Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer

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Abstract A quantitative solid phase immunoassay has been developed for the determination of the mass of electrophoretically separated prebeta apolipoprotein A-I (apoA-I) in human plasma. Conditions have been identified for the quantitative transfer and immunoblotting of the apolipoprotein in the absence of organic solvents or detergents. In normolipidemic plasma, the prebeta-migrating fraction of apoA-I represented $4.2 \pm 1.8\%$ of total apoA-I (61 ± 26 μg of apoA-I per ml of plasma). Significantly higher levels were found in hypercholesterolemia of genetic origin, in primary and secondary hypertriglyceridemia, and in congenital lecithin:cholesterol acyltransferase deficiency. In all cases prebeta-migrating apoA-I consisted in large part of low molecular weight lipoprotein species, compared to the size of the major, alpha-migrating apoA-I fraction.—**Ishida, B. Y., J. Frolich, and C. J. Fielding.** Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer. *J. Lipid Res.* 1987. **28:** 778–786.

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The observation that high density lipoprotein (HDL) levels are negatively correlated with coronary artery disease has suggested a role for HDL in tissue metabolism, in which these lipoproteins mediate a reduction in tissue cholesterol by an ability to transport such cholesterol from cell membranes into the plasma compartment (1,2). A particular subclass of HDL may be responsible for a major part of such cholesterol transport; it has been shown that when plasma was depleted of different lipoprotein species by immunoaffinity chromatography, HDL containing only apoA-I were identified as a carrier which can promote transfer of cholesterol from cell membranes to the plasma compartment (3). Such HDL are more effective in promoting cholesterol efflux from cell membranes than other HDL species, such as those containing apoE (3, 4). Experiments of this kind have recently led to renewed interest in the metabolism and functions of lipoprotein subfractions of the heterogeneous HDL density class. This interest has been further stimulated by findings indicating different proportions of HDL species in various pathological conditions associated with abnormal plasma lipid metabolism (5–7).

The finding that HDL species containing different associations of apoproteins are not segregated by flotation (8) and the observation that centrifugation can artefactually dissociate lipoprotein apoproteins (particularly apoA-I) from HDL (9) have led to a refining of alternative preparative techniques including affinity chromatography, isoelectric focusing, chromatofocusing, and electrophoresis (10–12).

Recent solid-phase immunological techniques permit the quantitation of antigens directly from a variety of semi-solid matrices. We here describe an immunoblotting technique to quantitate HDL species directly from agarose, and have used it to measure in normal and pathological plasma samples a subfraction of HDL containing apoA-I which, unlike the bulk of HDL, migrates with pre-beta mobility.

MATERIALS AND METHODS

Subjects and plasma collection

Normolipidemic subjects were selected from laboratory personnel. Hyperlipidemic plasma was obtained from pa-

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Abbreviations: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I, the major protein component of HDL; IgG, immunoglobulin G; LCAT, lecithin:cholesterol acyltransferase; PBS, phosphate-buffered saline.

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tients attending the Lipid Clinic of Shaughnessy and University Hospitals, University of British Columbia, Vancouver, B. C. These patients (with primary hypertriglyceridemia, familial hypercholesterolemia, and congenital lecithin:cholesterol acyltransferase deficiency) **(Table 1)** were investigated because of earlier reports (5–7) that in these groups there is an abnormal size distribution of HDL in plasma. Plasma was obtained from 18 hyperlipidemic patients and 16 normolipidemic control subjects who had fasted overnight. Seven of the patients had hypertriglyceridemia: 3 were from families with familial combined hyperlipidemia, two had insulin-independent (Type II) diabetes, one was deficient in lipoprotein lipase, and one in apoC-II. Nine patients were heterozygotes for familial hypercholesterolemia. Two patients had familial lecithin:cholesterol acyltransferase deficiency. Plasma was separated by centrifugation at 1500 *g* at 5°C for 30 min, and was either maintained in wet ice for up to 24 hr, or else frozen in dry ice/ethanol for up to 10 weeks, then thawed immediately before use. The effects of storage were shown to be without effect on subsequent analysis, as detailed below.

Electrophoresis of plasma

Flat-bed electrophoresis was carried out in 0.5% (w/v) agarose (Bio-Rad, M_r 0.13) (Bio-Rad, Richmond, CA) cast on glass and containing 0.25% human serum albumin (Sigma, St. Louis, MO) in 50 mM barbital buffer, pH 8.6. Electrophoresis was in the same buffer at 10°C. Plasma (1 ml) was initially depleted of endogeneous immunoglobulin G (IgG) by passage through columns (0.5 × 5 cm) of covalently complexed protein A-agarose (Pharmacia, Uppsala, Sweden) (13) at 3°C. Dilutions of plasma were prepared to contain 2–10 mg of protein/ml. As previously reported (3) recovery of apoA-I through immunoaffinity chromatography was complete, and the pattern of apoA-I was unchanged.

