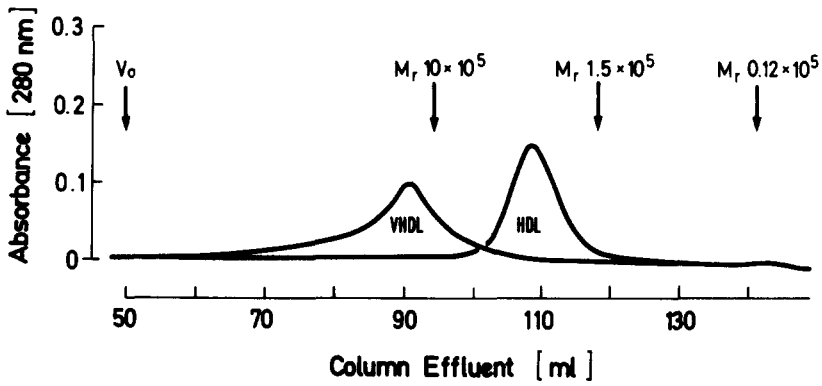

Fig. 4. Elution profiles of rat VHDL and HDL on a Sepharose 4B column, calibrated with reference proteins (glutamate dehydrogenase, HDL_3, and cytochrome C). Vo, void volume.

teins and virtually no radioactivity appeared in the zone of VHDL after centrifugation of ^{125}I-labeled chylomicrons, VLDL or HDL.

The decay of trace amounts of ^{125}I-labeled VHDL and ^{125}I-labeled HDL injected intravenously into intact rats is shown in **Fig. 9A and B**. ^{125}I-labeled VHDL was much more rapidly cleared from the circulation than ^{125}I-labeled HDL. Within 10 min 84% and 70% of the radioactivity of ^{125}I-labeled VHDL was removed from plasma of male and female rats, respectively, as opposed to only 21% and 10% in the case of ^{125}I-labeled HDL (Fig. 9A and B). In functionally eviscerated rats, ^{125}I-labeled VHDL left the plasma at a considerably lower rate (Fig. 9C). Under these conditions only 20% of the radioactivity had disappeared from the circulation within 10 min with little further decline thereafter.

Ninety-five percent of the radioactivity of VHDL, cleared from the plasma of intact rats within 10 min after injection, was found in the liver (**Fig. 10**). Neither muscle nor adipose tissue nor any of the parenchymal organs played a role in the uptake of ^{125}I-labeled VHDL comparable to that of the liver. Also, the radioactivity per gram of tissue was higher in the liver (**Table 2**), followed by the spleen and adrenals with a fourfold lower ratio.

Light microscopic radioautographs showed that 10 min after intravenous injection of ^{125}I-labeled VHDL into intact rats, silver grains representing the labeled apolipoproteins of VHDL were seen mainly over hepatocytes and near their sinusoidal surfaces (**Fig. 11**). There was no evidence of concentration of grains in v. Kupffer cells, as less than 1% of the grains were associated with these cells.

^{125}I-labeled VHDL bound to isolated rat hepatocytes was displaced by an excess of unlabeled VHDL (**Fig. 12A**). As with hepatocytes, VHDL bound to fibroblasts (Fig. 12B). Half-maximal inhibition of binding occurred at a tenfold excess of unlabeled VHDL. Also, increasing amounts of unlabeled VHDL competed progressively for the degradation of ^{125}I-labeled VHDL (Fig. 12C). Inhibition was about threefold more effective in human than in rat cells.

DISCUSSION

This study reports the isolation of a very high density lipoprotein from rat serum. Recently it was shown by rate zonal ultracentrifugation that HDL in this species comprises one major fraction, similar to the human HDL_2 (25). An additional minor fraction constituting about 10% of the total HDL, floats between the typical HDL and the lipoproteins of lower density (25) and resembles human HDL_c (26). Apparently rat HDL has a small, if any, HDL subpopulation comparable to human HDL_3 (26).

Recently it was shown that lipoprotein particles may exist with hydrated densities higher than that of HDL_3. Stimulation of lipolysis of triglyceride-rich lipoproteins in vivo generates small particles with a mean diameter of 58 Å and an average density of 1.23 g/ml in man (27). Laggner, Stabinger, and Kostner (28) claim the existence of a normal human lipoprotein with a density slightly higher than that of HDL_3.

