Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography

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Abstract A reproducible and quantitative subfractionation of human high density lipoproteins (HDL) by heparin-Sepharose affinity chromatography has been developed. Two elution methods (A and B) were used to subfractionate HDL_2 (d 1.063–1.125 g/ml) or total HDL (d 1.063–1.21 g/ml). Method A separated HDL_2 into three subclasses, each with distinct chemical properties and in vitro metabolic characteristics. The first subclass, referred to as HDL_2-without E, passed through the affinity column unretarded and represented approximately 85% of the HDL_2 lipoprotein protein. HDL_2-without E contained the A-I, A-II, and C apoproteins which characterize typical HDL. The second subclass eluted from the column (7–10% of the protein) contained, in addition to the A-I and A-II apoproteins, the E and (E—A-II) apoproteins, and was designated as HDL_2-with E. The B apoprotein was the major protein component of the third subclass eluted from the column (β lipoproteins). The β subclass accounted for approximately 3–8% of the HDL_2 protein and was similar to Lp(a) in composition and size. Method B further subdivided the β subclass into two fractions (β_1 and β_2) with slightly different electrophoretic mobilities. The various heparin-Sepharose subclasses were further characterized by their ability to compete with ^{125}I-labeled low density lipoproteins (LDL) for binding to cell surface receptor sites of fibroblasts. By virtue of the presence of the E apoprotein, HDL_2-with E competed effectively with ^{125}I-labeled LDL for binding to the cell surface receptors, whereas HDL_2-without E were ineffective in competing with LDL. The β subclass possessed binding capability similar to that of LDL. Subfractionation of HDL by heparin-Sepharose affinity column chromatography provides an attractive alternative to methods based solely on ultracentrifugation, in that it subfractionates HDL into subclasses with differing apoprotein contents that impart distinct metabolic characteristics to each class.—**Weisgraber, K. H., and R. W. Mahley.** Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* 1980. **21:** 316–325.

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The reported negative correlation between high density lipoprotein levels and coronary artery disease has generated a renewed interest in HDL (1–4). Until recently, the subclassification and separation of HDL (d 1.063–1.21 g/ml) were based on ultracentrifugal procedures (5, 6), with little emphasis placed on the metabolic properties of these subclasses. The most commonly described HDL subclasses isolated by preparative ultracentrifugation are HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) (5). Using density gradient ultracentrifugation, Anderson et al. (7, 8) further subdivided HDL_2 into two different groups of lipoproteins with different size ranges. These HDL_2 subclasses are referred to as HDL_{2b} (d 1.063–1.10 g/ml) and HDL_{2a} (d 1.10–1.125 g/ml) (7, 8). It is now apparent that HDL represents a heterogeneous class of plasma lipoproteins which are difficult to define strictly on the basis of flotation properties. In order to gain insight into overall HDL metabolism, we must begin to understand and, where possible, to define HDL subclasses in terms of their metabolic functions.

We have used Geon-Pevikon preparative block electrophoresis to subfractionate HDL_2 by electrophoretic mobility and have described two HDL subclasses, HDL-I and HDL-II. With respect to apoprotein content, HDL-I differs from HDL-II in that HDL-I contains, in addition to the A-I and A-II apoproteins, the E apoprotein and the apo(E—A-II) complex (9). The presence of the E apoprotein imparts a distinctive in vitro metabolic characteristic to HDL-I. Apo-E-containing lipoproteins can interact with and bind to the same cell surface receptors on cultured fibroblasts as do apo-B-containing lipoproteins (10). In fact, the binding ability of the E apoprotein is 10- to 100-fold greater than that of the B apoprotein (11). The A-I and A-II apoproteins do not interact with

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins.

the receptors. Thus HDL-I which contains variable but significant levels of the E apoprotein competes effectively with LDL for cell surface receptor binding sites, whereas HDL-II which lacks the E apoprotein does not bind to the receptors (12). The binding capability of HDL-I is solely due to the presence of the E apoprotein in the uncomplexed form; the apo(E—A-II) complex does not interact with the receptors (12). The occurrence of receptor-active and -inactive HDL subclasses has also been described in dogs and swine (13, 14). For example, apo-E-containing HDL_1 of control dogs and HDL_c of cholesterol-fed dogs binds to cell receptors, whereas HDL which lacks the E apoprotein does not bind.

In man, dietary cholesterol appears to alter the metabolic properties of HDL. A recent report from this laboratory demonstrated that egg feeding in humans increased the cell surface receptor binding ability of HDL (d 1.095–1.21 g/ml), regardless of whether the plasma cholesterol levels increased or not (15). The increased binding capacity of the HDL was correlated with the presence of the E apoprotein. These results underscore the need for a quantitative HDL subfractionation method which correlates with metabolic parameters. Because the various apoproteins differ in their degrees of interaction with heparin, we investigated the use of heparin-Sepharose affinity column chromatography as an alternate approach to subfractionating HDL, with the objective of isolating and quantitating the HDL subclass which contains the E apoprotein.

MATERIALS AND METHODS

Lipoprotein preparation and characterization

Plasma was obtained from fasted normal healthy subjects, and the various density fractions were obtained by preparative ultracentrifugation (16) in a 60 Ti rotor (Beckman Instruments, Palo Alto, CA) at 59,000 rpm. The d 1.063–1.125 g/ml and the d 1.063–1.21 g/ml fractions were centrifuged for 24 and 36 hr, respectively. Each fraction was recentrifuged for 24 hr at the appropriate density to minimize plasma protein contamination. HDL-I was isolated from the d 1.063–1.125 g/ml fraction by Geon-Pevikon block electrophoresis as described previously (9).

