Apolipoprotein A-II content of human plasma high density lipoproteins measured by radioimmunoassay

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Abstract A double antibody radioimmunoassay for human ApoA-II is reported. ApoA-II isolated from human plasma high density lipoprotein (HDL) by column chromatography migrated as a single band on polyacrylamide disc gel electrophoresis, had the appropriate amino acid composition, and provoked the production of monospecific antisera. ^{125}I-ApoA-II (iodinated by lactoperoxidase, purified by Sephadex G-75 chromatography) migrated with "cold" ApoA-II as a single band on disc gel electrophoresis in SDS. Its specific radioactivity was 5–12 mCi/μg. In assays, (0.05 M barbital buffer, 0.01% Triton X-100, pH 8.6) over 90% of ^{125}I-ApoA-II was bound by excess first antibody and over 95% was displaced by excess "cold" ApoA-II. Low density lipoprotein, very low density lipoprotein, ApoA-I, ApoC-II, and ApoC-III displaced no counts. Intraassay and interassay coefficients of variation for lipoprotein or plasma samples were 7 ± 4 and 11 ± 6%, respectively. As little as 1.0 ng of ApoA-II was detectable with a precision of 10%. ApoA-II made up 20–25% of the proteins of HDL (d 1.083–1.19), HDL_2 (d 1.083–1.124), and HDL_3 (d 1.124–1.19) on column chromatography. The ApoA-II contents of these HDL fractions were also 20–25% by radioimmunoassay. Similar results were obtained whether assays were carried out on intact or delipidated HDL samples. Thus, in contrast with ApoA-I (only 10% of which is detectable), all of the ApoA-II contents of intact HDL are detected with accuracy by this assay. Plasma levels of ApoA-II in young normolipemic subjects were approximately 40 mg/dl (n = 29). In these subjects, over 98% of ApoA-II was found in the d 1.063–1.21 density fractions.

Supplementary key words high density lipoprotein structure · density distribution of apolipoprotein A-II

High density lipoprotein (HDL) consists of approximately 50% protein (1, 2). About 60% of this protein is ApoA-I (3, 4) which, in addition to being important to the structure of HDL, is also an activator of lecithin–cholesterol acyl transferase (5). The amino acid sequence of ApoA-I is known (6). Ten percent of HDL protein is comprised of the ApoC proteins that modulate the activity of lipoprotein lipase (7) and are also present in VLDL (8) and chylomicrons (9). Perhaps 5% or less is contributed by ApoD, the function of which is not known (10). The remainder, approximately 20%, is ApoA-II. The amino acid sequence of this protein is also known (11). ApoA-II has no known activity in the modulation of enzymes; it appears to be primarily a structural protein that avidly binds lipids (12–15).

The development of assays for the apoproteins ApoA-I (16–20) and ApoB (21–24) has added important information to both the structure and the metabolism of lipoproteins. For example, it has been demonstrated that most of the ApoA-I in intact HDL is immunologically not reactive, suggesting that many of its antigenic determinants may be "masked" by lipid–protein or protein–protein interactions (16, 17, 25). We have also suggested that the NH_2-terminal region may be more "masked" than the COOH-terminal region (26).

Because of the probability that measurement of ApoA-II could further increase knowledge of HDL structure and metabolism, we undertook the development of a double antibody radioimmunoassay for human ApoA-II. Here we report on its application to structural studies of HDL. The data indicate that, in contrast to ApoA-I, all of the ApoA-II in HDL is immunologically reactive. A radioimmunoassay for ApoA-II based on a different method of separation of bound from free label has recently been reported by Mao, Gotto, and Jackson (27) and an electroimmunoassay was reported by Curry, Alaupovic, and Suerman (20). The present assay is suitable for the measurement of ApoA-II levels in human plasma.

Abbreviations: VLDL, LDL, and HDL are very low, low, and high density lipoproteins, respectively; Apo, apolipoprotein; RIA, radioimmunoassay; NIRS, non-immune rabbit serum, BSA, bovine serum albumin; ^{125}I-ApoA-II, ^{125}I-labeled ApoA-II; PC, phosphatidylcholine.

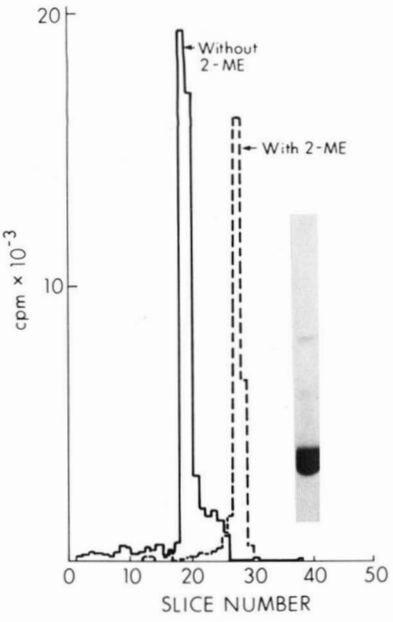

Fig. 1. Appearance of ApoA-II on SDS–polyacrylamide disc gel electrophoresis, following reduction with 2-mercaptoethanol. Migration is downward, and the gel was stained with Coomassie blue. Photometric scan performed at 550 nm is not shown. The major band is ApoA-II monomer (mol wt 8700). The peak at the top third of the gel corresponds to ApoA-II dimer (mol wt 17,400). The ill-defined band in between represents <<1% of the area scanned and is unidentified. Electrophoretic migration of ^{125}I-ApoA-II in SDS–polyacrylamide gels is also shown. ^{125}I-ApoA-II was mixed with unlabeled ApoA-II; one aliquot of the mixture was reduced with 2-mercaptoethanol (2-ME) and the other was not. Both aliquots were subjected to electrophoresis. Gels were stained, sliced into 2-mm segments and counted in a Packard Autogamma spectrometer. Radioactive peaks corresponded with stained areas.