In this investigation the rat was chosen as an animal model, which would permit experiments in vivo necessary to delineate the metabolic characteristics of isolated lipoproteins. Preference was given to female rats since in some centrifugations of serum from male rats a small peak was found in the position where HDL_3 would be expected to float and this might have interfered with the isolation of VHDL. Zone-broadening during prolonged centrifugation (29) turned out to be a problem in the isolation of VHDL. This difficulty was overcome by employing two consecutive centrifugations for 16 hr and 24 hr. At the same time this approach with a relatively short first

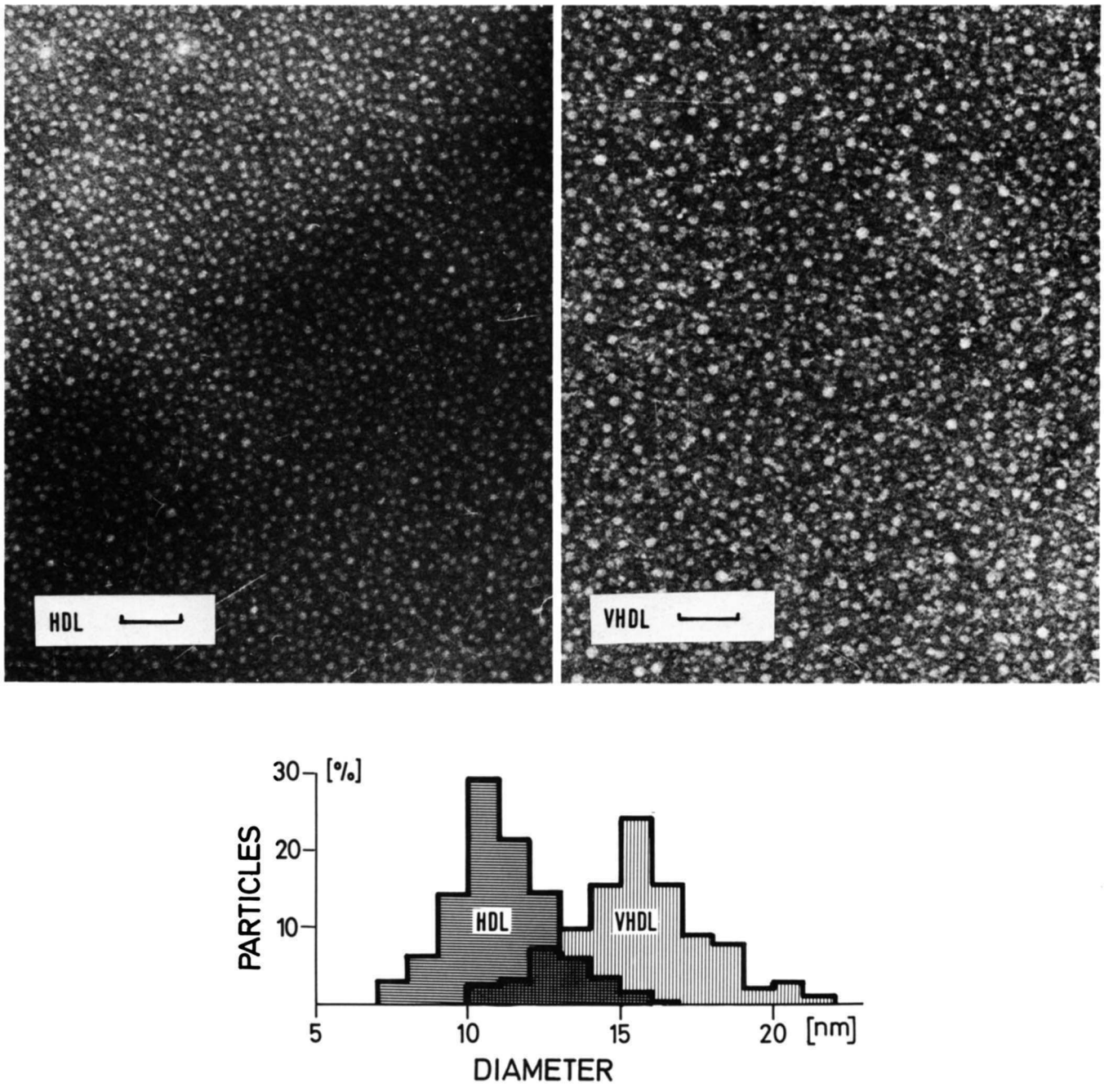

Fig. 5. Electron micrographs of negatively stained rat HDL and VHDL. Bar marker represents 1000 Å. For the distribution of particle diameters, 400 particles of each micrograph were measured.

