

The Structure of Human High Density Lipoprotein and the Levels of Apolipoprotein A-I in Plasma as Determined by Radioimmunoassay

GUSTAV SCHONFELD and BARBARA PFLEGER

From the Lipid Research Center, Departments of Preventive Medicine and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT The major apoprotein of high density lipoprotein is apolipoprotein A-I (ApoA-I). In addition to being a structural component of this class of lipoproteins, ApoA-I also has a physiologic role as an activator of lecithin-cholesterol acyl transferase, an enzyme important in the metabolism of all lipoproteins.

To measure ApoA-I content in human plasma, to assess its immunologic activity in hyperlipoproteinemia, and to carry out certain structural studies of high density lipoproteins, we have developed a double antibody radioimmunoassay. ApoA-I, isolated by gel filtration, was used to produce monospecific antisera. ApoA-I was iodinated by chloramine-T and the resulting [¹²⁵I]-ApoA-I was purified by gel filtration. > 85% of [¹²⁵I]-ApoA-I was precipitated by antibody, and 90% of bound [¹²⁵I]ApoA-I was displaced by "cold" ApoA-I. Other lipoproteins and apoproteins did not react.

Plasma and high density lipoprotein from normals and subjects with hyperlipoproteinemia displaced counts in parallel with ApoA-I, suggesting that the same antigenic determinants were reacting with antibody on lipid-free and lipid-associated ApoA-I. However, less than 5% of ApoA-I of high density lipoprotein reacted in the assay. Removal of the lipids by extraction increased the reactivity of ApoA-I in high density lipoprotein 15–20-fold; thus more than 95% of the ApoA-I molecules in "intact" high density lipoprotein are unreactive with antibody. Normal and hyperlipoproteinemic plasma and high density lipoproteins isolated from the same subjects continued to display parallelism with ApoA-I standard after lipid extraction, suggesting that ApoA-I of normal and hyperlipoproteinemic subjects are immunologically identical.

About 90% of ApoA-I was in the d 1.063–1.21 fractions of normal plasma, trace quantities were found in

the lipoproteins of $d < 1.063$, and the rest (about 10%) was in the $d > 1.21$ fraction. Normal plasma levels, assessed in extracted plasmas with a precision of 8%, were 100 ± 35 mg/dl. Levels were normal in small groups of subjects with types II and IV hyperlipoproteinemia and high in pregnancy. However, larger population studies need to be performed to determine the distribution of ApoA-I levels in the various hyperlipoproteinemias.

INTRODUCTION

Much has been learned recently about the structure and metabolism of the high density lipoproteins (HDL)¹ of plasma—the density class which carries most of the plasma phospholipids and a large portion of the cholesterol (1, 2). About 50% of HDL by weight is composed of several apoproteins (ApoHDL), the major class of which is apolipoprotein A (ApoA) (3). The latter consists of two proteins ApoA-I (R-Gln-I) and ApoA-II (R-Gln-II). ApoHDL also contains another class of apolipoproteins, ApoC, which is comprised of three proteins ApoC-I (R-Ser), ApoC-II (R-Glu), and ApoC-III (R-Ala). The third major class of apolipoproteins of plasma, ApoB, is found in chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL), but not in HDL (4–6).

¹ *Abbreviations used in this paper:* ApoA, B, C, apolipoproteins A, B, and C; ApoHDL, high density lipoprotein apoproteins; BSA, bovine serum albumin; Chol, cholesterol; EDTA-saline, 0.16 M NaCl, 1 mM in disodium EDTA; HDL, high density lipoproteins; LCAT, lecithin-cholesterol acyl transferase; LDL, low density lipoproteins; NIRS, nonimmune rabbit serum; RIA, radioimmunoassay; TCA, trichloroacetic acid; TG, triglyceride; Tris-EDTA, 0.1 M Tris-HCl, pH 8.6, 1 mM in disodium EDTA; VLDL, very low density lipoproteins.

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The apolipoproteins appear to have important structural and physiologic functions. For example, ApoC-II is an activator of lipoprotein lipase (7, 8) and ApoC-III is an inhibitor (9). ApoA-I is important in the structural integrity of HDL (5) as well as in the activation of lecithin-cholesterol acyl transferase (LCAT) (10) an enzyme of great importance in lipoprotein metabolism (11, 12). The consequences of ApoA-I deficiency are well exemplified in Tangier disease where both HDL structure and VLDL metabolism appear to be abnormal (6). It is possible that subtle qualitative or quantitative abnormalities of ApoA-I may play a role in the pathogenesis of some of the familial hyperlipoproteinemias as classified by Fredrickson, Levy, and Lees (13) or in some of the lipoprotein disorders secondary to other diseases. However, studies of ApoHDL in disease states and studies of the normal structure and metabolism of HDL have been hampered by the absence of suitable assays. We report, herein, the development of a radioimmunoassay (RIA) for the ApoA-I component of ApoHDL and the results obtained with it in some metabolic and structural studies. Some of these results have been reported previously in abstract form (14, 15). Recently, another assay for ApoA-I has been reported in abstract form (16).

METHODS

Isolation of HDL. HDL was isolated from the plasma of a 47-yr-old normal male donor, who was eating an ad lib diet, as follows: after a 16-h fast the patient donated 2 U of blood, which were collected in acid citrate dextrose solution. After separation, the red cells were reinfused, and the plasma was dialyzed against 0.16 M NaCl, 1 mM in disodium EDTA, pH 8.6 (EDTA-saline).

After dialysis, the background density of the plasma was raised with solid KBr to a calculated density of 1.18 (2). (Densities of salt solutions prepared in parallel were checked by pycnometry.) The first ultracentrifugation was carried out in a 60 Ti rotor in a Beckman L265B instrument (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 50,000 rpm, at 10°C for 20 h. The resulting supernate, containing nearly all of the lipoproteins ($d < 1.18$), was removed with a Pasteur pipette and transferred to new tubes. A solution of KBr ($d 1.18$, pH 8.6) was layered over the lipoproteins and they were recentrifuged as above. The resulting supernate, containing "washed" lipoproteins, was diluted with EDTA-saline to a calculated density of 1.090 and centrifuged under the same conditions described above. The infranate, which consisted of material of $d 1.090-1.18$, was dialyzed against EDTA-saline and analyzed immunologically. It contained only ApoA and ApoC by immunodiffusion against the appropriate antisera. No ApoB, albumin, or other serum proteins were detectable. The 1.090-1.18 density range was used only as the starting material for the preparation of ApoA-I, in order to avoid possible contamination by Lp(a) (17) and by non-lipoprotein proteins. For routine applications HDL was isolated between the densities 1.063 and 1.21.