METHODS

Isolation of ApoA-II

ApoA-II was isolated from plasma along with ApoA-I as previously described (3, 16). Briefly, HDL was isolated from plasma of normal volunteers by two ultracentrifugations for 3×10^8 g-min at 10°C at d 1.083 in a Beckman L265 ultracentrifuge using a 60 Ti rotor. The infranatant was recentrifuged twice at 1.195 and the supernatant was saved. The narrower density ranges were chosen (instead of 1.063–1.21) to minimize contamination either by lipoproteins, or by other plasma proteins. The resulting HDL did not react with antisera against human LDL or albumin in a double immunodiffusion system. HLD was dialyzed against 0.16 M NaCl–1 mM EDTA pH 8.0 (EDTA–saline); it was then lyophilized and delipidated with ether–ethanol 2:3 (v/v) (28). The lipid-free protein was solubilized in 0.01 M Tris–6 M urea, pH 8.6 (Tris–urea), and filtered on a 2.5 × 90 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and equilibrated with Tris–urea. ApoA-I eluted in peak II and ApoA-II in peak III (3, 16). Peak III was refiltered on the same column. The protein was dialyzed against EDTA–saline and lyophilized. ApoA-II preparations obtained as described were used as assay standards, as ^{125}I-labeled tracers, and as antigens for the production of antisera.

The identity of ApoA-II was confirmed by its behavior on polyacrylamide disc gel electrophoresis in 1% SDS (9) (**Fig. 1**) and by its amino acid composition, which was nearly identical with that reported by others (**Table 1**). The purity of the ApoA-II was greater than 99% as judged by polyacrylamide disc gel electrophoresis in SDS. The apparent ApoA-I (16), ApoB (19), and ApoC-II (30) contents of ApoA-II preparations measured by the appropriate radioimmunoassays did not exceed 0.5% for each component. Finally, most preparations of ApoA-II stimulated the production of monospecific antisera.

Protein concentrations were determined by the method of Lowry et al. (31) using crystalline bovine albumin standards (Sigma Chemical, Inc., St. Louis, MO). The mass of ApoA-II determined by amino acid analysis was 0.97–1.01 times the protein value (n = 4). (We are grateful to Dr. Ralph Bradshaw, Department of Biological Chemistry for the amino acid analyses.)

Iodination of ApoA-II

ApoA-II was iodinated in a small conical tube by the lactoperoxidase method (32) as modified for ApoA-I (26) using the following reagents: 0.5 mCi

TABLE 1. Amino acid composition of ApoA-II

	Present Report[a]	Jackson, et al. (42)
Lysine	8.6	8.3
Histidine		
Arginine		
Aspartate	3.6	3.1
Threonine	5.1	5.5
Serine	4.3	5.5
Glutamate	17.0	16.2
Proline	4.1	4.0
Glycine	3.7	3.0
Alanine	5.9	4.3
Half Cystine		0.2
Valine	6.0	5.6
Methionine	0.6	0.7
Isoleucine	1.1	0.8
Leucine	8.6	8.2
Tyrosine	3.1	3.8
Phenylalanine	4.1	3.7

[a] Mean of three determinations, results are given as mol amino acid per mol of protein assuming monomer molecular weight of 8690 (42).

carrier-free $Na^{125}I$ (Amersham-Searle, Chicago, IL) in 5–10 μl, 25 μl of 0.05 M Na_2HPO_4 (pH 7.6), 25 μl of ApoA-II (1 mg/ml), and 15 μl of a solution of lactoperoxidase (0.15 mg/ml, Calbiochem, Elk Grove, IL). The reaction was started by the addition of 2 μl of 0.44 M H_2O_2. At the end of 2 min, 500 μl of cold phosphate buffer was added and the contents of the reaction tube were immediately loaded onto a 0.9 × 30 cm column of Sephadex G-50 equilibrated with 0.05 M barbital buffer, pH 8.6. The labeled protein peak eluting between 11 and 15 ml (free iodine eluted at 18–25 ml) was made 5% with respect to bovine serum albumin (BSA, fraction V, Sigma Chemical Co.) with a 15% BSA barbital solution, and frozen in small aliquots. ^{125}I-ApoA-II was thawed and purified just before use by two filtrations on a 1.5 × 30 cm column of Sephadex G-75 equilibrated with 0.05 M barbital pH 8.6–0.01% Triton X-100 (Rohm and Haas, Philadelphia, PA). In some cases the Sephadex G-75 column was equilibrated with barbital–3% BSA (see below). Specific radioactivities of ^{125}I-ApoA-II were 5–12 μCi/μg. Iodinations were carried out every 4–6 weeks.