Density gradient ultracentrifugation of HDL_2 was performed in an SW41 rotor (Beckman Instruments, Palo Alto, CA) as follows. Solid KBr was added to 1 ml of HDL_2, containing 14 mg of lipoprotein protein, to raise the density from 1.006 to 1.125 g/ml. The mixture was transferred to a cellulose nitrate tube and mixed with 1 ml of NaCl–KBr solution (d 1.125 g/ml). The d 1.125 g/ml solution was then overlayed with 4.0 ml of d 1.10 g/ml, 2.5 ml of d 1.087 g/ml, 2.5 ml of d 1.080 g/ml, and ~1.5 ml of d 1.063 g/ml NaCl–KBr solutions. After centrifugation of the gradient for 48 hr at 40,000 rpm, 12 1-ml fractions were collected with the aid of a Fraction Recovery System (Beckman Instruments, Palo Alto, CA). The density of each fraction was determined by pycnometry.

HDL_2 (d 1.063–1.125 g/ml) was chromatographed on agarose in a glass column (2.5 × 200 cm; Kontes Glass Company, Vineland, NJ) packed with Bio-Gel A-5m, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA) (17). Approximately 50 mg of protein in 10 ml of 0.15 M NaCl (0.01% EDTA, pH 7.0) was applied to the column. The column flow rate was 9 ml/hr and 3-ml fractions were collected.

Chemical analyses of the lipoproteins included total (18) and esterified (19) cholesterol, phospholipid (20), triglyceride (21), sialic acid (22), and protein (23). Paper electrophoresis and negative staining electron microscopy were performed as described previously (24). SDS gel electrophoresis was carried out on 11% acrylamide gels as described previously (9) using the Tris/glycine buffer system of Stephens (25). Gradient gel electrophoresis was performed on a Pharmacia Electrophoresis Apparatus GE-4 with PAA 4/30 gradient gels (Pharmacia Fine Chemicals, Uppsala, Sweden). Approximately 30 μg of protein in 0.5% agarose was applied to each well. Electrophoresis was carried out at 150 V for 14 hr at approximately 15°C in a Tris/borate buffer (0.09 M Tris HCl, 0.08 M boric acid, 0.09% EDTA, pH 8.4). After electrophoresis the gels were fixed in 10% sulfosalicylic acid, stained with 1% Coomassie brilliant blue R, and destained by diffusion. A mixture of standard proteins (thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine albumin) obtained from Pharmacia Fine Chemicals, Inc., was run on each slab as a reference marker.

Tissue culture assays for binding at 4°C were performed according to Goldstein and Brown (26) and Goldstein et al. (27), with minor modifications as described previously (11). Cultures of human fibroblasts were obtained from a preputial specimen from a normal infant, and monolayers of the fibroblasts were maintained exactly as described (11).

Heparin-Sepharose affinity column chromatography

Heparin (Hynson, Westcott, Dunning, Inc., Baltimore, MD) was covalently attached to epichlorohydrin cross-linked Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) basically as described by Iverius and Oštund-Lindqvist (28). The amount of cyanogen bromide (Aldrich Chemical Company, Milwaukee, WI)

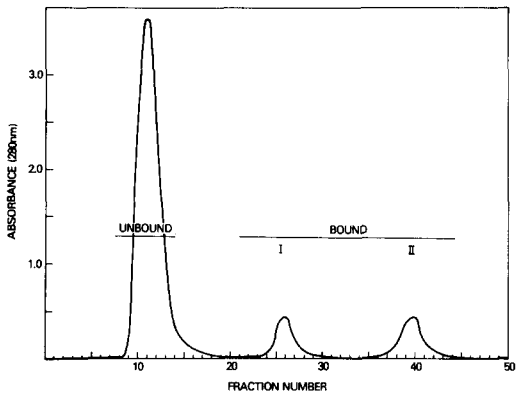

Fig. 1. Heparin-Sepharose affinity column chromatography of human HDL_2 (d 1.063–1.125 g/ml). Approximately 30 mg of HDL_2 protein in 0.005 M Tris-Cl, pH 7.4, containing 0.05 M NaCl and 0.025 M $MnCl_2$ was applied to the column (1.0 × 30 cm). The column was operated at a rate of 24 ml/hr and 3.6-ml fractions were collected. The unbound lipoproteins (fractions 8–16) were eluted in the column void volume with the Tris-NaCl Mn^{2+}-containing buffer. The column was then eluted in a stepwise manner (Method A). As fraction 12 was being collected, the eluting buffer was changed to a buffer containing 0.095 M NaCl and no Mn^{2+}. This resulted in the elution of the bound I lipoproteins (fractions 23–30). An increase in the NaCl concentration to 0.29 M at fraction 27 eluted bound II lipoproteins (fractions 35–45). Pooled fractions were dialyzed against 0.15 M NaCl, 0.01% Na_2 EDTA, pH 7.0.

used to activate the Sepharose was increased to 9 g of CNBr to 75 ml of sedimented gel.

Affinity chromatography was carried out at 4°C in 1.0 × 30 cm glass columns (Kontes Glass Company, Vineland, NJ) with the aid of a peristaltic pump operating at a rate of 24 ml/hr. The heparin-Sepharose was equilibrated with NaCl-Tris buffer (0.05 M NaCl, 0.005 M Tris, pH 7.4) containing 0.025 M $MnCl_2$. Approximately 30 mg of lipoprotein protein in 2.5–3.5 ml of NaCl-Tris buffer (0.05 M NaCl, 0.005 M Tris, pH 7.4) was fractionated in each run. Just before applying the sample to the column, solid $MnCl_2$ was added to the sample to give a final Mn^{2+} concentration of 0.025 M. After sample application, the tubing was rinsed with column buffer and the sample was allowed to equilibrate with the column overnight.