Isolation of ApoA-I. HDL was dialyzed against 0.1 M Tris-HCl, pH 8.6, 1 mM in disodium EDTA (Tris-EDTA)

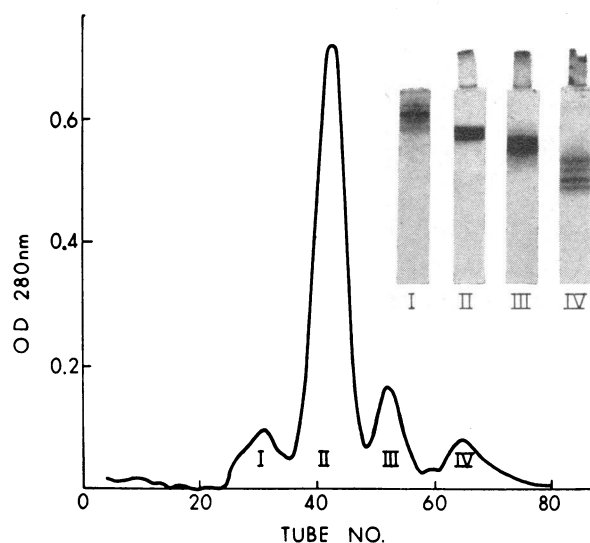


FIGURE 1 Isolation of ApoA-I. HDL ($d 1.090-1.18$) was delipidated and chromatographed on Sephadex G-200 in 0.1 M Tris-HCl, pH 8.6, 1 mM in Na₂ EDTA, and 8 M in urea (18, 19). Peaks were pooled and subjected to disk electrophoresis in 9% polyacrylamide, pH 8.9, 6 M urea and stained with Coomassie Blue. Peak II represents ApoA-I, peak III is ApoA-II, peak IV is ApoC. Peak I contains several slowly migrating proteins.

and delipidated with ether-ethanol solutions according to Scanu and Edelstein (18). 85-95% of the protein was recovered from this step. The resulting ApoHDL was solubilized in Tris-EDTA buffer and separated by gel chromatography on a 2.5 × 90-cm column containing Sephadex G-200 equilibrated with Tris-EDTA, 8 M in urea, as described by Scanu, Toth, Edelstein, Koga, and Stiller (19) (Fig. 1). 10-15 mg of protein were applied and recovery of protein averaged 95%. The ascending portion and the "top" of the peak corresponding to ApoA-I was dialyzed against 1 mM EDTA, pH 8.6, and lyophilized. To reduce the possibility of contamination by ApoA-II, the descending portion of the peak was not utilized.

ApoA-I prepared in this way migrated as a single band (Fig. 1) in the appropriate positions on disk electrophoresis (9% polyacrylamide, 6 M urea, pH 8.9) and yielded a single immunoprecipitin band on immunodiffusion and immunoelectrophoresis against anti-HDL antisera (20) (see also Fig. 7). It did not react with antisera raised against ApoA-II, ApoB, ApoC, or other serum proteins. When there were questions as to the purity of a given ApoA-I preparation, it was rechromatographed on Sephadex G-200 as above. Upon injection of ApoA-I into rabbits (see below) antisera were produced which did not react against ApoA-II or ApoC.

Some workers have found that Apo-I migrates as several bands on disk electrophoresis and elutes in at least three separate peaks on DEAE-cellulose chromatography (21). This microheterogeneity may be due to small differences in total amino acid composition. Others have not found a similar degree of heterogeneity (22). Our ApoA-I preparations were not chromatographed on DEAE-cellulose, thus some microheterogeneity may have been present in these preparations. However, there were no proteins other than ApoA-I present by the criteria outlined above.

TABLE I
Specificity of Anti-APOA-I Antisera

	Anti-ApoA-I					Anti-ApoA-II	Anti-ApoC	Anti-LDL
	1	2	3	4	5			
Whole plasma	+	+	+	+	+	+	+	+
VLDL	-	-	-	-	-	-	+	+
LDL	-	-	-	-	-	-	±	+
HDL	+	+	+	+	+	+	+	-
ApoA-I	+	+	+	+	+	-	-	-
ApoA-II	-	±	±	-	-	+	-	-
ApoC	-	-	-	-	-	-	+	-
>1.21	+	+	+	+	+	-	-	-

+, precipitin line present; -, no reaction; ±, reaction with some preparations, not with others. Antisera were produced in rabbits against the designated plasma fractions, and tested for reactivity by the Ouchterlony technique (20).

To quantify the various proteins of ApoHDL, the areas under the peaks of the elution profiles were measured by planimetry (Fig. 1). ApoA-I content, expressed as percent of total area under the curve, was $66 \pm 4\%$ in six HDL preparations (mean ± 1 SD). In two ApoHDL preparations, the protein content of each of the elution peaks was also determined by the Lowry procedure (23). ApoA-I represented 57 and 65% of the protein of HDL by planimetry, and 53 and 59% by Lowry, respectively.

Preparation of antisera. 0.5–1.0 mg amounts of ApoA-I were dissolved in 0.5 ml H₂O and emulsified in an equal amount of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Three rabbits were injected subcutaneously on three occasions 2 wk apart and bled 8–9 days after the last injection. Two of the rabbits were given "booster" injections and rebled. Sodium azide 0.01% was added to the antisera which were stored frozen at -20°C . The five different antisera were tested against a number of plasma fractions and apoproteins by immunodiffusion and immunoelectrophoresis (20) (Table I); they appeared to be monospecific. Anti-rabbit IgG antisera were produced in goats.

Iodination of ApoA-I. 1 mCi of Na¹²⁵I (Industrial Nuclear Co., Inc., St. Louis, Mo.) was dried under nitrogen in a small conical test tube. 100 μl of 0.5 M Na₂HPO₄, pH

8, was added next, followed by 25 μl of ApoA-I (20–25 μg in 0.5 M Na₂HPO₄). The reaction was started by the addition of 10 μl of chloramine-T (175 $\mu\text{g}/\text{ml}$ in 0.05 M phosphate buffer) (24). The reaction was allowed to proceed for 10 min, then the contents of the reaction tube were rapidly loaded onto a chromatographic column (0.9 \times 30 cm containing Sephadex G-50 equilibrated with 0.05 M barbital buffer, pH 8.6) and filtered. Two peaks were obtained, one corresponding to the void volume and containing iodinated protein and 15–20% of the ¹²⁵I counts and the other, presumably containing unreacted iodine (about 80% of the counts). The first peak was diluted 1:1.5 with barbital buffer, which contained 9 g of bovine serum albumin per 100 ml (BSA, Sigma Chemical Co., St. Louis, Mo.). It was then chromatographed on a 1.5 \times 30-cm column containing Sephadex G-75 equilibrated with barbital buffer containing 3 g BSA/100 ml (BSA-barbital). The elution profile (Fig. 2) consisted of a small peak (I) eluting with the void volume (16 ml), the major peak (II) which eluted in the same position as noniodinated ApoA-I (24 ml), and a smaller peak (III) which eluted in the same position as potassium chromate (44 ml). The major peak (¹²⁵I-ApoA-I) was stored at 4°C and was rechromatographed within 24 h of use in any assay. Both peaks I and III were generated from [¹²⁵I]ApoA-I (peak II) as it aged at 4°C over 2–3 wk. The calculated specific radioactivity of fresh [¹²⁵I]ApoA-I preparations ranged between 2 and 5 $\mu\text{Ci}/\mu\text{g}$ protein. Iodinations were carried out every 5–6 wk.

