Isolation of human serum HDL_1 by zonal ultracentrifugation

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Abstract High density lipoprotein subfraction-1 (HDL_1) is thought to interact with the high-affinity apoprotein B, E receptors of peripheral cells and may act as a modulator of LDL binding and uptake. In the present study the concentration and composition of HDL_1 in normal and hypercholesterolemic sera were studied using zonal ultracentrifugation. To permit separation of the HDL_1 from VLDL, LDL, and Lp(a), the apoB-containing lipoproteins were first precipitated from serum using the phosphotungstic acid/magnesium chloride ($PTA/MgCl_2$) method after which the supernatant fraction was subjected to zonal ultracentrifugation. It could be demonstrated that following $PTA/MgCl_2$ precipitation HDL_1 floats as a single peak at d 1.08–1.09 g/ml (NaBr) and is sufficiently separated from high density lipoprotein-2 (HDL_2) and high density lipoprotein-3 (HDL_3). The HDL_2/HDL_3 subfraction pattern was not affected by the precipitation method. As previously described, in vitro incubation of serum leads to the LCAT-dependent interconversion of HDL_3 or HDL_2. Using the technique described here, it was discovered that a simultaneous elevation of HDL_1 occurred. This increase in HDL_1 concentration could not be observed when LCAT was inhibited by heat inactivation or addition of Ellman's reagent. In normal fresh serum only a small HDL_1 peak could be detected, but in patients with familial hypercholesterolemia (apoB, E receptor deficiency) HDL_1 was elevated five to tenfold compared to normal values and further increased in concentration upon incubation of serum. On the other hand, in sera of patients with familial HDL deficiency (Tangier disease), HDL_1 was undetectable. Analysis of the HDL fractions in serum of a patient with abetalipoproteinemia revealed that following in vitro incubation there was formation of HDL_1 despite the lack of apoprotein B-containing lipoproteins.█ These data support the concept that HDL_1 formation occurs during LCAT-mediated HDL_3/HDL_2 interconversion in vitro.—**Schmitz, G., and G. Assmann.** Isolation of human serum HDL_1 by zonal ultracentrifugation. *J. Lipid Res.* 1982. **23:** 903–910.

Supplementary key words LCAT • HDL_2 • HDL_3 • apoE

Despite present understanding of the role of various lipoproteins in transporting cholesterol in blood, little is known about the factors that regulate the flux of cholesterol to and from the peripheral tissues. The homeostatic control of these two pathways may be critical in restricting cholesterol accumulation in tissues and thus of importance in preventing atherosclerosis. The bulk of the plasma cholesterol is transported in the LDL fraction. LDL transfers cholesterol to the peripheral tissues, where it is recognized by apoB, E receptor cells and, after cellular uptake, is degraded (1). Transfer from peripheral tissues to the liver is thought, on the other hand, to be mediated by high density lipoproteins (2, 3). Thus, the deposition of cholesterol in tissues theoretically may result from enhanced transfer by LDL or from a failure to eliminate cellular cholesterol via the HDL pathway.

The precise mechanism by which HDL removes cholesterol from peripheral cells is not yet known. In addition to the possible role of circulating HDL_3 as a cholesterol-acceptor macromolecule, tissue cholesterol may be alternatively removed by HDL precursors that are interconverted to HDL_2 by the activity of lecithin:cholesterol acyltransferase (LCAT) (4). These particles may then be taken up by the liver as intact particles (5), or the cholesterol ester moiety may be transferred to the hepatic cells with subsequent reconstitution to form an HDL_3 particle (6). A further HDL subfraction potentially involved in the transport of cholesterol is the apoprotein E-containing HDL_1 fraction. The present study provides evidence that HDL_1 is formed during the LCAT-mediated HDL_3/HDL_2 interconversion and that in the serum of patients with apoB, E receptor deficiency, HDL_1 is increased in concentration. It is suggested that the concentration of HDL_1 may be physiologically important in regulating LDL uptake by apoB, E receptor cells.

MATERIAL AND METHODS

Incubation studies

All incubations were performed in a shaking water-bath at 37°C with serum obtained from freshly drawn blood. The phosphotungstic acid/magnesium chloride

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Abbreviations: HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; HPTLC, high performance thin-layer chromatography.

(PTA/MgCl₂) method (7) was used to precipitate the apoprotein B-containing lipoproteins from fresh and incubated samples. Using the PTA/MgCl₂ precipitation method, the supernatant is free of apoB-containing lipoproteins, while the concentration of HDL is not affected (7). Coprecipitation studies of ^{125}I-labeled HDL₁ added to normal serum before PTA/MgCl₂ precipitation revealed a 90–95% recovery of the radioactivity in the supernatant. LCAT was inhibited either with Ellman's reagent (1.4 mmol/l) (2) or by heat-inactivation at 56°C for 30 min (9). Addition of Ellman's reagent resulted in 95% inhibition of LCAT activity, whereas heat-inactivation led to total inhibition.