Method A. Elution with the manganese-containing buffer (application buffer described above) was continued until fraction 11 had been collected (3.6 ml/fraction). At this point the NaCl concentration in the buffer applied to the top of the column was increased to 0.095 M and the manganese was deleted. Elution with this second buffer was continued until tube 27; then the NaCl concentration in the eluting buffer was increased to 0.29 M. A final increase in the NaCl concentration in the eluent to 0.6 M was made at fraction 37. On the basis of their absorption at 280 nm, the fractions were combined into appropriate pools and dialyzed against NaCl-EDTA (0.15 M NaCl, 0.01% EDTA, pH 7.0). Recoveries of protein applied to the column averaged 92.4%.

Method B. Elution with the manganese-containing buffer was continued until fraction 11 had been collected. At this point the NaCl concentration in the buffer applied to the top of the column was increased to 0.070 M and the manganese was deleted. Elution was continued until tube 27; then the NaCl buffer concentration was increased to 0.115 M. At tube 37 the NaCl concentration was increased to 0.29 M. The final buffer (0.6 M NaCl) was started at tube 47. Recoveries of protein applied to the column averaged 91.8%.

RESULTS

Affinity column chromatography of human HDL_2 (d 1.063–1.125 g/ml) on heparin-Sepharose resulted in the separation of three distinct subclasses (Method A). An unbound fraction eluted with the starting buffer (0.05 M NaCl, 0.005 M Tris, 0.025 M $MnCl_2$). Two bound subfractions, designated bound I and bound II, were eluted by omitting the $MnCl_2$ from the eluting buffer and increasing the NaCl concentration in a stepwise manner. Most of the HDL_2 was unretarded by the affinity column and eluted with the void volume, as shown for a typical subfractionation in **Fig. 1.** In this example the unbound subfraction accounted for 84.3% of the lipoprotein protein recovered. Total protein recovery from this column was 93%. Bound I subfraction was eluted when the NaCl concentration in the eluting buffer was increased to 0.095 M and the $MnCl_2$ was deleted. This subfraction accounted for 7.8% of the recovered lipoprotein protein. Bound II subfraction was eluted with 0.29 M NaCl and accounted for 8.6% of the recovered lipoprotein protein. No additional lipoproteins were detected in the eluate collected after the NaCl concentration was increased to 0.6 M.

SDS gel electrophoresis of the three subfractions revealed that the unbound and bound I subfractions contained the A-I and A-II apoproteins as major protein constituents (**Fig. 2**). Identity of the A-I and A-II apoproteins was determined by coelectrophoresis with authentic standards (not shown). However, unlike the unbound subfraction, bound I subfraction also contained the apo(E—A-II) complex as a major protein component as well as a significant amount of the E apoprotein. The identity of the apo(E—A-II) complex was confirmed by reduction of the bound I apoproteins with mercaptoethanol, which converted the apo(E—A-II) complex to the E and A-II apoproteins (Fig. 2). In addition, bound I lipoproteins contained no immunodetectable

B apoprotein. The major apoprotein component of bound II subfraction was the B apoprotein, as determined by SDS gel electrophoresis and immunochemical reactivity with a monospecific apo-B antiserum. Very low concentrations of the (E—A-II) and A-I apoproteins were observed in the bound II subfraction.

The three heparin-Sepharose subfractions were designated and defined on the basis of their respective apoprotein contents (Fig. 2): HDL_2-with E (HDL_2-c̄E), lipoproteins that eluted in the bound I subfraction and contained, in addition to the A-I and A-II apoproteins, the apo(E—A-II) complex and the E apoprotein; HDL_2-without E (HDL_2-s̄E), lipoproteins that eluted in the unbound subfraction and contained the A-I, A-II, and C apoproteins, but not the apo(E—A-II) complex or the E apoprotein; and β lipoproteins (β), lipoproteins that eluted in the bound II subfraction and contained the B apoprotein as the major protein component. On paper electrophoresis (**Fig. 3**) both HDL_2-with E and HDL_2-without E migrated in the α position, with HDL_2-with E migrating slightly more slowly than HDL_2-without E. The apo-B-containing β lipoproteins migrated as a broad band in a position slightly ahead of the β-migrating LDL (d 1.02–1.05 g/ml) (Fig. 3).

The conditions required for the stepwise elution of the heparin affinity column described above were established empirically in order to separate the HDL_2-with E from the β lipoproteins and from the HDL_2-without E. The NaCl concentrations required to separate the HDL_2-with E from the β-lipoproteins varied slightly with different heparin-Sepharose preparations, but once the conditions were established, the subfractionation was reproducible with a high degree of precision. Triplicate subfractionation of HDL_2 from a male subject showed that 85.1 ± 2.8%, 7.3 ± 0.3%, and 8.4 ± 1.7% of the recovered lipoprotein protein were contained in the HDL_2-without E (unbound), HDL_2-with E (bound I), and β (bound II) subfractions, respectively. Similar results were obtained from duplicate subfractionation of the d 1.063–1.125 g/ml fraction from a female subject, where the HDL_2-without E, HDL_2-with E, and β subfractions accounted for 83.6%, 9.7%, and 6.7% of the recovered protein, respectively.