Extraction of HDL and of plasma for ApoA-I assay. In order to assess the ApoA-I content of HDL and of plasma accurately, it was necessary to extract their lipids with organic solvents. Plasma and HDL preparations were diluted 1:10 with EDTA-saline, and extracted in 10 times their volume with water-saturated diethyl ether with shaking at 4°C overnight. The ether was aspirated and evaporated with nitrogen. The extracted samples were then further diluted 1:10 with 8 M urea and allowed to sit at 15°C for several hours. Alternatively, 0.1-ml solutions of HDL or plasma were extracted with 10 ml of ether-ethanol (2:3 vol/vol) overnight. The protein precipitates were washed twice with ether, dried under N₂, and solubilized in 8 M urea as above. To avoid any deleterious effects of urea on the assay itself, extracted samples were diluted 1:50 or more with BSA-barbital before assay. At similar dilu-

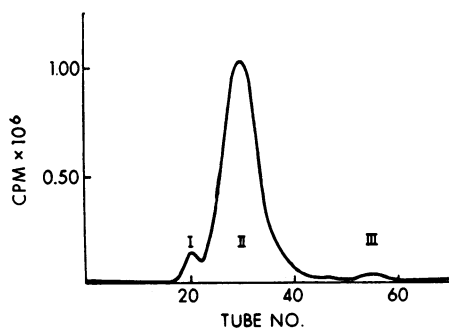


FIGURE 2 Purification of [¹²⁵I]ApoA-I. ApoA-I was iodinated with chloramine-T (24). The reaction mixture was first filtered on Sephadex G-50 and then on Sephadex G-75 (shown here). Peak II was used as the tracer.

tions, urea did not affect precipitability of counts in the assay. The latter procedure yielded more precise results and was therefore adopted for routine assays.

Other procedures. Triglycerides and cholesterol were determined by the Auto-analyzer II techniques standardized by the Core Laboratory of the Lipid Research Clinic (25). Phospholipids were assessed by the method of Bartlett (26) and proteins were determined according to Lowry, Rosebrough, Farr, and Randall (23), using BSA standards. Disk electrophoresis (27) (PAGE) was carried out at pH 8.9 in 6-inch tubes in a Canalco (Rockville, Md.) apparatus. Separating gels were made of 9% polyacrylamide, 6 M in urea. Gels were fixed in 12.5% trichloroacetic acid (TCA), stained with Coomassie Blue, and destained in 7% acetic acid (28).

The assay. Assays were carried out in 10 × 75-mm disposable test tubes which had been precoated with Siliclad (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). All dilutions were made in BSA-barbital (29). A typical tube contained 10–200 μl of sample, 50 μl of non-immune rabbit serum (NIRS, diluted 1:200), 100 μl of anti-ApoA-I (diluted 1:500 to 1:1,500), 100 μl [¹²⁵I]-ApoA-I (8,000–12,000 cpm), and sufficient BSA-barbital to bring the volume to 500 μl. Blank tubes which contained no anti-ApoA-I were also included to control for non-specific precipitation. (This averaged 3% of added counts, range 2–4%.) Other blank tubes contained only label and buffer, to control for the adsorption of label to glass. (This was consistently < 2.5% of added counts.)

In routine assays all reactants were added at the same time. Tubes were incubated for 42 h at 4°C, and second antibody was added for 18 h. (In one experiment [¹²⁵I]-ApoA-I was added later, see Results, ApoA-I in HDL. At the end of incubation, tubes were centrifuged at 2,500 rpm for 45 min. Supernates were aspirated; precipitates were resuspended with cold barbital buffer and recentrifuged. The "wash" was aspirated and precipitates were counted in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Results are expressed as the percent of the total counts of [¹²⁵I]ApoA-I added which were precipitated (%ppt). $B = [\text{125I}] \text{ApoA-I cpm precipitated} - \text{cpm in nonspecific precipitate}$; $T = \text{total } [\text{125I}] \text{ApoA-I cpm added} - \text{cpm in nonspecific precipitate}$. %ppt = B/T . $B_0 = [\text{125I}] \text{ApoA-I cpm precipitated (when no unlabeled ApoA-I is added to the system)} - \text{cpm in nonspecific precipitate}$. B/B_0 is sometimes used instead of B/T in the construction of standard curves.

RESULTS

Homogeneity of [¹²⁵I]ApoA-I. Three criteria were used to judge the homogeneity of [¹²⁵I]ApoA-I: (a) electrophoresis, (b) column chromatography, and (c) immunochemistry. In each instance the behavior of the labeled moiety was compared with unlabeled ApoA-I.

(a) [¹²⁵I]ApoA-I was added to a solution of ApoA-I and subjected to electrophoresis on paper in a Beckman Durrum Cell using 0.05 M barbital buffer, pH 8.6, for 16 h. At the end of electrophoresis the paper was dried in an oven, cut into 4-mm strips, and counted on a Packard gamma spectrometer. The strips were then stained with bromophenol blue. ApoA-I migrated to the alpha globulin area. The

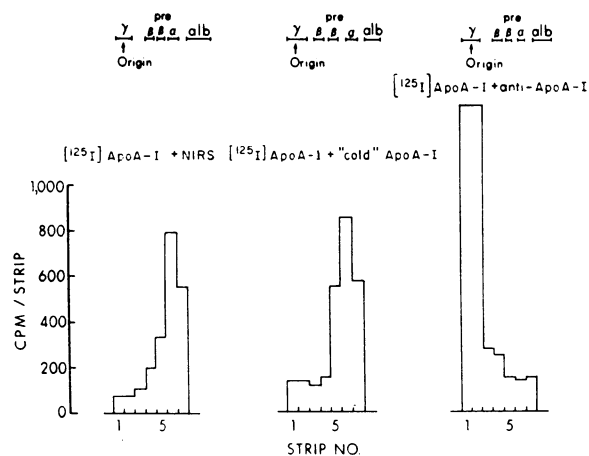


FIGURE 3 Electrophoretic mobility of [¹²⁵I]ApoA-I. [¹²⁵I]-labeled ApoA-I was mixed with NIRS, "cold" ApoA-I, or anti-ApoA-I antiserum and subjected to electrophoresis on 1% agarose. Electropherograms were cut into strips, radioactivity was determined in a gamma counter, location of proteins was ascertained with Amido Schwartz.

radioactivity due to [¹²⁵I]ApoA-I coincided with the stained band produced by ApoA-I.

A similar experiment was carried out using 1% agarose as the electrophoretic medium (Fig. 3). [¹²⁵I]ApoA-I was added to each one of the following and the resulting mixtures were subjected to electrophoresis: "cold" ApoA-I, NIRS, and rabbit anti-ApoA-I antiserum. 90% of [¹²⁵I]ApoA-I migrated with ApoA-I to the alpha globulin area. Migration was almost completely blocked by anti-ApoA-I antiserum but not by NIRS.

(b) [¹²⁵I]ApoA-I was added to ApoA-I and chromatographed on a 0.9 × 30-cm column containing Sephadex G-75 equilibrated with barbital buffer. The radioactive counts (95% of which were recovered) eluted in a single symmetrical peak which coincided with the protein (read at 280 nm) (Fig. 2).

(c) 80–90% of [¹²⁵I]ApoA-I was precipitated in the RIA in the presence of excess antibody. A similar percentage was precipitated by 10% TCA.

The assay curve. In the absence of "cold" ApoA-I, 80–90% of [¹²⁵I]ApoA-I counts were precipitated by each of five different anti-ApoA-I antisera. For routine use, antisera were diluted (1:500 to 1:1,500) to give precipitabilities of 70–75% in the absence of unlabeled ApoA-I (B_0/T). With these dilutions, as increasing amounts of "cold" ApoA-I were added (1–50 ng), a typical RIA curve was obtained (Fig. 4), in which more than 90% of bound label was displaced by "cold" ApoA-I.