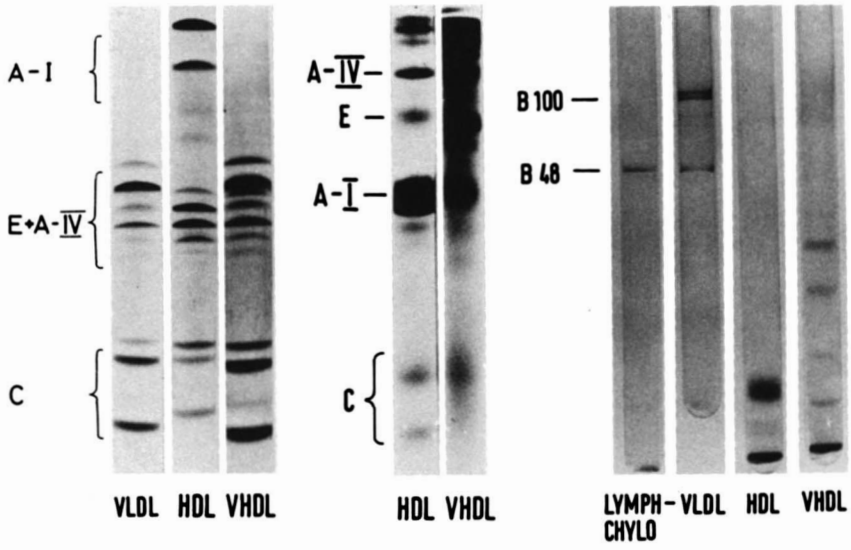

Fig. 6. Polyacrylamide gel electrophoretograms of apolipoproteins of rat lipoproteins. Left, isoelectric focusing (pH 3.5–7.0); middle, 5% SDS-gels; right, 3% SDS-gels.

spin for crude separation possibly reduced the chance of generating artificial complexes, inasmuch as apolipoproteins may dissociate and reaggregate with lipoproteins of different densities during centrifugation.

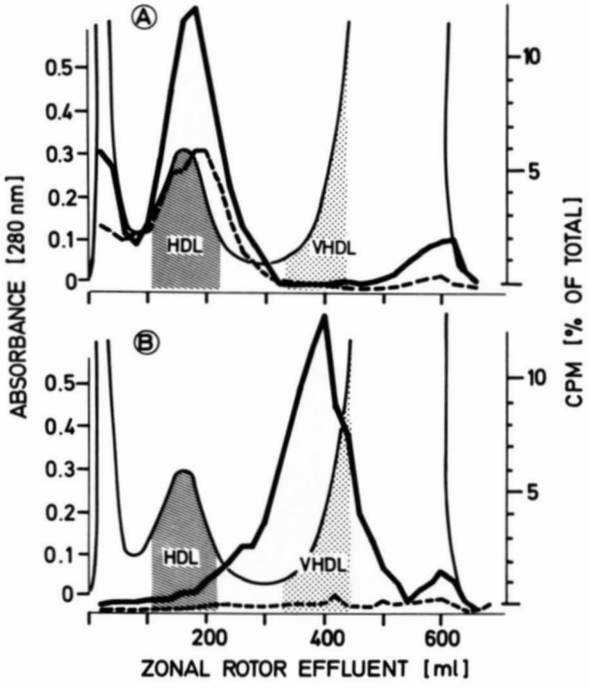

Fig. 7. Distribution of ^{125}I after rate zonal ultracentrifugation (16 hr) of ^{125}I-labeled HDL (A) or ^{125}I-labeled VHDL (B). Radioiodinated lipoproteins were either incubated at room temperature with 5 ml of female rat serum (——) or injected intravenously into rats (- -). After 60 min the lipoproteins were reisolated. Radioactivity is given as percent of the total radioactivity of the incubation mixture or of the injected dose. Absorbance 280 nm (——).