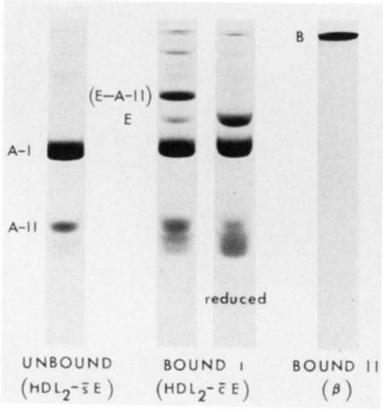

Fig. 2. SDS polyacrylamide gel electrophoresis of the heparin-Sepharose HDL_2 subclasses (Unbound, Bound I, Bound II) on 11% acrylamide gels. Apoprotein (35 μg) in 2.5 mM Tris-glycine (pH 8.3), 0.3% SDS, was applied to each gel. The apo-HDL_2-with E was reduced by incubation with mercaptoethanol (final concentration 1%) for 2 hr at 37°C. Gels were stained with Coomassie brilliant blue and diffusion destained. HDL_2-s̄E, HDL_2-without E; HDL_2-c̄E, HDL_2-with E; β, β subclass. The HDL_2-without E contains the typical HDL apoproteins, A-I and A-II, as the major apoprotein components. The HDL_2-with E contains, in addition to A-I and A-II, the E and (E—A-II) apoproteins. The major protein of the β lipoproteins is the B apoprotein.

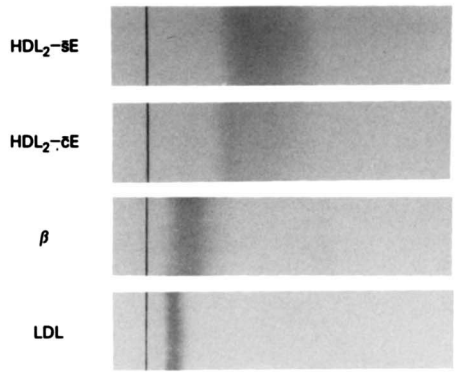

Fig. 3. Paper electrophoresis of the heparin-Sepharose subclassees (HDL_2-s̄E, HDL_2-c̄E, β) and LDL. Electrophoresis was conducted for 18 hr and the dried paper strips were stained with Oil Red O.

Further characterization of the heparin-Sepharose subfractions included chemical composition and particle size as determined by negative staining electron microscopy. As shown in **Table 1,** the HDL_2-with E and HDL_2-without E had similar compositions, with HDL_2-with E containing significantly more cholesterol and less protein than the HDL_2-without E. The HDL_2-with E was larger in diameter (122 ± 17 Å) than HDL_2-without E (95 ± 12 Å). The compositions and sizes of the HDL_2-with E and HDL_2-without E closely resembled those characteristics of the Geon-Pevikon d 1.063–1.125 g/ml subfractions HDL-I and HDL-II, respectively (9). The 205-Å particles in the β subfraction were composed of 5.6% triglyceride, 33.9% total cholesterol, 24.0% phospholipid, and 36.5% protein (Table 1). This composition is similar to that reported for Lp(a) (29). One characteristic of Lp(a) which distinguishes it from LDL is an increased sialic acid content (30). Determination of the sialic acid content of the β subfraction from six normal individuals gave a range of values (12.3–21.8 μg sialic acid/mg protein) intermediate between those of LDL

TABLE 1. Percent composition and particle size of the heparin-Sepharose subfractions

	Triglyceride TG	Total Cholesterol TC[a]	Phospholipid PL	Protein	Size[b]
		%			Å
HDL_2-without E[c]	3.3 ± 0.7	18.4 ± 2.0	32.8 ± 0.7	45.6 ± 1.9	95 ± 12 (65–115)
HDL_2-with E[d]	1.3 ± 0.5	25.8 ± 0.2	35.1 ± 0.9	36.8 ± 2.0	122 ± 17 (100–150)
β[d]	5.6 ± 2.8	33.9 ± 1.6	24.0 ± 3.7	36.5 ± 2.2	205 ± 19 (180–240)

[a] Cholesterol esters represented from 76.3 to 79.6% of the total cholesterol.
[b] The diameters of approximately 200 particles for each lipoprotein class were measured. Data are presented as the mean diameter and standard deviation with the range in parentheses.
[c] Average of single determinations on four preparations.
[d] Average of single determinations on three preparations.

(12.8 μg sialic acid/mg protein) and those expected for Lp(a). Thus it appears that the β subfraction isolated by Method A represents a variable mixture of LDL and Lp(a).

The three heparin-Sepharose HDL subfractions were further characterized by electrophoresis on a polyacrylamide gradient slab gel (**Fig. 4**). From the migration distances of the HDL_2-with E and HDL_2-without E subfractions and those of a set of standard proteins, it was possible to calculate the Stoke's diameter of the subfractions using the Stoke-Einstein equation, as described by Anderson et al. (7). The diameters were determined to be 105–115 Å and 110–135 Å for HDL_2-without E and HDL_2-with E, respectively. These are similar to the particle sizes determined by negative staining electron microscopy (Table 1).

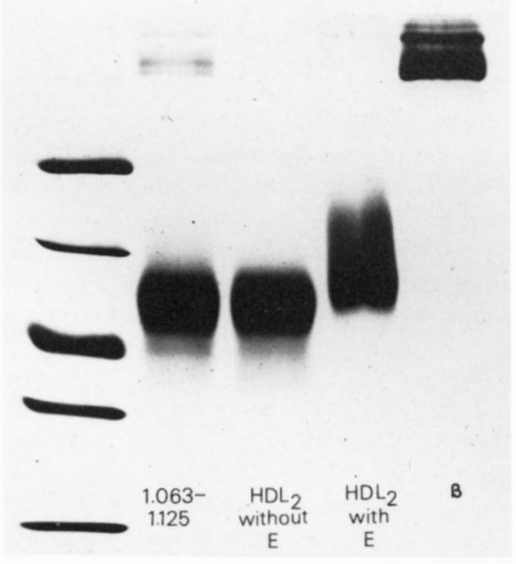

Fig. 4. Polyacrylamide gradient slab gel electrophoresis of the heparin-Sepharose subclasses (Fig. 2). Electrophoresis was performed on PAA 4/30 polyacrylamide gradient slabs (Pharmacia Fine Chemicals, Uppsala, Sweden) with a GE-4 Pharmacia Electrophoresis Apparatus. Approximately 25 μg of lipoprotein protein in 0.1% agarose solution was applied to each well. The first well (left) contained the reference markers thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine albumin. Electrophoresis was performed in a Tris-borate buffer (0.09 M Tris HCl, 0.08 M boric acid, pH 8.4) at 150 volts for 14 hr. The slabs were stained with 1% Coomassie brilliant blue and diffusion destained.