VLDL, twice ultracentrifuged at $d = 1.006$ and LDL ($d = 1.025$ – 1.050), when added in 200-fold the usual

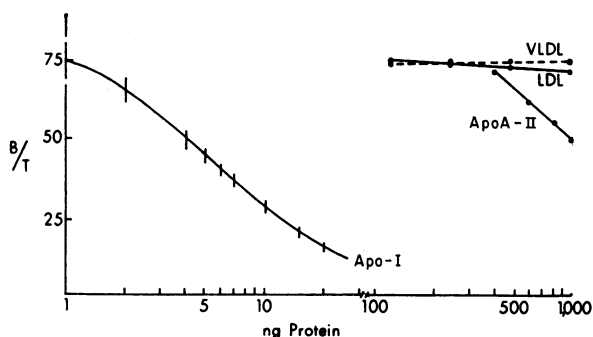


FIGURE 4 $[^{125}\text{I}]$ ApoA-I was displaced from anti-ApoA-I antiserum by ApoA-I, but not by VLDL or LDL. ApoA-II preparations purified by rechromatography did not displace any ^{125}I counts even in amounts which were 2000-fold greater than ApoA-I.

amounts of ApoA-I protein, produced no displacement of counts in this system. ApoC also displaced no counts. Five different preparations of ApoA-II displaced counts in parallel with ApoA-I when added in 80–1,000-fold excess (Fig. 4). ApoA-II repurified by column chromatography on Sephadex G-200 did not react even in 2,000-fold excess.

The precision of 48 points on standard curves each run in triplicate was 5.5 ± 3.3 (coefficient of variation, mean ± 1 SD). In 12 assays B_0/T was 75 ± 3.1 , and 9.7 ± 1.6 ng of "cold" ApoA-I were required to obtain a B/B_0 of 0.5 in the same assays. As little as 3 ng of ApoA-I could be reliably detected with a precision of 10%.

Each of the five anti-ApoA-I antisera and the two anti-HDL antisera which were produced yielded acceptable standard curves.

ApoA-I in HDL. HDL isolated from four normal, three type IV, and two type III subjects produced standard curves which paralleled those produced by ApoA-I. However, 20–90 times more ApoHDL than ApoA-I were needed to produce equal amounts of displacement ($n = 22$). The reactivity of HDL preparations diminished with storage at 4°C (preparations up to 8 wk old were tested), accounting for the variability of HDL preparations. Nevertheless even the "freshest" HDL had only about 1/20th the reactivity of ApoA-I. Assays utilizing another anti-ApoA-I antiserum (no. 1) yielded similar results.

The possible explanations for the relatively poor reactivity of HDL were: (a) that ApoA-I represented only a small fraction of ApoHDL, (b) that the rates of equilibration of ApoA-I and HDL with the rest of the RIA system differed because of differences in the sizes of ApoA-I and HDL, and (c) that the structure of HDL is such as to make most of its ApoA-I complement unreactive in this system.

Since ApoA-I comprised about 66% of ApoHDL by column chromatography (see Methods, Fig. 1, and Refs. 19, 21, 30) the first possibility was ruled out. To study the second question, i.e., the possible effects of differential reaction kinetics on the activities of HDL and ApoA-I relative to each other, five pairs of standard curves were set up, each pair consisting of an ApoA-I and an HDL curve. Each ApoA-I curve contained identical components with every other ApoA-I curve and each HDL curve was identical with every other HDL curve, but the time at which $[^{125}\text{I}]$ ApoA-I was added relative to the rest of the reactants was varied for each of the pairs. Thus, at 0 time $[^{125}\text{I}]$ ApoA-I was added to each member of the pair at the same time with the rest of the reactants; at 6 h label was added 6 h after the other reactants, etc. This experimental design allowed for increasing periods of preincubation of unlabeled ApoA-I or HDL with anti-ApoA-I antibody before the addition of $[^{125}\text{I}]$ ApoA-I, giving the unlabeled moieties a "competitive advantage" vis-à-vis the label. This would result in apparent increases in the reactivity of both ApoA-I and HDL (31). It was expected, that if the relative lack of reactivity of HDL was due to kinetic considerations, the increases in the reactivities of HDL and ApoA-I would not be parallel in time-course or magnitude.

It is evident (Fig. 5) that preincubation did produce the expected rises in the activities of both the standards.

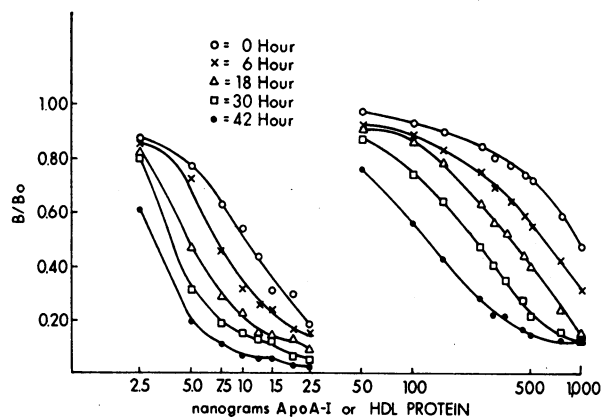


FIGURE 5 Changes in the activities of ApoA-I and HDL with increasing periods of preincubation. For 0 hour, all reactants of the assay were added at the same time; for 6 hours all reactants were added at the same time except for $[^{125}\text{I}]$ ApoA-I which was added 6 h later, etc. Thus, ApoA-I and HDL were incubated with anti-ApoA-I for increasingly longer periods of time compared with $[^{125}\text{I}]$ ApoA-I. This gave the unlabeled moieties a "competitive advantage" and appeared to increase their activities. Activity is defined as the mass of material required to produce a given amount of displacement; therefore a smaller mass means greater activity or a shift of the curves to the left. The activities of both ApoA-I and HDL were increased by preincubation.

TABLE II
Activities of APOA-I and HDL in the RIA for APOA-I*

Hours	ApoA-I	HDL	HDL/ApoA-I wt ratio
	<i>ng protein</i>		
0	10.7	950	91
6	6.9	600	87
18	4.8	380	76
30	3.9	232	60
42	3.6	170	47

* Values were obtained from Fig. 5, and results are expressed in nanograms of protein required to produce sufficient displacement of counts to produce $B/B_0 = 0.5$. Thus a smaller number denotes increased activity. Hours refer to the duration of preincubation of unlabelled ApoA-I or HDL before the addition of [125 I] ApoA-I.

(Activity is measured as the amount of ApoA-I or HDL required to produce a B/B_0 of 0.5. Increases in activity meant that less ApoA-I or HDL were required to produce a B/B_0 of 0.5.) The activity of HDL was increased from 950 to 380 ng at 18 h and to 170 ng at 42 h. ApoA-I activity during the same times rose from 10.4 to 4.8 and then to 3.6. In both cases most of the rises took place by 18 h; relatively little additional increase in activity was obtained at 42 h. The activities of ApoA-I relative to HDL at 0, 18, and 42 h were 91, 76, and 47, respectively (Table II). These findings suggest that differences in kinetics of reaction accounted for only a small fraction of the greater reactivity of ApoA-I compared with HDL.

To expose more of the ApoA-I in HDL, "intact" HDL was subjected to a variety of disruptive treatments, including repeated freezing and thawing, disaggregation with 8 M urea or 6 M guanidine hydrochloride, or delipidation with ether or ether-ethanol solutions followed by exposure to urea (see Methods).