Isolation of lipoproteins from incubated samples

Zonal ultracentrifugation. Two-ml aliquots of the samples before and after 12 hr incubation at 37°C were subjected to zonal ultracentrifugation for analysis of HDL subclasses. Ultracentrifugation was performed with a Beckman L8-70 ultracentrifuge and a Beckman Z-60 rotor at 59,000 rpm for 18 hr at 4°C. All zonal ultracentrifugations were carried out at constant $\omega^2 \times t$ values. A nonlinear three-step NaBr gradient modified for the Z-60 rotor was used for the separation of lipoproteins (10). With this procedure, lipoproteins of d 1.063 g/ml emerge in the first 30–40 ml of the rotor effluent, while the HDL range is maximally expanded. The sera were adjusted to d 1.4 g/ml NaBr prior to injection. After centrifugation the rotor was deloaded by displacing the content with a d 1.4 g/ml NaBr solution (flow rate of 10 ml/min) from the rotor periphery. The effluent was continuously monitored by absorbance at 280 nm, and collected in 5-ml fractions. The gradient of each 5-ml fraction was measured by sodium determination in a Beckman K-Li-Na-flame photometer and the density was calculated from the following formula: at 20°C d-NaBr (g/ml) = 2.307 × 10⁻³ × (Na⁺ (mmol/l)) + 1.00155, corrected for density at 4°C (11).

Isopycnic ultracentrifugation. For preparative purposes, HDL₁ was obtained from serum after phosphotungstic acid/magnesium chloride precipitation followed by ultracentrifugation for 24 hr at d 1.075 g/ml KBr, 4°C in a Beckman 70 Ti-rotor. The supernatant was dialyzed against 0.005 M Tris buffer, 0.15 M NaCl, pH 7.4. The final dialysis was performed in 0.005 M Tris buffer (pH 7.4) containing 0.005 M NaCl and 0.025 M MnCl₂. The dialyzed fractions were subsequently applied to a 0.8 × 40 cm heparin-Sepharose affinity column for separation of residual HDL-A (consisting primarily of apoA-I and apoA-II) from HDL-E (consisting of apoE in addition to apoA-I and apoA-II) (Fig. 8) according to the method described by Mahley and Weisgraber (12), using Sigma analytical-grade heparin.

Labeling of HDL-E-containing particles

HDL particles containing apoE were isolated either as described for the isopycnic ultracentrifugation of HDL₁ or from the HDL₃ subfraction derived from normal serum after zonal ultracentrifugation and subsequent heparin-Sepharose chromatography (12). The column effluent was concentrated in an Amicon micro-cell (XM-100 membrane) up to 15 mg protein/ml and labeled by the McFarlane method (13). The labeled lipoprotein was separated from excess iodine by dialysis against 0.005 M Tris-HCl, 0.15 M NaCl, pH 7.4. Radioactivity was measured in a Kontron gamma-counter Mod. MR 480. Four to six % of the radioactivity was found in the lipid fraction; the remainder was associated with the apoproteins. As demonstrated by SDS polyacrylamide gel electrophoresis (14), 55% of the protein-associated activity comigrated to apoA-I and 28% to apoE when β-mercaptoethanol and 1% decylsulfate (Eastman Kodak) were added to the samples.

Analysis of lipoproteins

The zonal rotor fractions were pooled and analyzed for lipids and apoproteins. Each pool was dialyzed exhaustively against normal saline, pH 7.4. After dialysis the fractions were concentrated to a volume of 0.5–1 ml of Amicon multiconcentration cells using XM-100 membranes. Free and esterified cholesterol, triglycerides, and phosphatidylcholine were analyzed either by enzymatic procedures (Boehringer Lot No.: 124 087; 274 119) or by quantitative high performance thin-layer chromatography (HPTLC) (15). Protein was measured by the procedure of Lowry et al. (16). Lipoproteins were delipidated with ethanol–ether 3:1 at −20°C as previously described (17). Apoprotein analysis was performed either by flat bed SDS polyacrylamide gel electrophoresis using the Neville system (14) in the one- or two-dimensional technique or by polyacrylamide gel isoelectric focusing in the pH range between 3.5 and 7 (18). Apoproteins were visualized by staining with Coomassie blue G-250 and quantitated by gel densitometry in a Shimadzu CS-910 Scanner. With respect to individual chromogenicity, calibration curves (four points) were prepared for each apoprotein on the same gel. The individual masses of polymorphic forms, as derived from the curves, were added to yield the total mass for each apoprotein. Identification of the apoprotein bands in isoelectric focusing and SDS gels were performed using the comigration technique of purified apoproteins in one- and two-dimensional SDS gels. Electron microscopy was carried out with a Hitachi H 500 electron microscope. The lipoprotein particles were visualized by negative staining using either phosphotungstic acid or uranyl acetate.