Nevertheless, artifacts of this kind had to be considered. However, experiments with radioiodinated chylomicrons, VLDL, and HDL indicated minimal dissociation of apolipoproteins or lipids, and of even more importance, virtually no radioactivity was recovered in the zone of VHDL. The validity of these experiments could have been compromised by the fact that the radioiodinated lipoproteins had already been centrifuged once, though for a short period. However, neither triglyceride-rich lipoproteins nor HDL isolated by gel permeation chromatography yielded VHDL upon zonal ultracentrifugation. On the other hand, VHDL could be demonstrated in a fraction of rat serum, from which HDL and the bulk of triglyceride-rich lipoproteins had been removed by gel

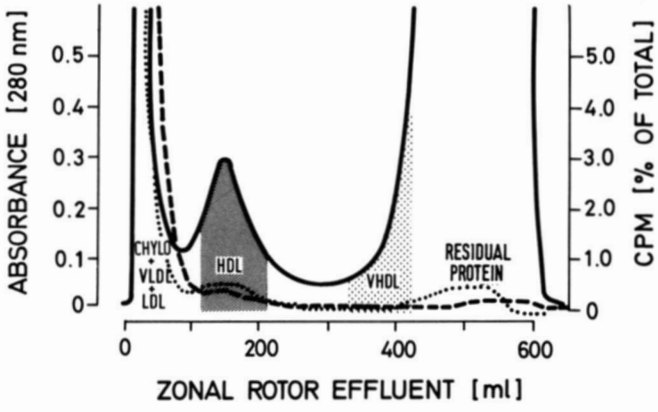

Fig. 8. Distribution of ^{125}I after zonal ultracentrifugation (16 hr) of ^{125}I-labeled chylomicrons (- - -) or ^{125}I-labeled VLDL (· · ·) incubated with 8 ml of female rat serum for 60 min at room temperature. Absorbance 280 nm (——).

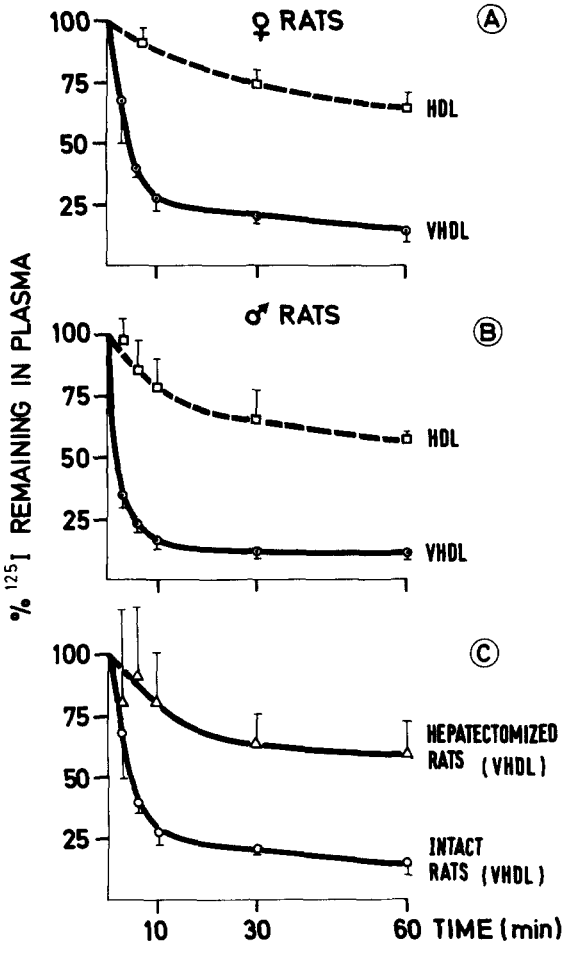

Fig. 9. Removal of ^{125}I from plasma after injection of ^{125}I-labeled HDL or ^{125}I-labeled VHDL into female (n = 3) (A) or male (n = 4) (B) intact rats. The removal of ^{125}I-labeled VHDL from plasma of female intact rats (n = 3) or hepatectomized rats (n = 5) is shown in (C). Bars indicate 1 SD.