HDL subjected to such treatments continued to displace counts in parallel with ApoA-I (Fig. 6). Freezing and thawing doubled the reactivity of HDL; urea and guanidine HCL each increased it fivefold. 12–20-fold rises in reactivity were obtained by ether and ether-ethanol extraction and treatment with 8 M urea (Fig. 6). Thus, disruption of HDL structure resulted in increased reactivity. It is worth noting that the age-related variability of "intact" HDL noted above disappeared after solvent extraction, i.e., fresh and aged HDL preparations displaced counts to the same extent after extraction.

A number of workers have shown that disruptive manipulations of HDL yield protein-containing "fragments" of variable sizes and densities (32–34). For example, Levy and Fredrickson (32) have produced a smaller less lipidated form of HDL by maneuvers very similar to those used here. Their smaller HDL migrated more slowly on immunoelectrophoresis (α LpB)

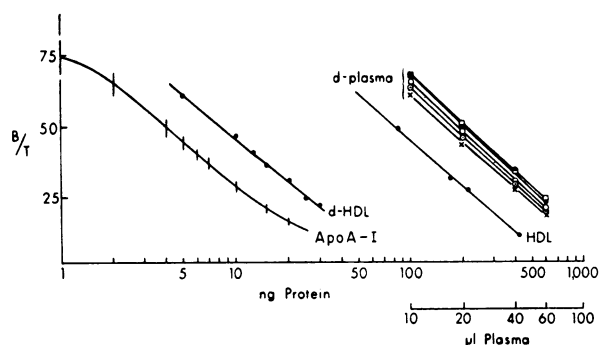


FIGURE 6 Extracted HDL (d-HDL) and extracted plasmas (d-plasma) displayed parallelism with ApoA-I and with each other. The microliter scale applies to plasma only, plasmas were diluted 1 : 5,000.

than did "native" HDL (α LpA). We also subjected ApoA-I, and "native" HDL, and ether and ether-ethanol-extracted HDL to immunoelectrophoresis utilizing our five anti-ApoA-I antisera (Fig. 7). With each antiserum HDL yielded a single arc which stained for lipid with Fat Red 7 B. ApoA-I also yielded a single arc which migrated more slowly anodally and which did not stain for lipid. Ether-extracted HDL yielded two arcs, one faster and the other migrating more slowly; both arcs were longer than the HDL arc. Neither stained well with the lipid dye. Ether-ethanol-extracted HDL gave rise to a single, nonstaining arc which migrated at about the same rate as ApoA-I and the slow component of ether-extracted HDL. Thus, the anti-ApoA-I antisera "recognized" ApoA-I whether it

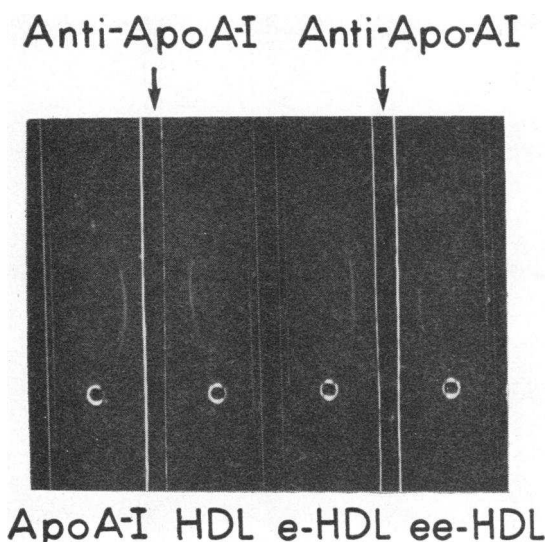


FIGURE 7 Immunoelectrophoresis of ApoA-I, HDL, and delipidated HDL against anti-ApoA-I. Delipidation of HDL altered the mobility of ApoA-I. e, ether extracted; ee, ether-ethanol extracted.

was associated with "intact" or variously degraded HDL. If one compares our results with those of Levy et al., native HDL consisted almost entirely of α LpA; purified ApoA-I and ether-ethanol-delipidated HDL behaved as α LpB; and ether-extracted-HDL appeared to produce at least two populations, both of which migrated more slowly than α LpA. Our findings greatly resemble those of Levy and Fredrickson (32).

After extraction of HDL, ApoA-I accounted for $51 \pm 9\%$ of ApoHDL ($n = 11$). Whereas, ApoA-I represented $66 \pm 4\%$ ($n = 6$) of ApoHDL by column chromatography. Thus, the RIA appeared to detect about 80% of the available ApoA-I compared to the column chromatographic procedure. (The results of analyses of plasma ApoA-I by RIA to be reported below have not been adjusted to reflect these discrepancies between the physical and immunologic procedures.)

ApoA-I distribution in plasma. The distribution of ApoA-I in the density fractions of two normal plasmas was determined. The plasmas were ultracentrifuged at the following densities: 1.006, 1.063, and 1.21. The ApoA-I content of the fractions was obtained by RIA after extraction. The $d < 1.063$, d 1.063–1.21, and $d > 1.21$ fractions contained < 1 , 93, and 7% of the total ApoA-I, respectively. Recovery of ApoA-I was 103–106%. ([Sum of ApoA-I in density fractions $\times 100$] / [ApoA-I in whole plasma]). An additional six normal plasmas were spun at $d = 1.21$. $92 \pm 8\%$ of ApoA-I was in the $d < 1.21$ and $8 \pm 1\%$ was in the $d > 1.21$ fractions.

The above plasmas and density fractions were also assayed by RIA before extraction. Extraction increased reactivity of whole plasma 12–13-fold, and of HDL 14–15-fold. By contrast, the reactivity of $d > 1.21$ fractions was increased only 2–3-fold. (The reactivity of

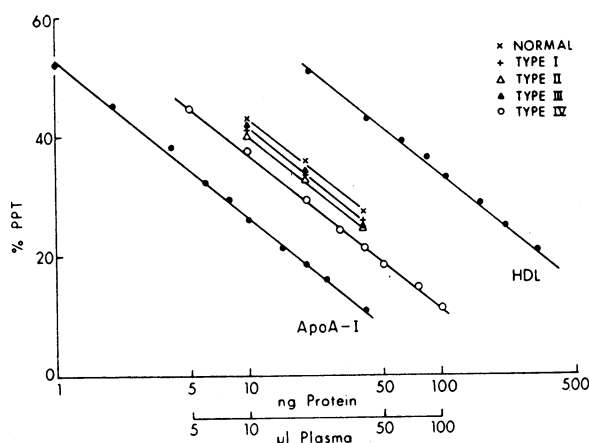


FIGURE 8. HDL and plasmas displayed parallelism with ApoA-I and with each other. HDL and plasma from normals and subjects with the various hyperlipoproteinemias were used. The microliter scale applies to plasma only. Plasmas were diluted 1:500 before assay.

TABLE III

APOA-I Contents of Plasma as Determined with Five Different Antisera

Subjects	Antisera				
	1	2	3	4	5
S. S.	89	93	63	74	82
A. M.		101	86	107	106
M. M.		138	118	149	144
D. B.	98	96	80	81	84
G. M.	82	88	68	78	77

Plasmas were extracted with ether, treated with 8 M urea. ApoA-I contents were determined in assays using the above antisera, [125 I]ApoA-I as the labeled moiety and ApoA-I standards.

one VHDL preparation (d 1.21–1.25) was increased fivefold.)