Patients

The normal sera were derived from ten normolipidemic male students. Other sera were obtained from two patients with the homozygous or heterozygous form of familial hypercholesterolemia (FH). Patient P.D. was a 12-year-old girl (total cholesterol, 21.98 mmol/l; triglyceride, 0.84 mmol/l) homozygous for FH; and patient G. Sch. was a 35-year-old woman (total cholesterol, 9.05 mmol/l; triglyceride, 0.49 mmol/l) heterozygous for FH. Both patients were known to have a deficiency of apoB, E receptors in fibroblasts. The patients affected with abetalipoproteinemia (M.K., 20 years old, male: total cholesterol, 0.70 mmol/l; triglyceride, 0.08 mmol/l), Tangier disease (E.G., 48 years old, female: total cholesterol, 2.46 mmol/l; triglyceride, 2.71 mmol/l; and J.S., 44 years old, male: total cholesterol, 1.29 mmol/l; triglyceride, 3.95 mmol/l) have been described in previous publications (19, 20).
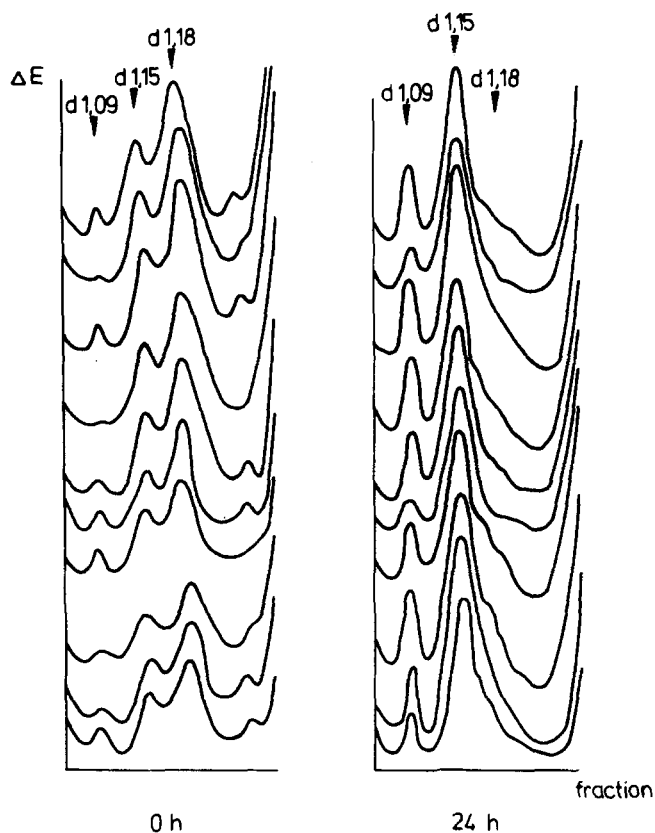

Fig. 1. Zonal ultracentrifugation analysis of 1.8 ml of PTA/MgCl₂-supernatant derived from sera of ten fasting normolipidemic male students. The left plot demonstrates the absorption profiles at 280 nm of supernatants from nonincubated sera (0 hr); the right plot reveals the absorption profiles of corresponding supernatant aliquots after 24 hr incubation of sera at 37°C. All zonal ultracentrifugations were carried out at 250,000 g, 4°C for 18 hr and constant $\omega^2 \times t$ values.
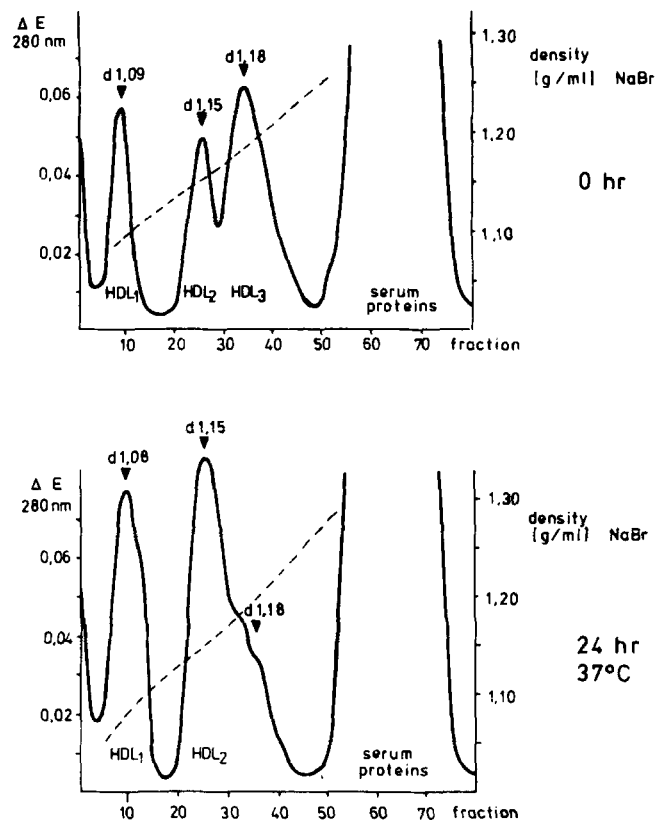