Antisera

Antisera were prepared in female New Zealand white rabbits weighing 2–3 kg. Solutions of ApoA-II, 0.5–1 mg/ml in EDTA–saline, were emulsified with equal volumes of complete Freund's adjuvant. Three injections of 0.5 mg each 2–3 weeks apart were given and the rabbits were bled 7–10 days after the last injection. Rabbits were also given "booster" injections several weeks after each blood drawing and bled 7–10 days later. Thus, more than one antiserum was obtained from each rabbit. NaN_3 was added (0.01% final concentration) and the antisera were stored frozen in aliquots. Non-immune rabbit serum (NIRS) was obtained from similar rabbits that had not been immunized. Whole antisera were used in routine assays, but for some assays IgG was isolated from antisera by ammonium sulfate precipitation (33). Anti-rabbit IgG was made in goats.

The assay

Routine assays were set up in 10 × 75 mm disposable tubes using barbital–0.01% Triton X-100. For some assays barbital–3% BSA was used; under those conditions tubes were coated with silicone (Siliclad, Persippany, NJ). The tubes contained the following: (*a*) 10–250 μl of sample or ApoA-II standard (0.05 μg/ml), each diluted in the appropriate buffer; (*b*) 100 μl of antiserum diluted appropriately (usually 1:1800, final dilution 1:9,900); (*c*) 500 μl of NIRS (diluted 1:2000); (*d*) 100 μl of ^{125}I-ApoA-II (15,000 cpm/tube, approximately 0.5–1 ng/tube); and (*e*) sufficient buffer to bring the volume to 500 μl. Tubes

TABLE 2. Apparent ApoA-I and ApoA-II contents of HDL fractions measured by radioimmunoassay

	Column Chromatography		RIA			
			Intact		Delipidated	
HDL Preparation	ApoA-I	ApoA-II	ApoA-I	ApoA-II	ApoA-I	ApoA-II
HDL #22	59.4	20.3	6.6	20.8 (24.0)	60.6	20.5 (26.8)
HDL_2 #22	58.7	20.2	3.1	21.9 (24.1)	55.6	23.0 (25.1)
HDL_3 #22	62.3	28.0	7.2	24.4 (27.2)	57.8	20.1 (25.3)
HDL_3 #21	64.9	25.6	7.5	24.8	64.3	22.2

The indicated HDL or HDL subfractions were lyophilyzed, delipidated, solubilized, and subjected to column chromatography on 2.5 × 90 cm columns of Sephadex G-200 (0.01 1 M Tris, 8 M urea, pH 8.6). Fractions from each peak were pooled, and protein contents were determined by the method of Lowry et al. (30), and by RIA for ApoA-I and ApoA-II. 96% of ApoA-I and 92% of ApoA-II eluted in peaks II and III, respectively. 3–5% of ApoA-I and ApoA-II were found in peak I. 2–4% of the protein of peak II was ApoA-II. Similar proportions of ApoA-I were present in peak III. Peak IV had no ApoA. The calculations of the ApoA-I and ApoA-II contents of HDL have been corrected to reflect the distributions of ApoA-I and ApoA-II. The ApoA-II contents of HDL fractions were determined by RIA in barbital–Triton, or in barbital–3% BSA (shown in parentheses), on intact or delipidated samples. ApoA-I in HDL was determined only in barbital–3% BSA. Each result is the mean of 2–3 determinations. Results are given as percent of total protein (e.g., ApoA-I × 100 ÷ total protein).

were incubated at 4°C for 42 hr. 50 μl of goat anti-rabbit IgG antiserum was then added (diluted 1:50) and incubation was continued for another 16 hr. Tubes were centrifuged at 2500 g for 45 min, supernatants were discarded, and the pellets were "washed" in buffer and recentrifuged. Tubes containing pellets were counted in a Packard Autogamma spectrometer. Tubes containing no anti-ApoA-II antisera were included with each assay to quantify non-specific precipitation. These counts were subtracted from the gross counts of the assay tubes to obtain net counts. Points on the standard curve were determined in triplicate, and samples were analyzed at two doses in duplicate. Assays were calculated with a Hewlett-Packard programmable tabletop calculator using a program provided by the vendor, which uses the logit transformation of B/B_0 and weights each point on the standard curve in calculating the slopes and intercepts of the standard curve (34). B_0 = net counts in tube in the absence of sample or ApoA-II standard; B = net counts in the presence of sample or ApoA-II standard. Logit $B/B_0 = \ln [B/B_0 \div (1 - B/B_0)]$. In plotting of standard curves, y = logit B/B_0, x = log dose protein in nanograms.

Electroimmunoassay

Plasma ApoA-II concentrations were also measured by the "rockets" technique (35, 36). Glass plates, 85 × 105 mm, were covered with 12 ml of 0.5% agarose (Seakem, Marine Colloids, Inc., Rockland, ME), dissolved in 0.1 M barbital buffer, pH 8.6, and 2.5 mM Ca lactate. The agarose contained 0.15 ml of anti-ApoA-II antiserum (R 132-2, 1:80 dil). Samples of 5-μl were applied. Plasmas were diluted 1:15 to 1:120. Concentration of HDL (HDL #22, **Table 2**) or ApoA-II standards ranged from 12.5 to 120 μg/ml. Electrophoreses were carried out in an LKB 2117 Multiphor Chamber, at 220 V, 60 m amps (or 2V/cm) for 16 hr at 15°C. At the end of the runs, plates were soaked for several hours in 0.16 M NaCl, followed by 1–2 hr in H_2O; they were then pressed, dried, and stained with amido black. Rocket heights were plotted against dose of ApoA-II protein. (Similar results were obtained when areas of rockets were used.) To obtain ApoA-II dose when HDL #22 was used as standard, HDL-protein was multiplied by 0.203. The amount of ApoA-II in HDL was 20.3% as determined by column chromatography (see HDL #22, Table 2). Contents of plasma were obtained from these standard curves.