For metabolic characterization of the various HDL_2 subfractions, the abilities of the subfractions to compete with ^{125}I-labeled LDL for fibroblast cell surface receptors were compared using a competitive assay performed at 4°C. As shown in **Fig. 5,** the HDL_2-without E was completely inactive at a protein concentration as high as 100 μg/ml. The apo-B-containing β lipoproteins produced a displacement curve which was identical to that of LDL (d 1.02–1.05 g/ml). They displaced approximately 50% of the ^{125}I-labeled LDL at a concentration of 5 to 6 μg/ml. Because of the presence of low, but significant, concentrations of the E apoprotein in the HDL_2-with E (Fig. 2), this sub-

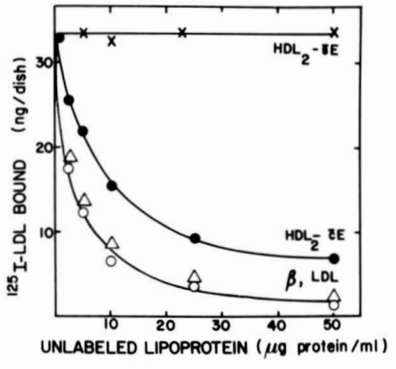

Fig. 5. Comparison of the ability of the heparin-Sepharose subclasses to displace human ^{125}I-labeled LDL from the cell surface receptors of human fibroblasts. The competitive binding assay was performed at 4°C as described previously.

class was an effective competitor with ^{125}I-labeled LDL for binding sites. The HDL_2-with E was capable of displacing 50% of the ^{125}I-labeled LDL at a protein concentration of approximately 16 μg/ml (Fig. 5).

Since the apoprotein content, chemical composition, size, and electrophoretic mobility of HDL_2-with E closely resemble those characteristics reported for HDL-I (9, 12), it was of interest to compare the elution properties of HDL-I on the heparin-Sepharose column. When HDL-I isolated by Geon-Pevikon block electrophoresis was subjected to heparin affinity chromatography, material eluted in both the unbound and bound I positions. In three HDL-I preparations, the HDL_2-with E (bound I) accounted for approximately half of the HDL-I. Thus HDL-I isolated by Geon-Pevikon electrophoresis represented a subfraction of the d 1.063–1.125 g/ml which was enriched in HDL_2-with E but which also contained HDL_2-without E.

It was also of interest to determine the limits of the density distribution of the B, (E—A-II), E, and A-I apoproteins within the d 1.063–1.125 g/ml range. This was accomplished by density gradient ultracentrifugation of the HDL_2 (d 1.063–1.125 g/ml), division of the density gradient into 12 fractions (1 ml each), and qualitative determination of the apoprotein content of each fraction by SDS polyacrylamide gel electrophoresis. As shown in **Fig. 6,** the B apoprotein was present in fractions 1 through 6, corresponding to d 1.055–1.085 g/ml. Fractions 1 through 10 (d 1.055–1.115 g/ml) contained the (E—A-II) and E apoproteins. Apo-A-I was present in all fractions. When fractions 1 through 8, which corresponded to the HDL_{2b} subfraction (d 1.063–1.10 g/ml) described by Anderson et al. (8), were combined and subjected to heparin-Sepharose chromatography, the elution profile shown in **Fig. 7** was obtained. All three heparin-Sepharose subclasses were present in the d 1.063–1.10 g/ml fraction. Affinity chromatography clearly indicates that HDL_{2b} is a heterogeneous class of lipoproteins.

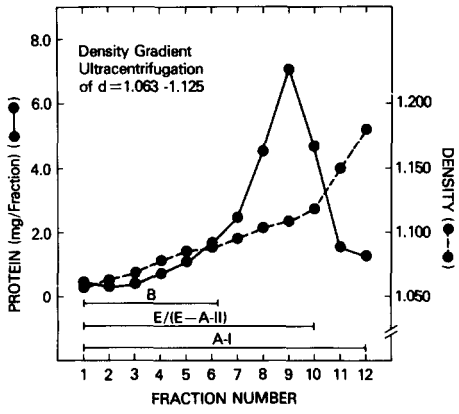

Fig. 6. Density gradient ultracentrifugation of human HDL_2 (d 1.063–1.125 g/ml). HDL_2 was subfractionated on a NaCl-KBr density gradient in an SW41 rotor. After centrifugation, the gradient was divided into 12 1-ml fractions, and the density of each fraction was determined by pycnometry. The distribution of the B, E, (E—A-II), and A-I apoproteins in the fractions was determined qualitatively by SDS polyacrylamide gel electrophoresis (30 μg of lipoprotein protein applied to each gel).