Fig. 2. Zonal ultracentrifugation analysis of fresh (upper) and incubated (lower) serum of a patient with homozygous familial hypercholesterolemia (P.D., homozygote FH). After 0 hr and 24 hr incubation at 37°C, serum was treated with PTA/MgCl₂ and the supernatant was subjected to zonal ultracentrifugation.

RESULTS

To study the HDL subfraction distribution in sera of normolipidemic patients and of patients with various forms of dyslipoproteinemia, supernatant fractions of PTA/MgCl₂ precipitations were subjected to zonal ultracentrifugation. As shown in **Fig. 1,** two major fractions were resolved in normolipidemic serum. These fractions were identified by chemical composition and electron microscopy as HDL_2 and HDL_3. The subfraction distribution of HDL_2 and HDL_3, as obtained with native serum (not shown), was not affected by the precipitation method employed (HDL_2, d 1.15 g/ml NaBr and HDL_3, d 1.19 g/ml NaBr, respectively). Furthermore, the chemical and morphologic characteristics of the HDL_2 and HDL_3 obtained after PTA/MgCl₂ precipitation and subsequent zonal centrifugation were indistinguishable from reference preparations obtained from zonal centrifugation fractions of fresh serum. In addition to the two major subfractions, another small peak was detected in the density range of approximately 1.08 g/ml NaBr
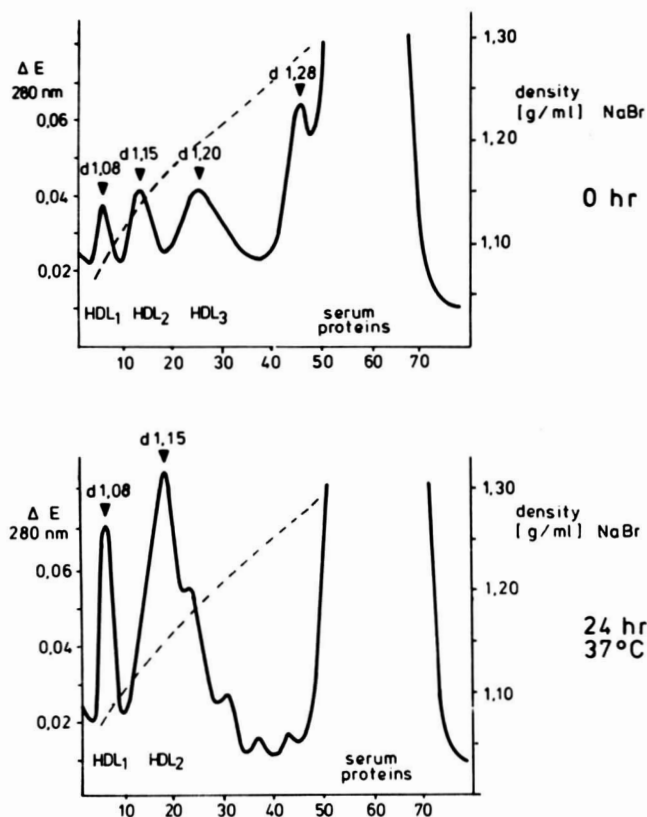
Fig. 3. Zonal ultracentrifugation analysis of fresh (upper) and incubated (lower) serum of a patient with heterozygous familial hypercholesterolemia (G. Sch.; heterozygous for FH). After 0 hr and 24 hr incubation at 37°C, serum was treated with PTA/MgCl$_2$ and the supernatants were subjected to zonal ultracentrifugation.

(Fig. 1, left). During incubation of the serum at 37°C for 24 hr (Fig. 1, right), this fraction increased in concentration simultaneously with the LCAT-mediated interconversion of HDL$_3$ to HDL$_2$ (4).