The distribution of ApoA-I by molecular size was assessed by filtering fresh normolipemic plasma on a 2.5×90 -cm column of Sephadex G-200 equilibrated with 0.05 M Tris buffer, pH 8.0. The column was calibrated for molecular weight (mol wt) determinations with the use of calibrators purchased from Pharmacia Fine Chemicals, Inc., (Piscataway, N. J.) and with potassium chromate. A plot of K_{av} vs. log mol wt was prepared. In addition, the elution position of IgM, α_2 M, fibrinogen, IgG, and albumin were determined by the Ouchterlony technique (20) using commercial antisera (Hoechst Pharmaceutical Co., Woodley, N. J.). The position of ApoB was assessed by RIA (35).

Fresh plasma yielded three protein peaks on column chromatography (OD at 280 nm): the first peak contained ApoB, α_2 M, and IgM, the second, IgG, and the third, albumin. ApoA-I was eluted in three broad peaks corresponding to approximate mol wts of 320,000, 155,000, and 50,000. The third peak was rechromatographed on Sephadex G-75; ApoA-I activity, together with albumin, eluted in a single peak near the void volume, ApoA-I activity was not detected in any smaller mol wt fractions. When $d > 1.21$ fractions of plasma were chromatographed on Sephadex G-200, three protein peaks were again obtained, but ApoA-I was detectable only in the 50,000 mol wt peak.

Each of the Sephadex G-200 peaks was pooled, extracted with ether, and assayed for ApoA-I by RIA. Peaks 1, 2, and 3 contained 42, 45, and 13% of the total ApoA-I, respectively. 87% of applied ApoA-I was recovered from the column (sum of pools $\times 100$ /whole plasma).

The three pooled peaks were also assayed before extraction to ascertain the increases in reactivity produced by the extraction procedure. The increases for peaks 1, 2, and 3 were 17-, 14-, and 2-fold, respectively. Thus, fractionation of plasma by ultracentrifugal and column chromatographic procedures both yielded

TABLE IV
ApoA-I Levels in Human Plasma

Diagnostic group	ApoA-I level*	Correlation
		ApoA-I vs. HDL-Chol
	<i>mg/dl</i>	
Normolipemic		
Men [37, 13-58]	100±35 (41)	0.78 (14) <i>P</i> < 0.001
Women [29, 16-58]	104±34 (34)	0.52 (16) <i>P</i> < 0.05
Type II		
Men [36, 12-62]	93±31 (21)	0.61 (18) <i>P</i> < 0.01
Women [48, 5-61]	100±35 (27)	0.70 (27) <i>P</i> < 0.001
Type IV		
Men [49, 31-57]	97±28 (24)	0.76 (21) <i>P</i> < 0.001
Women [45, 31-58]	103±30 (5)	0.81 (4)
Pregnant women [21, 18-27]	197±36 (22)	0.49 (22) <i>P</i> < 0.02

* Mean ± 1 SD; (), number of subjects; [], mean ages in years, range.

Patients were referred to the Washington University Lipid Research Center with known or suspected hyperlipoproteinemia or premature atherosclerosis. Normals included laboratory personnel, students, and patients found to be normolipemic. Pregnant women were in their second and third trimesters. Normal subjects had total TG and LDL-Chol levels of <200 mg/dl. Type II had LDL-Chol >200 mg/dl and total TG <200 mg/dl. Type IV had total TG >200 and LDL-Chol <200 mg/dl.

large mol wt fractions, the reactivity of which was greatly increased by extraction, and a 50,000 mol wt fraction which sedimented at $d = 1.21$. This fraction contained about 10% of the ApoA-I in normolipemic plasma and was much less affected by extraction.

ApoA-I levels in plasma. Plasmas drawn from one type I, five type II, two type III, six type IV, and one type V patients displayed parallelism with ApoA-I (Fig. 8). The apparent ApoA-I concentrations of untreated plasmas ranged from 4.8-14.4 mg/dl in assays using ApoA-I standards. These results were considerably lower than expected (36). Therefore, plasmas were extracted with ether-ethanol and treated with urea as described above for HDL. Parallelism with ApoA-I was retained (Fig. 6) and the reactivity of the plasmas was increased 10.5±2.1-fold ($n = 30$).

To check further on the ability of the assay to detect all of the ApoA-I present in plasma, ApoA-I or HDL were added to plasma, and the mixtures were extracted and processed as above. Recovery of added ApoA-I was 95±10% ($n = 12$). An ApoA-I-plasma mixture was also assayed without extraction; 99±3% ($n = 3$) of added ApoA-I was recovered. Finally, five extracted plasmas were assayed with five different anti-ApoA-I antisera (Table III). Four of the five antisera yielded comparable results. Assays using one antiserum (no. 3) consistently gave lower results. (Antiserum no. 5 was used routinely.)

The mean ApoA-I contents of normal whole plasmas,

measured after extraction, were about 100 mg/dl. Major departures from normal were found in pregnant women (twice normal) but not in familial types II and IV hyperlipoproteinemia (Table IV). We also measured the plasma ApoA-I contents of a 47-yr-old white man in whom hyperlipemia was found on a routine medical examination. Medical history, physical examinations, and routine chemical screen for thyroid, hepatic, and renal disease were normal. He had been started on Clofibrate, 0.5 g four times a day, by his physician 6 mo before referral. Fasting plasma lipoprotein levels on Clofibrate on four separate occasions 6 wk apart were as follows (mean±1 SD, mg/dl: VLDL-triglyceride (TG), 205±79; VLDL-Cholesterol (Chol) 47±7; LDL-TG, 123±9; LDL-Chol, 270±16; HDL-TG, 3±2; HDL-Chol, 10±7. Lipoprotein electrophoresis showed only a trace of alpha lipoprotein. Plasma ApoA-I levels were 15±2. Significant positive correlations were found in all patient groups between HDL-Chol and ApoA-I levels (Table IV), whereas correlations between total Chol and ApoA-II were not significant (not shown).

DISCUSSION

The assay. The HDL transport most of the phospholipids of plasma; thus they provide most of the lecithin—substrate for the LCAT reaction (11). The ApoA component, ApoA-I is an activator of LCAT (10), while ApoC contains proteins which both activate

(ApoC-II) and inhibit (ApoC-III) lipoprotein lipase (7-9). Gross distortions of lipoprotein structure and metabolism follow deficiencies of either LCAT (12) or of ApoHDL (6). Thus both the lipid and protein components of HDL are important physiologically and structurally. In spite of this, it has not been possible to quantify ApoHDL in plasma except by very laborious procedures involving isolation of the lipoproteins from plasma, followed by extraction and isolation of the individual apoproteins by column chromatography (19, 21, 30). Therefore, the development of a reliable assay for ApoA-I, the major protein of ApoHDL, was thought to be a useful addition to the available methodology for studying lipoprotein metabolism.

Any assay must meet criteria of specificity, precision, accuracy, and sensitivity before it can be deemed useful (37). In RIA, *specificity* is determined by the purity of the tracer (31). Our ApoA-I preparations met several of the commonly accepted criteria of purity, including the most stringent—the ability to stimulate the production of monospecific antisera. Iodination did not affect the reactivity of ApoA-I since [¹²⁵I]ApoA-I preparations behaved as did “cold” ApoA-I in several physical systems. Of even greater importance for the assay, [¹²⁵I]ApoA-I was 90% precipitable by excess anti-ApoA-I, and “cold” ApoA-I competed with the tracer for limiting amounts of antibody, displacing >90% of bound [¹²⁵I]ApoA-I. In assays which utilized [¹²⁵I]-ApoA-I, ApoA-containing plasma fractions ($d > 1.063$) displaced counts in parallel with ApoA-I, while LDL, ApoC, and ApoA-II did not react. The plasma of a man with hypoalphalipoproteinemia contained only about 10–15% of the usual levels of ApoA-I. Thus the assay appears to be specific.