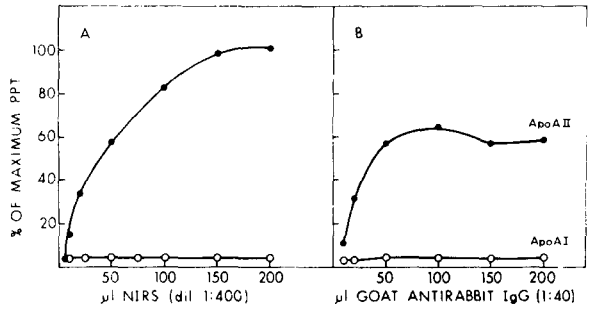

Fig. 2. Effect of increasing amounts of NIRS (non-immune rabbit serum) and anti-rabbit IgG on non-specific precipitation of ApoA-II and ApoA-I. In panel *A*, all tubes contained 50 μl of anti-rabbit IgG (dil 1:40); increasing amounts of NIRS were added as shown. In panel *B*, all tubes contained 50 μl of NIRS (dil 1:400); anti IgG was varied as shown. Maximum precipitability of label was 50% of that added (i.e., ~7500 cpm). The marked coprecipitation of ^{125}I-ApoA-II with the non-specific rabbit IgG–anti IgG complexes is evident. ^{125}I-ApoA-I by contrast was precipitated only minimally (<2% of added counts) and independently of the extent of non-specific complex formation.

RESULTS

^{125}I-ApoA-II

When thawed ^{125}I-ApoA-II was filtered on Sephadex G-75 columns equilibrated with barbital–BSA, two peaks were obtained. The first, containing ^{125}I-ApoA-II, eluted near the position of chymotrypsinogen (mol wt 25,000). This peak was 90–95% precipitable by 10% TCA. The second peak eluted with potassium chromate and only 5% was precipitable by TCA. Upon refiltration on the same column, ^{125}I-ApoA-II eluted in the same position. When a Sephadex G-75 column equilibrated with barbital–0.01% Triton X-100 was used, ^{125}I-ApoA-II again eluted near chymotrypsinogen. However, on refiltration, the label eluted near the void volume. This material too was 90% precipitable by 10% TCA.

The purity of ApoA-II label was tested by polyacrylamide disc gel electrophoresis in 1% SDS (Fig. 1). The ^{125}I-labeled protein was mixed with 50 μg of "cold" ApoA-II and split into two aliquots. In one aliquot the protein was reduced by incubating it for 1 hr at 37°C with 2-mercaptoethanol before electrophoresis; in the other aliquot, 2-mercaptoethanol was omitted. At the end of electrophoresis the gels were stained with Coomassie blue, sliced into 2-mm segments, and counted in the gamma counter. Single radioactive peaks were produced in both gels. The reduced protein moved faster than the nonreduced protein. Peaks of radioactivity corresponded with maximum staining. Molecular weight standards were included in the run and apparent molecular weights of the labeled components were computed (29). These were 8,500 and 17,000 for reduced and nonreduced labeled protein, respectively.

The assay

The assay was initially set up in siliconized tubes in barbital–3% BSA using 50 μl of NIRS diluted 1:200, and 50 μl of goat anti-rabbit IgG diluted 1:20 (16, 19). In this system, about 50% of ^{125}I-ApoA-II precipitated in the absence of anti-ApoA-II antisera; about 70% precipitated in the presence of anti-ApoA-II. When only label and the barbital–3% BSA buffer were added to the tubes, 3% of the added counts remained in the tubes after routine processing. Thus, the label was not sticking to the glass. To test whether the label was binding to and precipitating other proteins in the rabbit or goat sera, increasing amounts of either NIRS or goat anti-rabbit IgG antiserum was added to the tubes. When the sera were thus added singly, only 3% of the added counts remained after processing. Finally, NIRS and anti-IgG were added to the tubes together (**Fig. 2a** and **2b**). In the first case, anti-IgG was held constant and increasing amounts of NIRS were added. In the second case, the order was reversed. Clearly the amount of non-specific precipitation depended on the presence of both NIRS and anti-IgG, (i.e., on the formation of rabbit IgG–goat-anti-IgG complexes). However, if the amounts of both were held sufficiently low, non-specific precipitation of ApoA-II could be held to less than 3% of the added radioactivity. The contrasting behavior of ^{125}I-ApoA-I is clearly seen (Figs. 2a and 2b). With this label, non-specific precipitation was low and independent of the same amount of either NIRS or of rabbit anti-IgG in the system.

Having demonstrated that precipitation in the absence of anti-ApoA-I could be kept low, the proportions of NIRS and anti-IgG were adjusted to keep this precipitation at a minimum, and at the same time precipitate all of the ^{125}I-ApoA-II-anti-ApoA-II complexes in the assay. To determine the desired proportions, the amounts of NIRS and anti-IgG were varied simultaneously over a wide range (NIRS 1:200–1:8000, anti IgG 1:40–1:160). The IgG fraction of anti-ApoA-II was added to one set of tubes and omitted from the other set. (The IgG fraction was used in hopes of increasing "specific" precipitation.) With 50 μl of the goat anti-IgG antiserum (diluted 1:50) and 100 μl of NIRS (diluted 1:2000) between 3 and 10% of added radioactivity precipitated. The extent of precipitation was nearly independent of the amounts of "cold" ApoA-II present. (For example, in one experiment, 83% of ApoA-II was precipitated in the complete system. The tubes from which anti ApoA-II antiserum and "cold" ApoA-II were omitted contained 3% of the added counts. When 1–10 ng of ApoA-II were included in these "background" tubes, 2% of the added ApoA-II counts were still precipitated.) Up to 93% of ^{125}I-ApoA-II precipitated in the presence of anti-ApoA-II IgG, indicating that sufficient anti-IgG was present to separate all of the "bound" from the "free" ^{125}I-ApoA-II. It was later found that anti-ApoA-II antisera and anti-ApoA-II IgG gave similar degrees of precipitation.