Fig. 2 and Fig. 3 demonstrate the zonal ultracentrifugation pattern of patients with homozygous and heterozygous forms of familial hypercholesterolemia. In the fresh sera of these patients the d 1.08 g/ml NaBr fraction was elevated five to tenfold as compared with normal sera (compare Figs. 2 and 3, top with Fig. 1, left), when the PTA/MgCl$_2$ supernatants of sera from patients with familial hypercholesterolemia were analyzed by zonal ultracentrifugation before and after in vitro incubation of native sera at 37°C. In addition to the HDL$_3$/HDL$_2$-interconversion, a further peak increase in the d 1.08 g/ml NaBr fraction was observed.

Zonal ultracentrifugation analysis of serum from a patient with abetalipoproteinemia (**Fig. 4**) revealed the near absence of HDL$_1$ and an abnormal HDL$_2$/HDL$_3$ subfraction distribution in fresh serum. However, after in vitro incubation, a substantial peak at d 1.08 g/ml
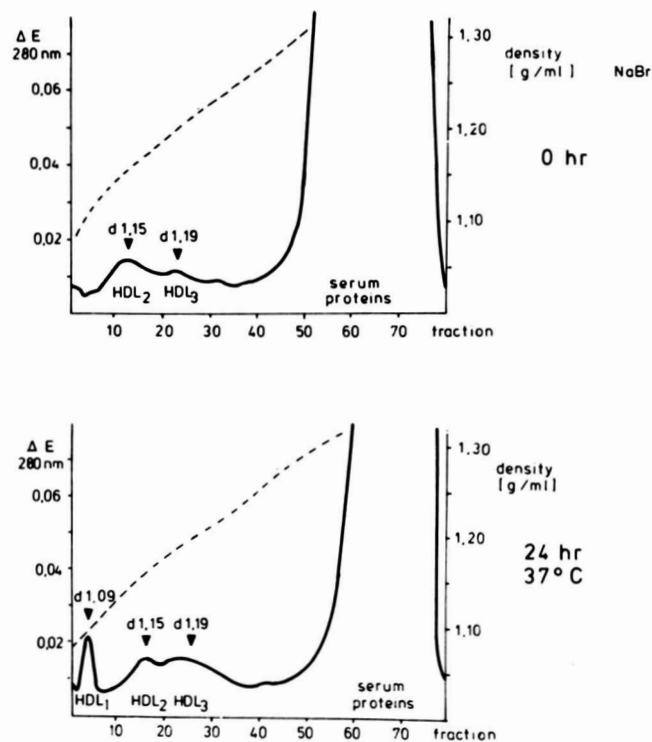

Fig. 4. Zonal ultracentrifugation analysis of fresh (upper) and incubated (lower) serum derived from a patient with recessive abetalipoproteinemia (K.M.). After 0 hr and 24 hr incubation at 37°C, serum was directly subjected to zonal ultracentrifugation.

NaBr could be detected. On the other hand, in sera of patients with familial HDL deficiency (Tangier disease), the fraction at d 1.08 g/ml was undetectable both before and after incubation (not shown).

In fresh normal serum the fraction floating at d 1.06–1.09 g/ml NaBr exhibited α_2-mobility in agarose electrophoresis (**Fig. 5**); electron microscopy of this fraction
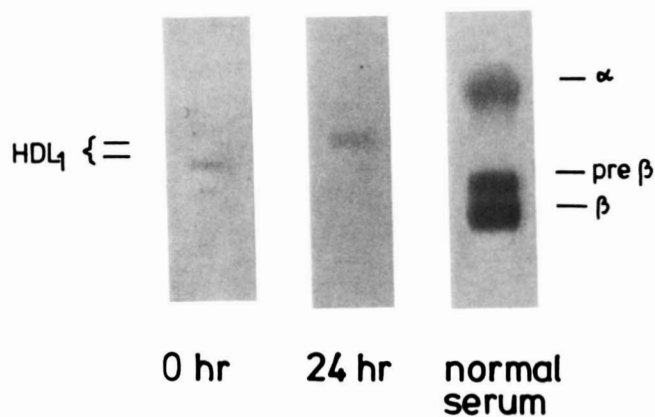