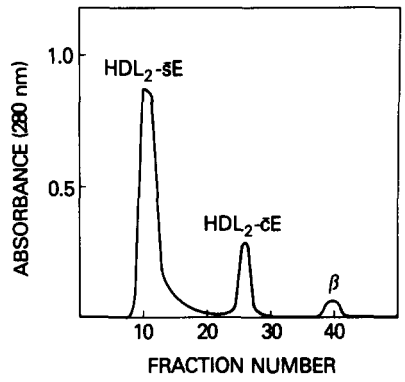

Fig. 7. Heparin-Sepharose affinity chromatography of human HDL_{2b} (d 1.063–1.10 g/ml). Fractions 1–8 (d 1.063–1.10 g/ml) from the HDL_2 density gradient subfractionation (Fig. 5) were pooled and subjected to heparin-Sepharose separation as described in Fig. 1.

Since the three heparin-Sepharose subclasses differed with respect to size (Table 1), subfractionation of HDL_2 by agarose column chromatography was investigated (**Fig. 8**). In order to maximize resolution, chromatography was carried out on a 2-meter column. The effectiveness of the separation was evaluated by SDS gel electrophoresis of various fractions (inset, Fig. 8). There was considerable overlap in the apoprotein distribution across the elution profile, particularly between the B- and E/(E—A-II)-containing lipoproteins, indicating that agarose column chromatography did not effectively separate an easily quantifiable HDL_2 subclass containing the E and (E—A-II) apoproteins from the apo-B-containing lipoproteins.

Heparin affinity column chromatography was also used to subfractionate the total HDL density range (d 1.063–1.21 g/ml), and the elution profile was qualitatively similar to that shown in Fig. 1 for the d 1.063–1.125 g/ml fraction. For subfractionation of total HDL, the subclasses were designated HDL-without E (unbound), HDL-with E (bound I), and β (bound II). The protein distribution among the three subclasses differed from that obtained with HDL_2 subfractionation, notably in an increased protein content in the HDL-without E. This increase in the percent of total protein in the unbound fraction (HDL-without E) is consistent with the observation that there was little detectable apo(E—A-II) or E in the 1.125–1.21 g/ml density fraction (9). The HDL-

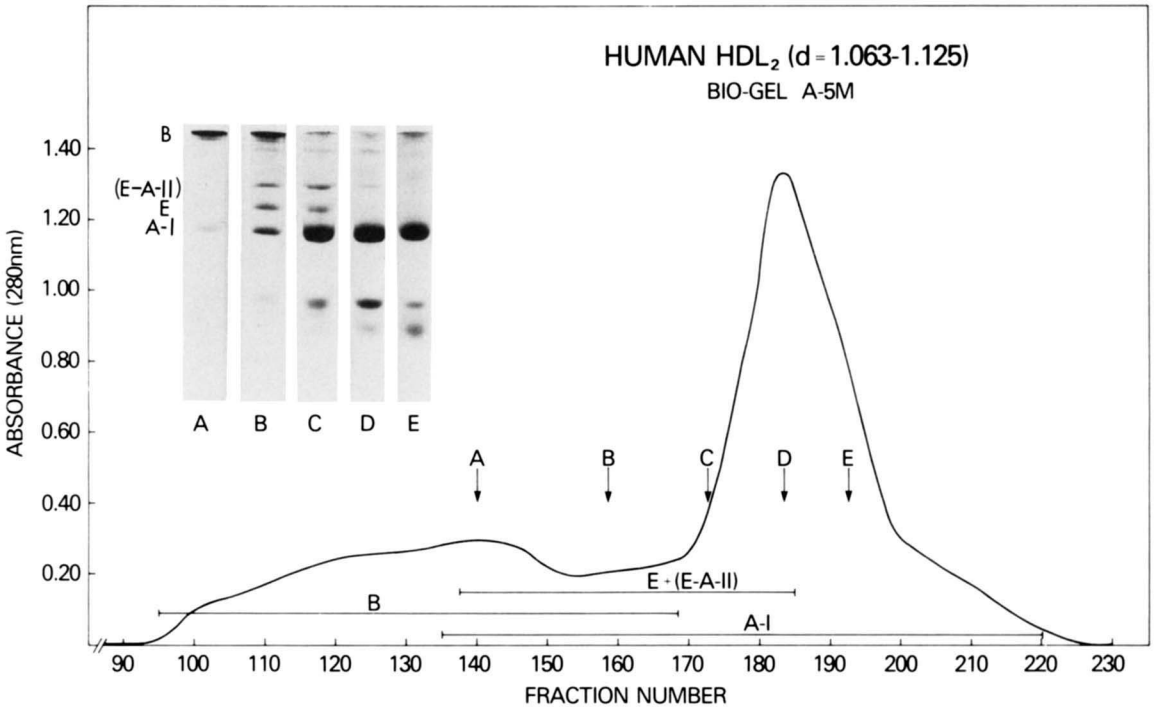

Fig. 8. Agarose gel chromatography of human HDL_2 (d 1.063–1.125 g/ml). HDL_2 was fractionated on a Bio-Gel A-5m column (2.5 × 200 cm) with 0.15 M NaCl, pH 7.0, containing 0.01% Na_2 EDTA at a rate of 12 ml/hr. Each fraction contained 3.5 ml. The distribution of the B, E, (E—A-II), and A-I apoproteins across the agarose elution profile was determined by SDS polyacrylamide gel electrophoresis. The inset shows the SDS polyacrylamide gels of the fractions designated A–E.

with E was identical with the HDL_2-with E obtained from the 1.063–1.125 g/ml density fraction with respect to apoprotein content, particle size, electrophoretic mobility, and chemical composition. The total HDL (d 1.063–1.21 g/ml) from six normal adult subjects (ages 25 to 38) were subfractionated. The average of the six subfractionations showed that the HDL-without E, HDL-with E, and β lipoproteins accounted for 91.8 ± 2.2%, 6.3 ± 1.6%, and 2.0 ± 1.3% of the HDL protein, respectively. This corresponded to an average plasma level of 109.3 ± 19.6, 7.4 ± 2.3, and 2.2 ± 1.2 mg/dl of HDL-without E, HDL-with E, and β lipoprotein, respectively, for these individuals.