In order to reduce precipitation in the absence of anti-ApoA-II even further, assays using 50 μl of anti-IgG (dil 1:50) and 100 μl of NIRS (dil 1:2000) were set up in several buffers: 0.5% BSA in 0.5 M borate, pH 8.6; 0.5% BSA in phosphosaline, pH 7.4; barbital, pH 8.6, alone; barbital with 0.5%, 1.0%, and 1.5% BSA; and barbital–0.01% Triton X-100. Only the last was better than the original system. Precipitation was consistently reduced to <3% without any changes in precipitation in the presence of anti-ApoA-II IgG or antisera. Thus, assays could be carried out in either barbital–3% BSA or barbital–Triton X-100, albeit the latter appeared preferable because of its consistency and lower cost.

The addition of increasing amounts of unlabeled ApoA-II to the complete system resulted in the production of typical radioactivity displacement curves (**Fig. 3**). More than 90% of added ^{125}I-ApoA-II was precipitated by most of these antisera. Ninety-two to ninety-four percent of the label was precipitated by 10% TCA. More than 95% of the ^{125}I-ApoA-II was displaced by unlabeled ApoA-II. Several monospecific antisera were produced that contained sufficient antibodies to make them useful in the assay. All of the antisera yielded useful displacement curves (Fig. 3). Antisera R 133-2 was chosen as the antiserum for routine use. In contrast with ApoA-II, either undetectable or negligible amounts (<1%) of displacement of label were produced by ApoA-I, ApoC, and isolated VLDL and LDL.

ApoA-II contents of natural and reconstituted HDL fractions

HDL (d 1.083–1.21), HDL_2 (d 1.083–1.124), and HDL_3 (d 1.124–1.195) each produced displacement curves that paralleled those produced by ApoA-II standards. Slopes of displacement curves on HDL fractions and ApoA-II standards were -3.169 ± 0.329 (mean ± ISD, n = 9) and -3.047 ± 0.332 (n = 11), respectively. Intact and delipidated plasmas also produced parallel curves (slope -3.091 ± 0.313, n = 5). The apparent ApoA-II contents of these HDL fractions ranged between 20.1 and 24.8 (Table 2). These values agreed well with the contents for

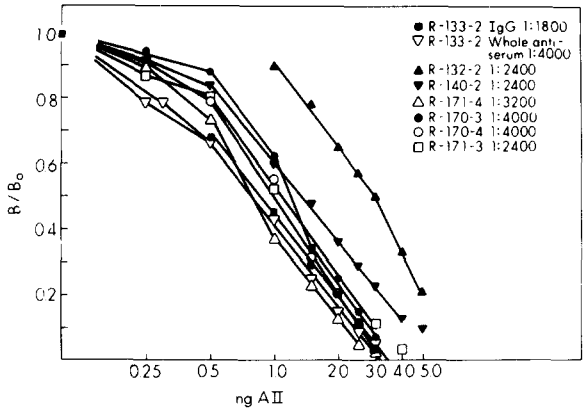

Fig. 3. ApoA-II radioimmunoassay. Displacement curves produced with several different anti-ApoA-II antisera.

ApoA-II obtained by column chromatography. (In addition to the HDL preparations shown, the ApoA-II contents of 16 other preparations of "normal" HDL have been assessed over the past three years by column chromatography. ApoA-II contents averaged 21% ± 4, mean ± 1 SD.) HDL samples, run either delipidated or intact in barbital–Triton buffer, yielded similar values. Assays run in barbital–3% BSA yielded slightly higher results (Table 2). Thus, virtually all of the ApoA-II in HDL, HDL_2, and HDL_3 was immunologically reactive in the assays run in either buffer. This is in contrast with ApoA-I where the apparent ApoA-I content of HDL_2 assayed in barbital–BSA or barbital–Triton was 5–10% instead of the expected 65% (16, 23).

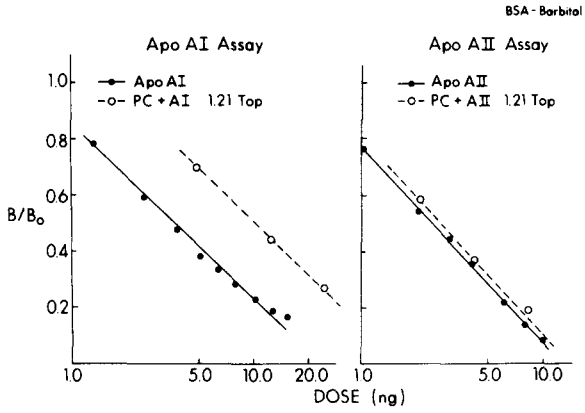

Fig. 4. Displacement curves produced by phosphatidylcholine–apoprotein complexes (PC, isolated from egg yolk). PC vesicles were prepared by sonication of 2 ml of PC (0.5 mg/ml) in 0.01 M Tris pH 7.4 containing 20 mM NaCl, 0.01% EDTA, and 0.02% NaN_3. Equal volumes of ApoA-I or ApoA-II (0.5 mg/ml) were added and sonication was continued for another 2 min (see ref. 23). Complexes were isolated by ultracentrifugation at d 1.063–1.25. PC/apoprotein ratios (mol/mol) of the ApoA-I–PC + ApoA-II–PC complexes were 25 and 16, respectively. Lipid-free ApoA-I and ApoA-II were not sonicated. Immunoreactivity of the ApoA-I–PC complex was only ~30% of the lipid-free ApoA-I. Immunoreactivity of the ApoA-II–PC complex did not differ from that of lipid-free ApoA-II. This assay was repeated in barbital–Triton buffer with similar results.