The *precision* of the assay compares favorably with the RIA for ApoB (35), and with other RIAs (38). Since samples are assayed after extraction, they may be stored frozen or at 4°C (unextracted) for several weeks without loss of potency.

Sensitivity is not a limiting factor in the plasma assay since 3 ng of ApoA-I are routinely detectable. This degree of sensitivity should prove to be useful for studies where small sample sizes are a consideration, e.g., in vitro or in infants. The necessity for extraction of plasma makes the procedure a little cumbersome; however, it is possible for one technician to assay 120 samples in triplicate in a routine week.

To assess *accuracy*, the ApoA-I contents of HDL obtained with RIA were compared to those obtained by chromatography. Similar comparisons could not be made for $d > 1.21$ fractions, since ApoA-I has not been isolated from them. (ApoA-I was also detected in extracted VLDL and LDL of normal plasma, representing <0.1% of ApoVLDL and ApoLDL. These small amounts are apparently not detected by other methods.)

ApoA-I represented 51% of ApoHDL by RIA compared to 66% by chromatography. This discrepancy may reflect an underestimate of the ApoA-I content of HDL by RIA, which could occur if the extraction and urea treatments do not expose all of the ApoA-I to antibody. (It is unlikely that ApoA-I determinants are destroyed since the ApoA-I standard is isolated in the same way.) On the other hand, physical methods may overestimate ApoA-I contents, if disproportionate losses of protein are sustained during the complicated processes of purification. Thus it is difficult to decide which results are the more valid. However, reasonable agreement exists between the immunologic and physical methods. In addition, the ApoA-I levels obtained here are not too different from those obtained by direct analysis of isolated HDL fractions (36) and by another RIA recently reported in abstract form (16). The assay, therefore, represents a distinct improvement over available methods.

ApoA-I in HDL. HDL was much less reactive in the assay than was expected from its ApoA-I content by column chromatography. This could have been due to causes related (a) to the technique of the assay, (b) to the characteristics of the immunologic reactions, or (c) to the structure of HDL itself.

The kinetic experiment showed that while there were some slight differences in the kinetic behavior of ApoA-I and HDL in the RIA, these were probably not of sufficient magnitude to account for the large discrepancies in activities of ApoA-I and HDL. Thus, while the assay as routinely performed may have overestimated the differences between ApoA-I and HDL 2-3-fold, >40-fold differences still remained even after 42 h of preincubation in this particular experiment.

It is also possible that the immunologic technique affected these results. HDL by virtue of its greater size could have altered the sizes of the immune complexes formed by HDL and anti-ApoA-I so as to effect their precipitability by the anti-rabbit IgG antibody. This possibility is unlikely. Chylomicrons and VLDL are much larger than HDL (1-4) yet all of the expected ApoB content of those particles is detectable by a similar double antibody RIA for ApoB (35). One more caution needs to be added; the activities of the HDL preparations were inversely related to their ages. However, these differences disappeared after extraction of the lipids of HDL. Furthermore, even the “freshest” preparations had only 1/20th of the activity of ApoA-I. Thus age may have introduced an artefact of scale but real differences remained. If differences in kinetics and size do not explain the disparate reactivities of ApoA-I and HDL, the most likely explanation must lie in their structural differences.

Two proposals have been put forth for the structure of HDL. According to Forte et al. (39, 40), HDL by

electron microscopy consists of a number of subunits surrounding one central subunit. The alternative structural model is based on studies using the small angle X-ray scattering technique (4, 41, 42). According to this model, HDL is spherical, with apoproteins covering its outer surfaces. The accessibility of nearly all the side chains of amino acids for chemical alteration is taken as supporting this formulation (4). The ApoB-containing lipoprotein (chylomicrons, VLDL, and LDL) are also thought to be spherical with the proteins placed on or near the surface of the spheres (1, 3). Our findings of the poor reactivity of HDL are compatible with both models.

Taking the Forte model first, if ApoA-I were uniformly arranged on the surface of the subunits, about 40% of it should be exposed and reactive in our system. Since only 5% or less is reactive, it may be that ApoA-I is preferentially arranged on the "interior" surface of each subunit with perhaps the greatest amount of ApoA-I being on the central subunit.

In the spherical model, protein subunits are disposed on the surface of the HDL spheres (4, 41, 42). Yet in spite of their surface location only 5% or less of ApoA-I is accessible to antibody. This is in contrast to ApoB all of which appears to be detectable in VLDL and chylomicrons by a similar double antibody RIA system (35). The unavailability of ApoA-I suggests that most of it is complexed with lipid or other Apo-HDL so as to make it unavailable to antibody in spite of its location on the surface of the spheres. (The accessibility of amino acid side chains to much smaller chemicals (4) does not alter the validity of this argument.)

In normal plasma about 90% of ApoA-I is associated with $d < 1.21$ and about 10% is in the $d > 1.21$ fractions. (Trace amounts are also found in the $d < 1.063$ fractions.) In the $d > 1.21$ fractions, ApoA-I seems to be part of a molecule with mol wt of 50,000. VHDL and $d > 1.21$ and the smaller mol wt fractions exhibited parallelism with ApoA-I but were activated to a much lesser extent by extraction than was HDL. This implies a simpler structure for these lipoproteins.

Intact HDL isolated from normal and hyperlipoproteinemic subjects displaced [125 I]ApoA-I in parallel with lipid-free ApoA-I standard with each other. One can conclude, that the same antigenic determinants were being detected in intact HDL and in purified ApoA-I, and that the HDL of normals and those with hyperlipoproteinemia are immunologically indistinguishable. Furthermore, *extracted* plasmas and HDL from normal and patients with hyperlipoproteinemia displaced counts in parallel with each other. This suggests that the ApoA-I of normal and hyperlipoproteinemic subjects is also immunologically identical. (The above conclusions are of course subject to the qualification that rabbit

antisera may not detect subtle differences between human proteins. Such relative insensitivity of heterologous antisera has been observed with LDL allotypes (43, 44).)

Total plasma ApoA-I levels were similar in normal and type II and type IV hyperlipoproteinemic subjects. However, more population studies need to be done to define the distribution of ApoA-I levels in normal subjects and in the various dyslipoproteinemias. The high levels of ApoA-I in pregnancy indicate that metabolic and/or hormonal factors play important roles in altering ApoA-I concentrations.

Note added in proof. A new molecular model for HDL has appeared recently (Assmann, G., and H. B. Brewer, Jr. 1974. A molecular model of high density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 71: 1534-1538.) which proposes that HDL is spherical and that the apoproteins are disposed on the surface as "icebergs" in a "sea" of lipid. Each "iceberg" consists of ApoA-I surrounded by ApoA-II. This model based on results obtained by means of nuclear magnetic resonance and circular dichroism predicts that most of ApoA-I in intact HDL would not be available to react in the RIA.