We examined, in addition to HDL from normal subjects, HDL (d 1.063–1.21 g/ml) from eight hypercholesterolemic patients (plasma cholesterol levels 220–345 mg/dl; elevated LDL). The d 1.063–1.21 g/ml fractions from four of these patients contained α- and β-migrating lipoproteins and a distinct pre-β-migrating lipoprotein band which was not a prominent component of the HDL from the six normal subjects or of the HDL from the other four hypercholesterolemic patients. After heparin-Sepharose subfractionation of these HDL, the pre-β-migrating lipoprotein was found to be approximately equally distributed between the HDL-with E and β subclasses, resulting in the contamination of the HDL-with E by the B apoprotein. To avoid this cross-contamination, a second elution procedure, Method B, was developed. Method B is identical to Method A up to the point of eluting HDL_2-with E. It differs from Method A in that the HDL-with E subclass is eluted with a slightly lower NaCl concentration (0.070 M) than used in Method A and the β subclass lipoproteins are eluted with two buffers (0.115 M and 0.29 M NaCl), separating them into two peaks.

The effectiveness of Method B in subfractionating HDL (d 1.063–1.21 g/ml) was tested with a second group of eight normolipidemic subjects (ages 17 to 50), four of whose HDL contained the prominent pre-β band. A typical heparin-Sepharose elution profile, obtained using Method B, and the SDS gels of the HDL subfractions are shown in **Fig. 9.** Use of Method B resulted in the separation of an HDL-with E uncontaminated by either the pre-β-migrating band or apo-B. The properties of the HDL-with E were identical to those of the HDL-with E obtained by Method A. Further, the pre-β- and β-migrating bands were separated from each other (**Fig. 10**), with the β_1 subclass containing the former and the β_2 subclass the latter. Determination of the sialic acid contents of the

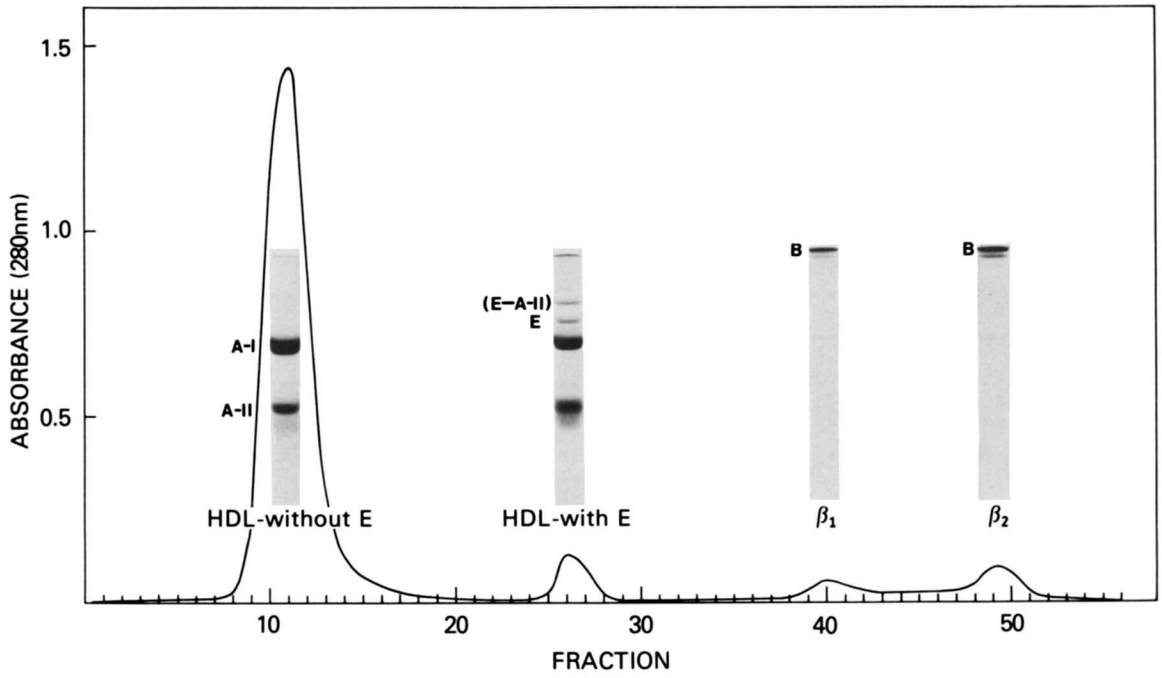

Fig. 9. Heparin-Sepharose column chromatography of human HDL (d 1.063–1.21 g/ml) and SDS polyacrylamide gel electrophoresis of the heparin-Sepharose subclasses. The column was equilibrated and operated as described in Fig. 1. The stepwise elution was according to Method B. The HDL-without E was eluted as with Method A (0.05 M NaCl, 0.025 M $MnCl_2$, 0.005 M Tris-Cl, pH 7.4). As fraction 12 was being collected, the eluting buffer was changed to a buffer containing 0.070 M NaCl and no Mn^{2+}. This resulted in the elution of HDL-with E. An increase in the NaCl concentration to 0.115 M at fraction 27 eluted the β_1 subclass. At fraction 37 the NaCl concentration was increased to 0.29 M, which eluted the β_2 subclass. Pooled fractions were dialyzed against 0.15 M NaCl, 0.01% Na_2 EDTA, pH 7.0. SDS polyacrylamide gel electrophoresis was performed as described in Fig. 2.

pooled β_1 subclasses and the pooled β_2 subclasses from these eight subjects showed that the β_1 and β_2 lipoproteins contained 35.3 and 27.6 μg sialic acid/mg, respectively. Since both β subclasses contained more sialic acid than is present in LDL from d 1.019–1.063 g/ml (12.8 μg sialic acid/mg), these lipoproteins may represent Lp(a) or Lp(a)-like lipoproteins present in the HDL of these subjects. The plasma levels of the various heparin-Sepharose subclasses in this group of subjects were 105.9 ± 16.2, 5.0 ± 1.7, 2.4 ± 1.3, and 3.9 ± 2.5 mg/dl of HDL-without E, HDL-with E, β_1 subclass, and β_2 subclass, respectively.