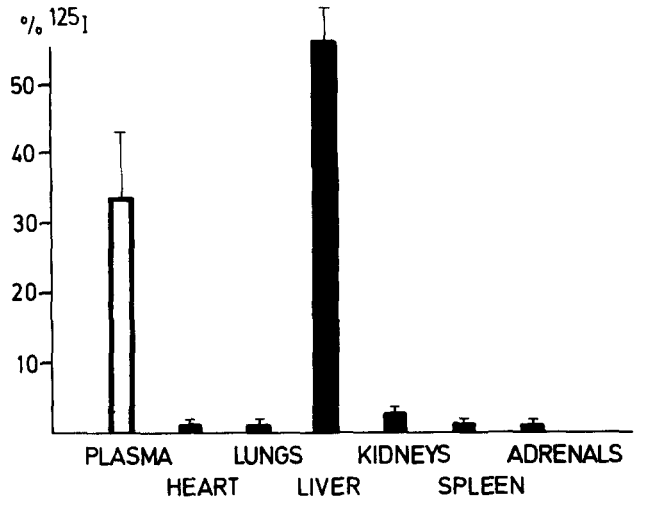

Fig. 10. Organ distribution of ^{125}I 10 min after intravenous injection of ^{125}I-labeled VHDL into female rats. Means of four experiments ± SD are given.

chromatography. Likewise, the chemical composition of VHDL with its high content of cholesteryl esters argues against the possibility that VHDL is derived from dissociated surface components of other lipoproteins.

There was no difference in the amount of VHDL recovered from fresh serum or serum that had been stored at 4°C for up to 2 weeks. Hence these lipoproteins are not likely to be formed spontaneously with time. Rather they exist as stable particles. This is demonstrated by the lack of significant loss of ^{125}I-labeled apolipoproteins from VHDL during centrifugation as well as by the appearance of one single peak upon permeation chromatography or repeated centrifugation.

From the chemical composition of VHDL, an average hydrated density of 1.28 g/ml was calculated, which is in good agreement with that determined by equilibrium centrifugation. Also, the experimental values of hydrated density and apparent molecular weight gave a theoretical particle diameter of 152 Å, which is close to that obtained by electron microscopy. These calculations validate the experimental data and the techniques that were used.

Although the results of this investigation suggest that VHDL is a genuine and stable particle, its quarternary structure cannot readily be derived from its physical and chemical properties. Based on the current concept of lipoproteins as pseudomicellar structures, VHDL obviously contains an excess of surface material relative to core components. One might, however, envisage a particle where the hydrophobic core is occupied in part by the apolar regions of apolipoproteins. The high protein content of VHDL is not likely to be due to contaminating proteins such as albumin, since albumin could not be detected in gel electrophoresis and recentrifugation of ^{125}I-labeled VHDL did not yield significant amounts of radioactivity in the zone of the residual plasma proteins.

VHDL is rapidly cleared from the plasma and is almost

TABLE 2. Percent of radioactivity per gram of tissue 10 min after injection of ^{125}I-labeled VHDL[a]

	Percent ± SD[b]
Liver	6.99 ± 0.64
Spleen	1.96 ± 0.55
Adrenals	1.66 ± 0.62
Lungs	0.71 ± 0.24
Kidneys	0.34 ± 0.09
Heart	0.11 ± 0.03
Muscle	0.06 ± 0.04
Adipose tissue	0.04 ± 0.01

[a] Recovery of injected ^{125}I in indicated organs and plasma without muscle and adipose tissue was 92.10 ± 5.72%.
[b] Mean of four experiments.