Fig. 5. Lipoprotein electrophoresis of d 1.08 g/ml NaBr fraction derived from zonal ultracentrifugation of serum after PTA/MgCl$_2$ precipitation of apoprotein B-containing lipoproteins.
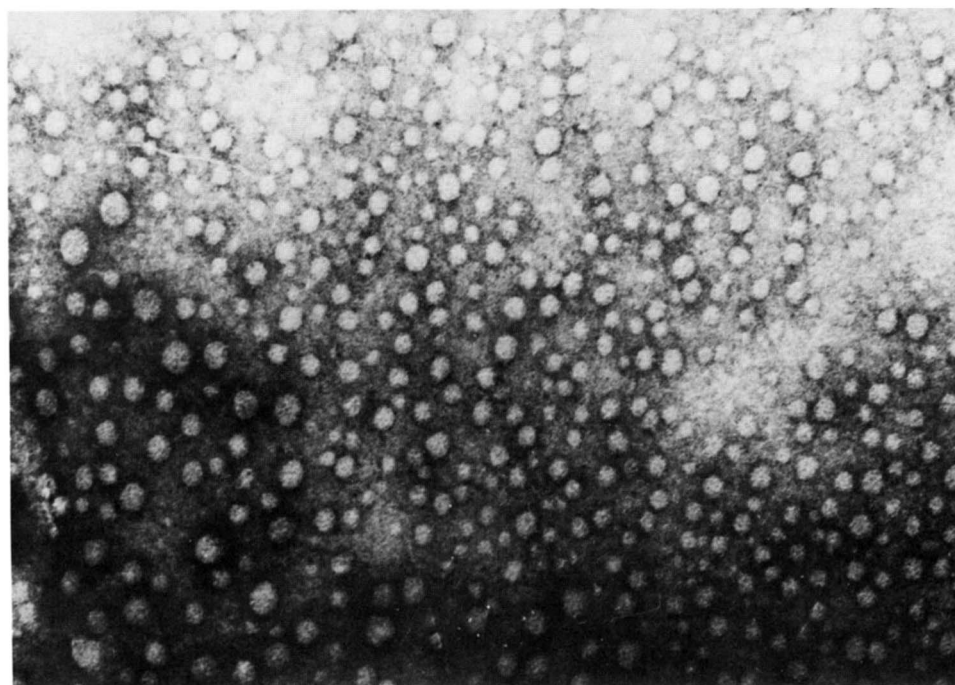
Fig. 6. Electron microscopy of the d 1.08 g/ml NaBr fraction derived from zonal ultracentrifugation of serum after PTA/MgCl₂ precipitation of apoprotein B-containing lipoproteins.

revealed spherical particles with a diameter of 13–19 nm (**Fig. 6**). Analysis of the fraction after in vitro incubation of normal serum resulted in a slight charge shift in agarose electrophoresis (Fig. 5), but no significant change in size (not shown).

Heparin-Sepharose affinity chromatography was performed to further identify the nature of the lipoprotein floating at d 1.085 g/ml NaBr and here designated HDL_1. As shown in **Fig . 7,** the d 1.08–1.09 g/ml NaBr fraction bound to the column and was eluted with increasing concentrations of NaCl at 0.15 M. Two minor fractions were also resolved and eluted at 0.07 M and 0.3 M NaCl. The nature of these minor fractions was not further investigated.

The apolipoprotein composition of the d 1.08–1.09 g/ml NaBr fraction, analyzed by SDS gel electrophoresis and isoelectric focusing, revealed apoA-I, apoA-II, and apoE as the major apolipoproteins in fresh (**Fig. 8, Table 1**) and incubated sera (not shown). The fraction isolated from fresh serum also contained C-apolipoproteins, whereas in the fraction derived from incubated serum C-peptides were barely detectable.

Quantitative analysis of lipids by HPTLC of the d 1.08–1.09 g/ml NaBr fraction revealed cholesterol, mainly cholesteryl esters, as the predominant lipid moiety; di- and triglycerides were also present (Table 1). The phospholipid concentration was low with a rel-

ative phosphatidylcholine/sphingomyelin molar ratio of about 4.1:1. Lipid analysis of the d 1.08–1.09 g/ml NaBr fraction after in vitro incubation of fresh serum revealed an increased concentration of cholesteryl esters, mainly cholesteryl linoleate, and a decreased concentration of free cholesterol and triglycerides (Table 1).