We have demonstrated previously that the immunologic reactivity of ApoA-I could be greatly diminished by cosonicating the protein with lipids (25). For this report, ApoA-I and ApoA-II were each separately cosonicated with phosphatidylcholine (PC) under identical conditions (**Fig. 4**). The resulting lipid–protein complexes (ApoA-I-PC and ApoA-II-PC) were each isolated by ultracentrifugation (d 1.063–1.24) and assayed in the ApoA-I and ApoA-II assays, respectively. Lipid-free apoproteins were not sonicated or subjected to ultracentrifugation. The immunoreactivity of the ApoA-I-PC complex was about 30% of that of lipid-free ApoA-I, whereas the reactivity of the ApoA-II-PC could not be distinguished from that of lipid-free ApoA-II (Fig. 4).

ApoA-II contents of normal plasma

To ascertain that all of the ApoA-II in plasma was detectable, recovery experiments were done. Known amounts of ApoA-II were added to plasma, and mixtures of ApoA-II and plasma were assayed before and after delipidation (**Table 3**). Delipidation of isolated ApoA-II did not result in any appreciable losses of the protein, or in any recognizable immunologic alterations. Ninety-eight percent of the expected protein was detected. When ApoA-II levels of plasma were determined for this experiment, levels were slightly higher in delipidated than in intact plasma. ApoA-II levels were nearly identical for other intact vs. delipidated plasmas (see below). In plasma + ApoA-II mixtures, assay values were 102% and 93% of expected values for the intact and delipidated mixtures, respectively.

To assess whether the hyperlipoproteinemic state affected the measurement of ApoA-II in plasma, 39 plasma samples were assayed before and after delipidation. Included were plasmas from 14 normolipemic subjects, 9 with Type II, 5 with Type III, and 7 with Type IV hyperlipoproteinemia. Overall mean values were 31.8 ± 7.4 and 32.3 ± 7.6 mg/dl of ApoA-II for the intact and delipidated plasmas, respectively. The correlation coefficient for intact vs. delipidated pairs was 0.90 and the regression formula was $y = 3.1 \pm 0.92\ x$ (x, intact; y, delipidated). Storage at 4°C for 2–3 months or freezing and thawing of samples did not affect the results systematically. The interassay coefficient of variation was assessed by use of six overlap samples, three of which were replaced every month, and two frozen samples of small aliquots that were freshly thawed before each assay. ApoA-II values of samples ranged

TABLE 3. Detectability of ApoA-II in plasma by radioimmunoassay

Sample Assayed	Added	Found	Recovery	Added	Found	Recovery
		ng	%		*ng*	%
ApoA-II	1.2 ng	1.2	100	1.2 ng	1.2	100
	2.4 ng	2.4	100	2.4 ng	2.5	104
	3.6 ng	3.6	100	3.6 ng	3.3	92
Plasma (dil 1:20,000)	80 μl	0.76	Not applicable	80 μl	0.90	Not applicable
	100 μl	1.00		100 μl	1.16	
	120 μl	1.15		120 μl	1.34	
	160 μl	1.47		160 μl	1.79	
	200 μl	1.81		200 μl	2.24	
Plasma + ApoA-II	1.00 +1.20			1.16 1.20		
	2.20 ng	2.49	113	2.36 ng	2.19	93
	1.00 +2.40			1.16 2.40		
	3.40 ng	3.19	94	3.56 ng	3.26	92
	1.00 +3.60			1.16 +3.60		
	4.60 ng	4.60	100	4.76 ng	4.43	93

To ascertain whether all of the ApoA-II added to plasma was detectable, ApoA-II standard and delipidated ApoA-II standard were added to intact and delipidated plasmas in the amounts indicated. Individual components and mixtures were assayed in barbital–Triton. This experiment was repeated on two separate occasions with similar results.

from 28 to 50 mg/dl, and the coefficient of variation averaged 11% ± 3 (mean ± 1 SD). ApoA-II values of 9 normolipemic women and 20 normolipemic men were 41.8 ± 10.7 and 39.2 ± 7.0, respectively (**Table 4**). Four plasmas were assayed using four different anti-ApoA-II antisera (**Table 5**). Similar values were obtained with three antisera; the fourth antiserum gave higher values. The ApoA-II contents of three plasma samples taken from fasting patients with Tangier disease (courtesy Dr. R. I. Levy, National Heart, Lung, and Blood Institute) averaged <10% of normal.