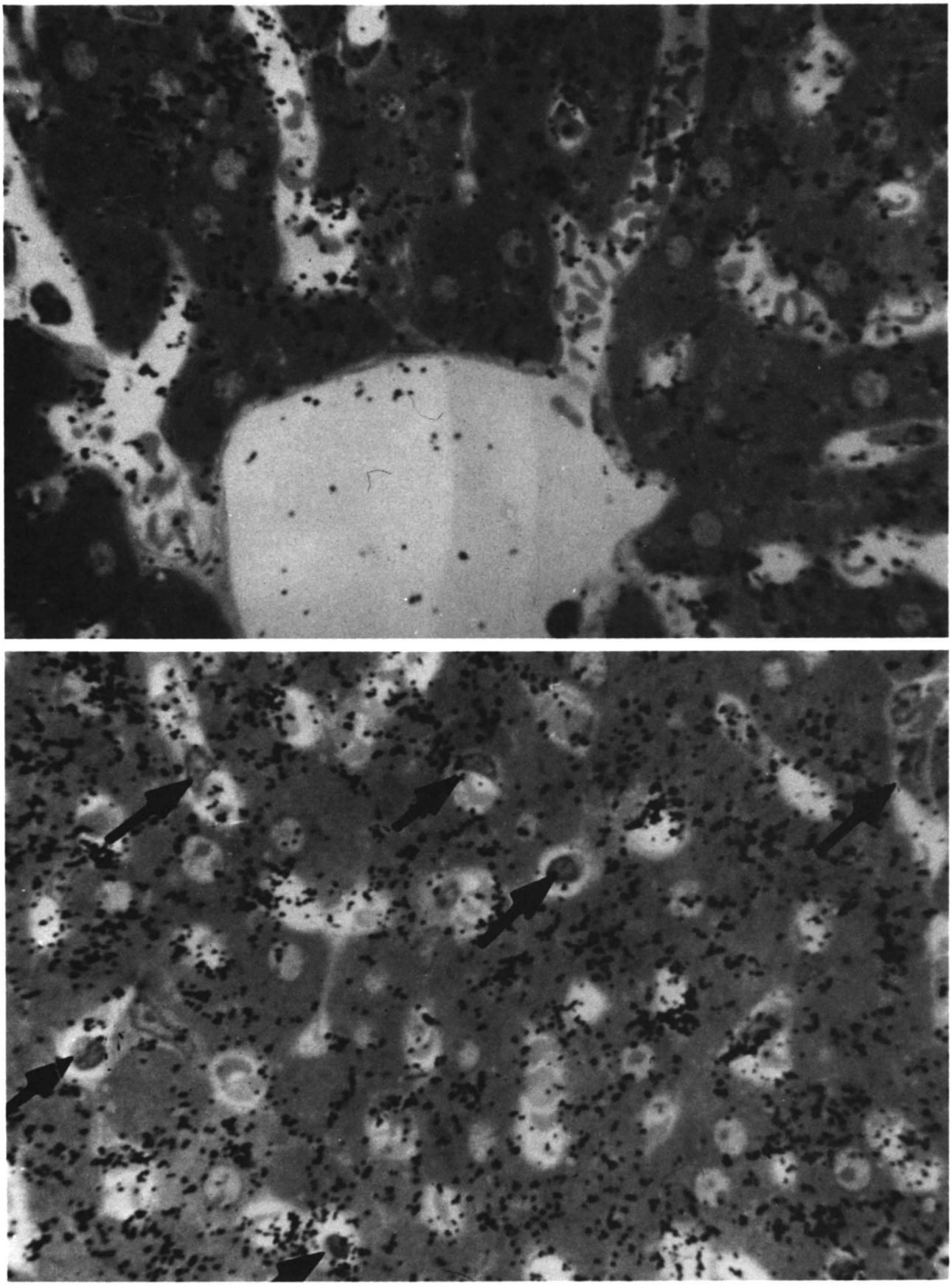

Fig. 11. Light microscopic radioautographs of thick sections of rat liver 10 min after intravenous injection of ^{125}I-labeled VHDL. More than 99% of the silver grains representing ^{125}I-labeled VHDL are associated with hepatocytes. Arrows indicate nonparenchymal cells.