To investigate the metabolic origin of the HDL_1 subfraction, HDL-E-containing particles were isolated from the HDL_3 fraction of fresh serum (which was obtained by ultracentrifugation) by means of heparin-affinity chromatography. After ^{125}I-labeling, the ^{125}I-HDL-E was again added to fresh normal serum and subjected to zonal ultracentrifugation. In fresh serum, the major peak of radioactivity was detected in the HDL_3-fraction
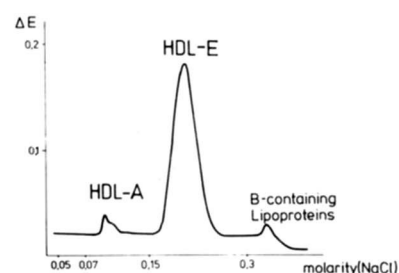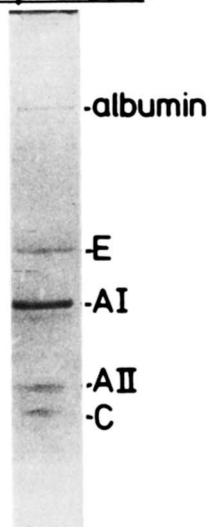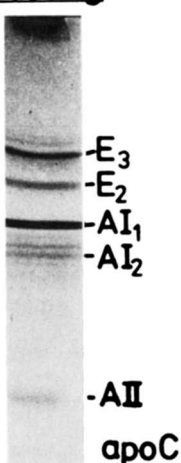

Fig. 7. Heparin-Sepharose chromatography of d 1.08 g/ml NaBr fraction of serum after PTA/MgCl₂ precipitation of apolipoprotein B-containing lipoproteins.

SDS-electrophoresis:
- -albumin
- -E
- -AI
- -AII
- -C

isoelectric focusing:
- -E$_3$
- -E$_2$
- -AI$_1$
- -AI$_2$
- -AII
- apoC

two-dimensional electrophoresis:
1. isoelectric focusing
2. SDS electrophoresis

mw std.
- -94,000
- -67,000
- -43,000
- -30,000
- -20,000
- -14,400

E$_1$E$_2$E$_3$
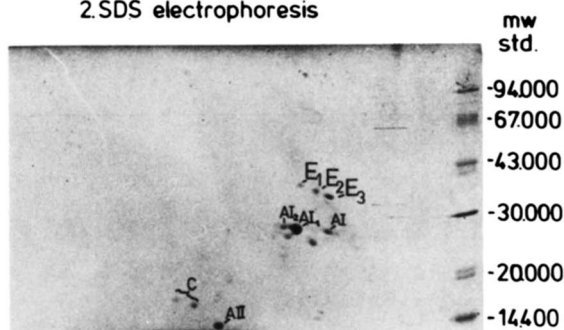AI$_1$AI$_2$ AI
C
AII

Fig. 8. Density 1.08 g/ml NaBr fraction obtained by zonal ultracentrifugation and analyzed for apoprotein composition by SDS polyacrylamide gel electrophoresis (left) and isoelectric focusing (pH 3.5–7) (middle) in conjunction with SDS gel electrophoresis (right).

(**Fig. 9**). When serum was incubated a significant portion of the radioactivity shifted to the lower densities coinciding with the HDL$_1$ fraction. When LCAT was inhibited (not shown) the major radioactivity peak remained in the HDL$_3$ range following incubation.

DISCUSSION

The present studies indicate that a lipoprotein with the compositional (apoE as major apoprotein), electrophoretic (α_2-mobility), and morphologic (13–19 nm diameter) characteristics of HDL$_1$ can be isolated from the PTA/MgCl$_2$ supernatants of fresh normolipidemic sera by zonal ultracentrifugation in the density range of 1.08–1.09 g/ml NaBr. This ultracentrifugal fraction, in contrast to the major fractions HDL$_2$ and HDL$_3$, binds to a heparin-Sepharose affinity column and can be eluted by increasing concentrations of NaCl. Thus, this fraction has characteristics similar to the lipoprotein HDL$_c$, previously characterized by Mahley (21) as a major lipoprotein in cholesterol-fed animals. This lipoprotein increases in concentration upon incubation of serum at 37°C. It is present in high concentrations in fresh sera of patients with familial hypercholesterolemia and could not be detected in native or incubated sera of patients with Tangier disease. However, it was found to be present in the incubated serum of a patient with abetalipoproteinemia.

We have previously demonstrated that LCAT participates in the interconversion of HDL$_3$ to HDL$_2$ (4). In those studies, only whole sera were analyzed by zonal centrifugation and the d 1.08–1.09 g/ml NaBr fraction could not be resolved due to the overlap of apoB-containing lipoproteins in the ultracentrifugal flotation profile. The HDL$_1$ subfraction, as shown in the present studies, could only be separated by zonal ultracentrifugation after prior precipitation of apoB-containing lipoproteins.