TABLE 4. ApoA-II contents of normal whole plasmas and plasma subfractions

Normolipemic Subjects	Total Plasma	Plasma Fractions (n = 9)	ApoA-II Contents
			mg/dl
Women (9)	39.2 ± 7	d < 1.006	0.04 ± 0.01
		d < 1.063	0.08 ± 0.02
Men (20)	41.8 ± 10.7	d < 1.25	33.8 ± 8.3
		d > 1.25	0.12 ± 0.03
		Whole	34.2 ± 7.7

ApoA-II contents of total intact plasma were determined by RIA. Plasmas were collected from normolipemic fasting subjects aged 21–57 years, after 14-hr fasts. Cholesterol and TG values were <240 and <140 mg/dl, respectively. Separate aliquots of nine plasmas were subjected to one ultracentrifugation each at the indicated densities and the ApoA-II levels of supernate and infranates were measured. More than 98% of the ApoA-II was found in the d 1.063–1.25 fractions.

Comparison of RIA and rockets assay

The accuracy of the RIA was tested further by comparing it to the rockets method (**Fig. 5**). First we needed to know whether the rockets technique could detect the ApoA-II contents of HDL accurately. When isolated ApoA-II was used as the standard in the rockets assay, the apparent ApoA-II contents of intact HDL ranged from 77 to 93%, clearly an overestimate. This suggested that isolated ApoA-II and HDL migrated at different rates and that ApoA-II could not be used as the standard for assessing the ApoA-II contents of plasma under the conditions employed. When intact HDL #22 (which contained

TABLE 5. ApoA-II levels of human plasma using different anti-ApoA-II antisera

Plasma	R 133-2	R 140-2	R 170-4	R 171-3
1	44, 48	60	46	43
2	52, 52	74	49	49
3	31, 35	43	31	31
4	33, 34	50	31	34

Each plasma sample was assayed by the radioimmunoassay for ApoA-II in barbital–Triton buffer, using four different antisera. Results are given in mg/dl. Plasmas were assayed on two separate occasions with R 133-2.

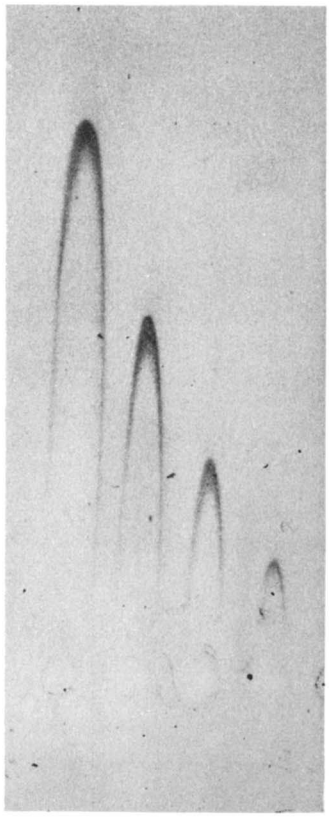

Fig. 5. Determination of ApoA-II contents of plasma by electroimmunodiffusion. 85 × 105 mm glass plates were covered with 12 ml of 0.5% agarose containing 0.15 ml of anti ApoA-II antiserum R 132-2 in 0.1 M barbital pH 8.6, 3.5 mM Ca lactate. A 5-μl portion of each sample was applied. Stain is Amido black. Rockets produced by HDL #22 are shown (left to right; 120, 60, 30, and 15 μg HDL protein/ml).

20.3% of ApoA-II, Table 2) was used as the standard with intact plasmas, the results of the two assays were close (**Table 6**). Similar results were obtained using delipidated HDL and delipidated plasmas, although these rockets were less satisfactory because they were shorter and tended to be rounded rather than peaked at their leading edges. Delipidated HDL could not be used with intact plasma or vice versa, because delipidated samples and HDL standards migrated at much reduced rates. The reduced electrophoretic migration of delipidated HDL has been noted before (16, 36).

DISCUSSION

ApoA-II is important in the structure of HDL since it seems to bind phospholipids much more avidly than ApoA-I alone. Indeed, when ApoA-II is added to ApoA-I, the binding of lipid by both is enhanced (14, 15). Thus, the two apoproteins seem to work synergistically in maintaining the structure of HDL. In view of this relationship it appeared important to develop a means of quantifying ApoA-II, because the addition of an ApoA-II assay to the already existing ApoA-I assay would magnify the value of both measurements for structural and metabolic studies.

The present assay appears to be as specific and precise for ApoA-II as are the previously described assays for ApoA-I (16–19). The specificity of most radioimmunoassays depends on the purity of the label. The ^{125}I-ApoA-II met several criteria of purity, including the ability to stimulate the production of monospecific antisera. ApoA-II in aqueous buffer appeared to be present either as the monomer, mol wt 17,400, or as the dimer. The relative proportions of each depend upon the concentration of ApoA-II in solution (38). ^{125}I-ApoA-II eluted from columns with barbital–BSA seemed to be primarily in monomer form. Labeled ApoA-II eluted with barbital–Triton ought be have been primarily in monomer form at the concentrations present in the filtrate. Yet, on the second pass, it eluted at V_0. This could have been due to self association but it is more likely that ^{125}I-ApoA-II may have associated with micelles of Triton X-100. In spite of the differences in the apparent sizes of ^{125}I-ApoA-II filtered in barbital–BSA and barbital–Triton, the results of the assay run with the two buffers were quite comparable. The accuracy of the measurements of ApoA-II in HDL appeared to be adequate, as evaluated by comparing the results obtained by column chromatography with those obtained by RIA (Table 2). Assays run in barbital–Triton were preferable to those run in barbital–BSA because, in barbital–Triton, (*a*) precision was better, (*b*) non-specific precipitation was consistently lower, and (*c*) the assays were cheaper to run.