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REFERENCES

- Schumaker, V. N., and G. H. Adams. 1969. Circulating lipoproteins. *Annu. Rev. Biochem.* 38: 113-136.
- Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* 6: 1-68.
- Alaupovic, P. 1972. Studies on the composition and structure of plasma lipoproteins. Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. *Biochim. Biophys. Acta.* 260: 689-707.
- Scanu, A. M., and C. Wisdom. 1972. Serum lipoproteins structure and function. *Annu. Rev. Biochem.* 41: 703-730.
- Scanu, A. M. 1972. Structural studies on serum lipoproteins. *Biochim. Biophys. Acta.* 265: 471-508.
- Fredrickson, D. S., A. M. Gotto, and R. I. Levy. 1972. Familial lipoprotein deficiencies. (Abetalipoproteinemia, hypobetalipoproteinemia, and Tangier disease.) In *Metabolic Basis of Inherited Diseases*. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw Hill Book Company, New York. 3rd edition. 493-530.
- Havel, R. J., V. G. Shore, B. Shore, and D. M. Bier. 1970. Role of specific glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase. *Circ. Res.* 27: 595-600.

8. Ganesan, D., R. H. Bradford, P. Alaupovic, and W. J. McConathy. 1971. Differential activation of lipoprotein lipase from human post-heparin plasma, milk, and adipose tissue by polypeptides of human serum apolipoprotein C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **15**: 205-208.
9. Brown, W. V., and M. L. Baginsky. 1972. Inhibition of lipoprotein lipase by an apoprotein of human very low density lipoprotein. *Biochem. Biophys. Res. Commun.* **46**: 375-382.
10. Fielding, C. J., C. T. Lim, and A. M. Scanu. 1970. A protein component of serum high density lipoprotein with co-factor activity against purified lipoprotein lipase. *Biochem. Biophys. Res. Commun.* **39**: 889-894.
11. Glomset, J. A. 1968. The plasma lecithin: cholesterol acyltransferase reaction. *J. Lipid Res.* **9**: 155-167.
12. Norum, K., R. J. A. Glomset, and E. Gjone. 1972. Familial lecithin: cholesterol acyltransferase deficiency. In *Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw Hill Book Company, New York. 3rd edition. 531-544.
13. Fredrickson, D. S., R. I. Levy, and R. Lees. 1967. Fat transport in lipoproteins—An integrated approach to mechanisms and disorders. *N. Engl. J. Med.* **276**: 215-225.
14. Schonfeld, G., and B. Pflieger. 1973. Immunology and plasma levels of APO R-GLN-I in hyperlipoproteinemia and in pregnancy. *Circ.* **48**: IV. 216.
15. Schonfeld, G., and B. Pflieger. 1973. Radioimmunoassay (RIA) for apolipoprotein R-GLN-I of human high-density lipoprotein (HDL). *Clin. Res.* **21**: 638. (Abstr.).
16. Starr, J. I., D. J. Juhn, M. E. Mako, M. Guerin, A. M. Scanu, A. H. Rubenstein. 1973. Circulating high density lipoprotein (HDL): Measurement of an apoprotein component by radioimmunoassay. *J. Clin. Invest.* **52**: 81a.
17. Ehnholm, C., H. Garoff, K. Simons, and H. Aro. 1971. Purification and quantitation of the human plasma lipoprotein carrying the Lp(a) antigen. *Biochim. Biophys. Acta.* **236**: 431-439.
18. Scanu, A. M., and C. Edelstein. 1971. Solubility in aqueous solutions of ethanol of the small molecular weight peptides of the serum very low density and high density lipoproteins: relevance to the recovery problem during delipidation of serum lipoproteins. *Anal. Biochem.* **44**: 576-588.
19. Scanu, A., J. Toth, C. Edelstein, S. Koga, and E. Stiller. 1969. Fractionation of human serum high density lipoprotein in urea solutions. Evidence for polypeptide heterogeneity. *Biochemistry.* **8**: 3309-3316.
20. Ouchterlony, O. 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor-Humphrey Science Publishers, Ann Arbor, Mich.
21. Shore, B., and V. Shore. 1968. Heterogeneity in protein subunits of human serum high-density lipoproteins. *Biochemistry.* **7**: 2773-2777.
22. Baker, H. N., R. L. Jackson, A. M. Gotto, Jr. 1973. Isolation and characterization of the cyanogen bromide fragments of high-density apolipoprotein glutamine I. *Biochemistry.* **12**: 3866-3871.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
24. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ¹²⁵I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**: 114-123.
25. ———. 1972. Manual of Laboratory Methods. Lipid Research Clinics Program. University of North Carolina, Chapel Hill, N. C.
26. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**: 466-468.
27. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
28. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Anal. Biochem.* **20**: 150-154.
29. Eaton, R. P., and D. M. Kipnis. 1969. Radioimmunoassay of beta lipoprotein-protein in rat serum. *J. Clin. Invest.* **48**: 1387-1396.
30. Lux, S. E., K. M. John, and H. B. Brewer, Jr. 1972. Isolation and characterization of ApoLp-Gln-II (ApoA-II), a plasma high density apolipoprotein containing two identical polypeptide chains. *J. Biol. Chem.* **247**: 7510-7518.
31. Berson, S. A., and R. S. Yalow. 1964. Immunoassay of protein hormones. *Hormones.* **4**: 557-630.
32. Levy, R. I., and D. S. Fredrickson. 1965. Heterogeneity of plasma high density lipoproteins. *J. Clin. Invest.* **44**: 426-441.
33. Scanu, A. 1966. Forms of human serum high density lipoprotein protein. *J. Lipid Res.* **7**: 295-306.
34. Borut, T. C., and F. Aladjem. 1971. Immunochemical heterogeneity of human high density serum lipoproteins. *Immunochemistry.* **8**: 851-863.
35. Schonfeld, G., B. Pflieger, and R. S. Lees. 1974. Assay of total plasma apolipoprotein B concentration in human subjects. *J. Clin. Invest.* **53**: 1458-1467.
36. Lux, S. E., R. I. Levy, A. M. Gotto, and D. S. Fredrickson, 1972. Studies on the protein defect in Tangier disease. *J. Clin. Invest.* **51**: 2505-2519.
37. Midgley, A. R., Jr., G. D. Niswender, and R. W. Rebar. 1969. Principles for the assessment of the reliability of radioimmunoassay methods. *Acta Endocrinol. Suppl.* **142**: 163-184.
38. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. Plasma insulin levels of normal subdiabetic and diabetic rats. *Diabetes.* **12**: 115-126.
39. Forte, T., K. R. Norum, J. A. Glomset, and A. V. Nichols. 1971. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. *J. Clin. Invest.* **50**: 1141-1148.
40. Forte, T. M., A. V. Nichols, E. L. Gong, S. Lux, and R. I. Levy. 1971. Electron microscopic study on reassembly of plasma high density apoprotein with various lipids. *Biochem. Biophys. Acta.* **248**: 381-386.
41. Laggner, P., O. Kratky, G. Kostner, J. Sattler, and A. Holasek. 1972. Small angle x-ray scattering of LpA, the major lipoprotein family of human plasma high density lipoprotein HDL_a. *FEBS (Fed. Eur. Biochem Soc.) Lett.* **27**: 1: 53-57.
42. Laggner, P., K. Müller, O. Kratky, G. Kostner, and A. Holasek. 1973. Studies on the structure of lipoprotein A of human high density lipoprotein HDL_a: the spherically averaged electron density distribution. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **33**: 1: 77-80.
43. Büttler, R. 1967. Polymorphisms of the human-low density lipoproteins. *Vox Sang.* **12**: 2-17.
44. Blumberg, B. S., D. Bernanke, and A. C. Allison. 1962. A human lipoprotein polymorphism. *J. Clin. Invest.* **41**: 1936-1944.