The term HDL$_1$ has been used in the past by different authors for the apoprotein E-containing HDL (21–23). As shown by Mahley and collaborators, HDL$_1$ increases

TABLE 1. Lipid and apoprotein composition of HDL

Composition	Native Serum (0 hr)	Incubated Serum (24 hr)
	%	%
Cholesteryl esters	22 ± 2.6	32 ± 2.9
Free cholesterol	16 ± 2.2	11 ± 1.8
Triglycerides	8 ± 1.1	5 ± 1.7
Phospholipids	14 ± 1.2	12 ± 1.8
Protein	40 ± 2.1	40 ± 2.9
ApoA-I	50	50
ApoA-II	15	10
ApoC's	5	2
ApoE	30	38

Relative lipid and apoprotein composition of HDL$_1$ before (0 hr) and after incubation (24 hr) at 37°C as calculated by quantitative HPTLC and polyacrylamide gel densitometry (15). The phosphatidylcholine/sphingomyelin ratio decreased from 4.1:1 (m/m) in native serum to 3.3:1 (m/m) in incubated serum.
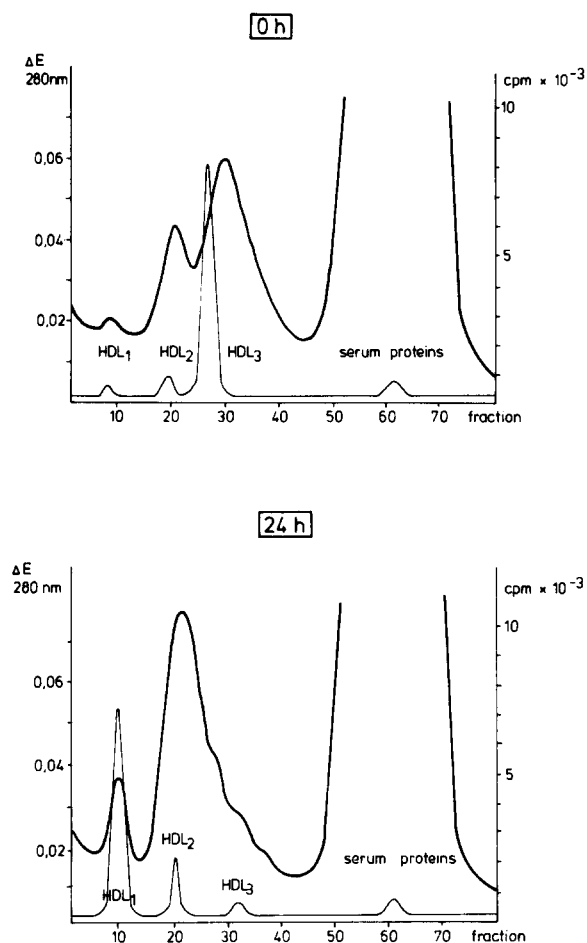
Fig. 9. Zonal ultracentrifugation analysis of the radioactivity distribution (lower curves) of ^{125}I-labeled HDL-E incubated with normal serum. HDL-E was isolated from the HDL$_3$ fraction of a FH-homozygote (D.P.), labeled with ^{125}I, and a total radioactivity of 400,000 cpm was added to 1.8 ml of normal serum. Immediately (0 hr) and after 24 hr incubation at 37°C the serum was treated with PTA/MgCl$_2$. Subsequent to the precipitation of the apo B-containing lipoproteins, the supernatant was subjected to zonal ultracentrifugation.

in cholesterol-fed animals and man in concentration and is designated HDL$_c$ (21). Innerarity et al. (24) have demonstrated that HDL$_c$ binds to the apoB, E receptor of peripheral cells and modulates the uptake of LDL.

The observations in this study of increased HDL$_1$ concentrations in sera of patients with familial hypercholesterolemia are in accord with current concepts of the apoB, E receptors in the recognition of LDL and HDL$_1$ and suggest that apoB, E receptors are important in the regulation of HDL$_1$ concentrations in plasma. Our data are in agreement with observations of Havekes et al. (25) who have demonstrated that the LDL fractions of patients heterozygote and homozygote for FH contain higher absolute concentrations of apoE as compared to normals. Also, Blum, (26) found higher absolute apoE

concentrations in the sera of hypercholesterolemia patients.

The precise metabolic origin of HDL$_1$ in human plasma remains unknown. However, the fact that HDL$_1$ was undetectable in fresh and incubated Tangier serum, but present in incubated serum of a patient with abetalipoproteinemia, suggests a metabolic relationship to the major HDL subfractions normally present in serum. Furthermore, the involvement of LCAT not only in the interconversion of HDL$_3$ to HDL$_2$ (4), but also in the formation of HDL$_1$, is supported by our data. Whether HDL$_1$ is generated from either HDL$_3$ or HDL$_2$, or alternatively serves as a substrate for LCAT or an acceptor for newly formed cholesteryl esters, remains to be clarified. Elucidation of the potential role of HDL$_1$ as a lipoprotein involved in the clearance of cholesterol from peripheral cells and/or delivery of cholesterol to the liver also awaits further studies.∎

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