It is interesting that all of the ApoA-II in HDL fractions is detectable in this assay and that removal from or addition of lipid to ApoA-II did not affect

TABLE 6. Detection of ApoA-II in plasma by radioimmunoassay and electroimmunoassay/rockets

	RIA		Rockets	
Plasma	Intact	Delipidated	Intact	Delipidated
	mg/dl			
M T	33	32	37	39
J L	31	33	34	34
B S	34	36	37	39
S W	35	34	35	36

The above plasmas taken from normal subjects were assayed intact and following delipidation by RIA and by the rockets using antiserum R 133-2 assays. RIA was carried out as described under methods. For the rockets, intact and delipidated HDL #22 were used as standards with the respective samples. The ApoA-II content of HDL #22 was taken as the Lowry protein × 0.203 (see Table 4).

its immunoreactivity. This is in marked contrast to the behavior of ApoA-I, most of which is not detectable in HDL (16, 17, 25). Similar findings for ApoA-II have been published by Mao, Gotto, and Jackson (27).

The complete reactivity of ApoA-II may be interpreted in several ways. One possibility is that all of the immunogenic sites of ApoA-II are exposed on the surface of HDL, whereas most of the immunogenic sites of ApoA-I are not. Another possibility, perhaps related to the first, may be that the conformations of the immunologically reactive sites on lipid-free ApoA-II are very similar to the conformations of the sites on lipid-associated ApoA-II. By contrast, conformations of the reactive sites on lipid-free and lipid-associated ApoA-I may differ from each other markedly. Differences in the conformation of proteins have been detected by immunologic means in other systems (39, 40). Indeed, there is immunologic evidence for changes in the conformation of ApoA-I. Antisera produced against HDL (lipid-associated ApoA-I) differ appreciably from antisera produced against lipid-free ApoA-I in their specificities for the NH_2- and COOH-terminal regions of ApoA-I[1]. These observed differences in the behavior of ApoA-I and ApoA-II may not be detectable by some of the physical spectroscopic methods used at the present time.

The suitability of the assay for measuring the ApoA-II in plasma is attested to by its specificity (e.g., Tangier plasmas contain very little ApoA-II) and acceptable precision. Accuracy is a difficult question, although two facts are helpful. More than 98% of ApoA-II, and 90% of ApoA-I are found in the d 1.063–1.25 fraction, (this fraction corresponds fairly closely to HDL). Thus, if one can determine the ApoA-II contents of HDL accurately, one has determined 98% of the plasma content of ApoA-II accurately. (Interference by "plasma factors" is unlikely to affect the assay since the plasma is diluted several thousand-fold before assay.) Most workers find the weight ratio of ApoA-I:ApoA-II in HDL to be between 3:1 and 4:1 (2–4, 40, 41). Given the density distribution of ApoA-I and ApoA-II, this ought to be close to the weight ratio of plasma. If the normal range of ApoA-I is 100–125 mg/dl (16–20), the ApoA-II values of 35–40 mg/dl obtained by the present assay yield ApoA-I:ApoA-II weight ratios that approach 3:1 for whole plasma. Thus, the results are acceptable on theoretical grounds. Added credence is lent to the results by the good agreement between results on delipidated and intact samples, by the recovery experiments (Table 3), and by the agreement between the rockets assay (20) and the RIA (Table 6). In our hands the RIA is preferable to the rockets assay because, in the latter, isolated ApoA-II is inadequate as a standard, whereas ApoA-II is a good standard for the RIA. In addition, in the rockets assay, delipidation alters the conditions of the assay. In the RIA, delipidation makes no difference. Thus, isolated peptides and lipid-bearing fractions can be assayed simultaneously.

The reasons for the higher values obtained by antiserum R140-2 (Table 5) are not clear; however, one antiserum that gave unusual values for ApoA-I was also found in the RIA for ApoA-I (16). The specificities and affinities of these antisera may be unusual.

Curry, Alaupovic, and Suernam (20), using a rockets assay, have reported plasma ApoA-II levels twice as large as ours and ApoA-I:ApoA-II ratios of 2:1. They found that isolated ApoA-II and delipidated and intact plasma samples migrate satisfactorily in their rockets assay. The reasons for the difference between the two assays are not clear. With respect to electrophoretic migration, Levy and Fredrickson (37) and we (16) noted that ultracentrifugation, freezing–thawing, and delipidation altered the migration of HDL in agarose electrophoresis. Perhaps the addition of Dextran T10 to the agarose by Curry et al. (20) abolished the differences between intact and manipulated HDL. With respect to the differences in plasma values, it may be that their antisera and ours differ. For example, if their antisera resemble our R 140-2, the differences in absolute values would be less. However, for the reasons stated, we believe the results obtained with the other antisera to be more accurate.

It is expected that ApoA-I and ApoA-II assays, carried out on the same samples simultaneously, will add considerably to the knowledge of HDL metabolism.

ADDENDUM

Since the submission of this manuscript, a paper by Assmann, G., E. Smootz, K. Adler, A. Capurso, and K. Oette entitled "Lipoprotein Abnormality in Tangier Disease—Quantitation of A Apoproteins" has appeared in the *J. Clin. Invest.* **59**(3): 565–575, 1977. A double antibody radioimmunoassay for ApoA-II is reported; plasma levels averaged 35 and 41 mg/dl for normal men and women, respectively.

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