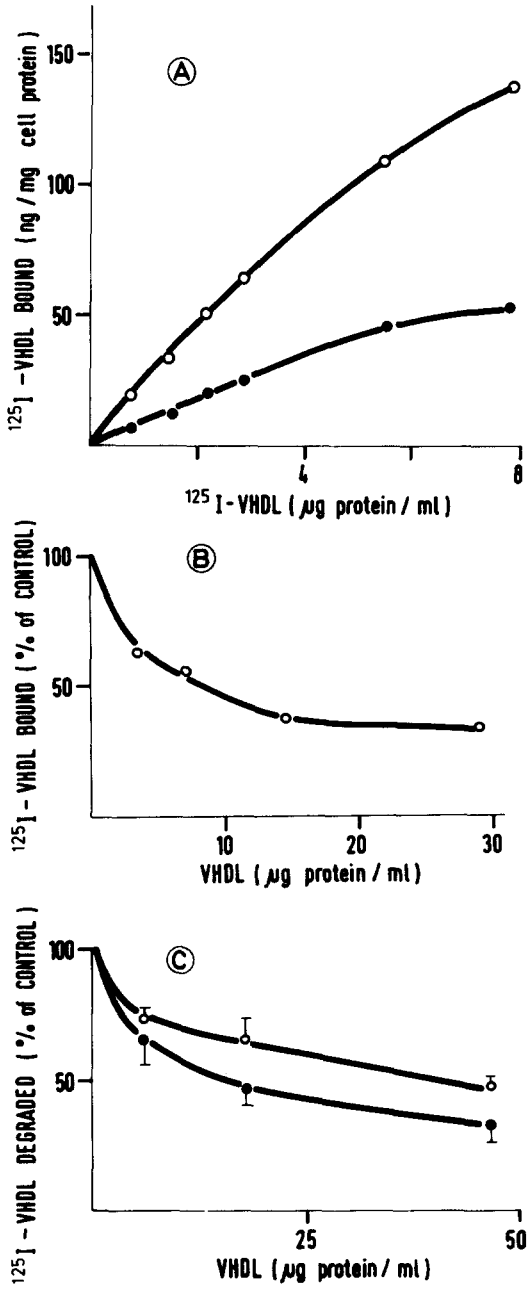

Fig. 12. Binding of ^{125}I-labeled VHDL in the absence (○) or presence (●) of a 50-fold excess of unlabeled VHDL by rat hepatocytes. The mean of two assays carried out in triplicate at 4°C for 60 min is shown (A). Binding of ^{125}I-labeled VHDL (0.7 µg of protein/ml) in the presence of increasing amounts of unlabeled VHDL by human skin fibroblasts. ^{125}I-labeled VHDL bound in the absence of unlabeled lipoprotein (35 ng/mg of cell protein) was taken as 100%. The mean of two assays carried out in triplicate at 4°C for 2 hr is shown (B). Degradation of ^{125}I-labeled VHDL (0.6 µg of protein/ml) in the presence of increasing amounts of unlabeled VHDL by human (○) and rat (●) skin fibroblasts. ^{125}I-labeled VHDL degraded in the absence of unlabeled lipoprotein (692 ± 89, human or 632 ± 100, rat, ng/mg of cell protein) was taken as 100%. The means and 1 SD of three assays carried out in triplicate at 37°C for 6 hr are shown (C).

exclusively taken up by hepatocytes. These results were obtained with ^{125}I as a label, which predominantly represents the protein moiety of VHDL. Nevertheless, ^{125}I can be considered a reliable marker of the whole VHDL particle, since there is no evidence for exchange of the label with other lipoproteins during incubation in vitro or after injection into rats. The results also suggest that VHDL is not converted to a lipoprotein of different density within the vascular space. Transfer of ^{125}I-labeled VHDL components to lipoproteins with rapid turnover, however, is not excluded, although this possibility is unlikely for the following reason. In the case of HDL, considerable transfer of ^{125}I-labeled constituents onto VLDL does occur but, in spite of the very short half-life of VLDL of less than 4 min (12), radioactivity was still found in the VLDL zone. Moreover, the distribution of radioactivity in the in vitro study was similar to that in the in vivo experiment where VLDL is continously degraded.

Because of this exchange of label, the decline of radioactivity in HDL does not necessarily reflect the catabolism of the HDL particles. Considering that this may result in an overestimation of the turnover of HDL, the actual difference in the rate of removal between VHDL and HDL appears to be even more pronounced. The half-life of VHDL, as derived from our data, should be less than 5 min, which is similar to that of VLDL (12). In comparison with the reported half-life of rat HDL of about 10 hr (30), this would mean a more than 100-fold faster turnover of VHDL. As VHDL cholesterol accounted for about 2% of the cholesterol in HDL, the cholesterol transport capacity of VHDL may be 2 times higher than that of HDL. In contrast to HDL, virtually all of the cholesterol is carried to the liver.

As shown by radioautography, VHDL is predominantly taken up by hepatocytes, which are able to excrete cholesterol of degraded lipoproteins in the bile. This uptake may be mediated by specific cell surface receptors, which is strongly suggested by the demonstration of specific binding of VHDL to isolated rat hepatocytes. Competitive inhibition of binding and degradation of VHDL can also be shown in rat and human skin fibroblasts. In comparison to LDL and even chylomicron remnants, VHDL is more avidly bound and degraded at a much higher rate (17). The predominant uptake of VHDL by the liver is probably due to the free access to hepatocytes via the sinusoidal fenestrae as is the case of other short-lived lipoproteins like remnant particles.

Taken together, it appears that VHDL, although a minor fraction of the total HDL, may play an important role in cholesterol transport. Obviously, however, knowledge about the site of synthesis of VHDL is critical for an understanding of its physiological function.

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