DISCUSSION

Because the various apoproteins interact with heparin and manganese to different degrees, it was possible to subfractionate HDL_2 (d 1.063–1.125 g/ml) or total HDL (d 1.063–1.21 g/ml) on heparin-Sepharose affinity columns into distinct subclasses according to their apoprotein contents. Elution of the affinity column with the stepwise buffer system of Method A separated HDL into three subclasses. The first subclass, unretarded by the column, represented >85% of the lipoprotein protein and was designated HDL_2-without E (from HDL_2 of d 1.063–1.125 g/ml) or HDL-without E (from the total HDL range, d 1.063–1.21 g/ml). The second subclass eluted from the affinity column accounted for approximately 7–10% (HDL_2-with E, from d 1.063–1.125 g/ml) or 7% (HDL-with E, from d 1.063–1.21 g/ml) of the protein. The HDL_2-with E differed from the HDL_2-without E in

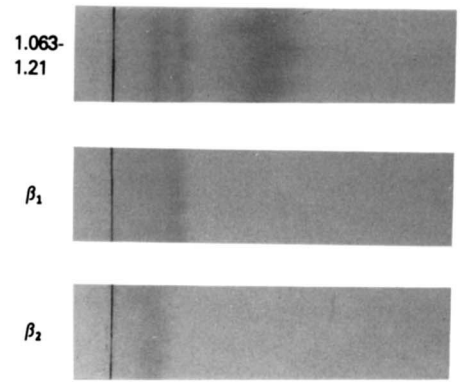

Fig. 10. Paper electrophoresis of the d 1.063–1.21 g/ml fraction and the β_1 and β_2 heparin-Sepharose subclasses (Fig. 9). Electrophoresis was conducted for 18 hr and the dried paper strips were stained with Oil Red O.

that HDL_2-with E contained, in addition to the A-I and A-II apoproteins, the apo(E—A-II) complex and the E apoprotein. Apo-E has been shown to interact with heparin both in the presence and absence of manganese (13, 31, 32). The ability of apo(E—A-II) to interact with heparin has not been studied, but this complex may also be capable of interacting with heparin to some degree. The third heparin-Sepharose subclass, referred to as the β subclass, contained the B apoprotein, which also has been shown to interact with heparin. Some HDL contained not only β-migrating lipoproteins but also a prominent pre-β-migrating band which partially eluted with the HDL-with E when Method A was used. This contamination of HDL-with E by an apo-B-containing lipoprotein could be avoided by adjusting the NaCl concentrations in the eluting buffers (Method B). Method B also separated the pre-β- and β-migrating lipoproteins. The presence of low concentrations of apo-B-containing lipoproteins, either LDL or LDL-like, in the high-density range of human plasma (d > 1.063 g/ml) has been demonstrated by Geon-Pevikon block subfractionation of the 1.063–1.125 g/ml density fraction (9). The β subclass also appears to contain Lp(a), which has been shown to occur partially in this density range (29). Studies are underway to determine the quantitative distribution of the heparin-Sepharose subclasses in defined age populations of normal male and female subjects.

The heparin-Sepharose affinity column procedure is highly reproducible, with near quantitative recovery of lipoprotein protein, and provides an attractive alternative to subfractionation of HDL by ultracentrifugation or agarose column chromatography. Recently, HDL_2 (d 1.063–1.125 g/ml) have been further subdivided into two ultracentrifugal subclasses, HDL_{2b} (d. 1063–1.10 g/ml) and HDL_{2a} (d 1.10–1.125 g/ml), and several correlations have been drawn between the levels of these subfractions and the total HDL levels (8). However, from density gradients ultracentrifugation of the d 1.063–1.125 g/ml and apoprotein analysis of the fractions, it appears that the HDL_{2b} class is heterogeneous with respect to apoprotein content. HDL_{2b} contains all three heparin-Sepharose subclasses: the apo-B-containing β lipoproteins, HDL_2-with E, and HDL_2-without E. Low levels of HDL_2-with E are also present in HDL_{2a} (data not shown).

While important correlations may be drawn among levels of HDL_{2a}, HDL_{2b}, HDL_3, and total HDL, it may be more meaningful, in terms of functional metabolism, to subdivide HDL based on apoprotein content rather than on size and flotation properties. In this regard, the report that egg feeding in man resulted in increased cell surface receptor binding activity of the HDL (d 1.09–1.21 g/ml) is particularly important. This increased binding activity appears to correlate with the presence of the E apoprotein. It is reasonable to speculate that the increased E content of the HDL after cholesterol feeding may be associated with the subclass of HDL described in the present study, the HDL-with E. This would be similar to the situation in animals whose response to cholesterol feeding is an increase in the plasma levels of the apo-E-containing HDL_1 (referred to as HDL_c after cholesterol feeding) (10, 14). Animal HDL_1 (dog and rat (14, 33), and swine (34)) and human HDL_2-with E have many properties in common and may represent equivalent classes of lipoproteins. Each contains the E apoprotein, interacts with heparin, and is responsible for the cell surface receptor binding activity of the high density lipoproteins in these species.

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