Plasma from which both IgG and apoA-I had been completely removed was prepared from this fraction by passing it through a second affinity column containing rabbit polyclonal antibody to human apolipoprotein A-I. Removal of apoA-I was >99% relative to the original plasma, as determined by quantitative radial immunodiffusion assay (13). ApoA-I was isolated from normolipemic fasting human plasma. Total HDL was isolated by preparative ultracentrifugation (14), delipidated with ethanol and diethyl ether at −20°C, dissolved in 0.05 M Tris-HCl buffer, pH 8.3, containing 6 M urea, and purified from detectable levels of other apolipoproteins by molecular sieve and DEAE-cellulose anion exchange chromatography (15). Isolated apoA-I migrated as a single band of prebeta mobility by agarose gel electrophoresis.

Samples (10–15 μl) of IgG-depleted plasma (diluted 6- to 30-fold from native plasma) and known concentrations of purified apoA-I (determined by radial immunodiffusion) (13) added to plasma depleted of apoA-I and IgG (prepared as described above) were applied to the precast wells of the agarose slab gel assembled in a flat-bed elec-

TABLE 1. Lipid and apoA-I characteristics of normal and hyperlipidemic plasma

	Total Cholesterol	Total Triglyceride	Total ApoA-I	Prebeta ApoA-I	% Prebeta ApoA-I
1. Heterozygous FH	378	63	87	20	23
2. Heterozygous FH	290	89	166	43	26
3. Heterozygous FH	243	42	150	27	18
4. Heterozygous FH	340	85	186	34	18
5. Heterozygous FH	229	53	159	27	17
6. Heterozygous FH	440	112	73	15	21
7. Heterozygous FH	247	54	143	29	20
8. Heterozygous FH	276	117	152	30	20
9. Heterozygous FH + familial CH	844	1950	138	73	53
10. LPL deficiency	166	262	83	25	30
11. ApoC-II deficiency	220	1242	85	17	20
12. LCAT deficiency	82	82	54	23	43
13. LCAT deficiency + renal failure	218	738	88	55	63
14. Type II diabetes	515	4695	150	30	20
15. Type II diabetes	369	1470	122	51	42
16. Familial CH	338	1115	109	42	39
17. Familial CH	292	2376	107	44	41
18. Familial CH	316	1020	119	46	39
Normolipidemia (n = 16)	178 ± 72	94 ± 31	142 ± 30	6 ± 3	4 ± 2

Concentrations are given as mg/dl^{-1}. Heterozygous familial hypercholesterolemia (FH) was diagnosed on the basis of at least one afflicted family member with hypercholesterolemia and/or tendon xanthomatosis and, in the case of #9, genetic analysis indicating a deletion in the LDL receptor gene. LCAT, LPL, and apoC-II deficiencies were diagnosed on the basis of the complete absence, determined immunologically, of the corresponding plasma protein. Type II (noninsulin-dependent) diabetes mellitus was diagnosed using the criteria of the National Diabetes Data Group, with hyperglycemia without ketosis or ketonuria. The diagnosis of familial combined hyperlipidemia (CH) was diagnosed on the basis of at least one afflicted family member. Total apoA-I was determined by radial immunodiffusion assay. The proportion of prebeta-migrating apoA-I was determined as described under Methods. All plasma samples were from donors whose blood was collected after an overnight fast.

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trophoresis unit (Multiphor, LKB, Bromma, Sweden). Electrophoresis was carried out at 10°C at 25 volts/cm until a bromophenol blue–bovine serum albumin marker had migrated 8 cm.

Radioiodination of proteins

One hundred μg of purified protein A (capacity 11–14 mg of human IgG per mg of solid) (Sigma, St. Louis, MO), or high molecular weight reference proteins (Pharmacia) (20 μl) were radioiodinated with carrier-free $Na^{125}I$ (500 mCi/ml) (New England Nuclear, Boston, MA) with chloroamine T-derivatized polystyrene beads (Iodobeads, Pierce Chemical Company, Rockford, IL) (16) on ice for 15 min. Protein A was reacted with 7.5 mCi of $Na^{125}I$ in the presence of two beads, while the molecular weight reference proteins were reacted with 1 mCi of $Na^{125}I$ and one bead, both incubations in a total volume of 200 μl of phosphate-buffered saline (PBS, 0.0 1 M sodium phosphate + 0.15 M NaCl, pH 7.4, containing 0.05% sodium azide solution). The reaction was terminated by transferring the reaction mixture to another tube containing 100 μl of 5 M KI. Free radioisotope was removed by molecular sieve chromatography using a column (1 × 10 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The specific activity of the radiolabeled protein was about 40 mCi/mg of protein. The radiolabeled molecular weight reference protein pool was diluted to 20% glycerol and stored in 125-μl aliquots at −70°C for up to 2 months. Radioiodination and storage at −70°C had no effect on the mobility of these proteins.

Electroblotting of separated proteins

Electrophoretically separated proteins on the agarose gel were electrophoretically transferred (TransBlot, Hoefer Scientific, San Francisco, CA) to nitrocellulose membranes (0.45 μm, Sartorius, Hayward, CA) in 0.025 M Tris + 0.2M glycine, pH 8.3 (17), without methanol or sodium dodecyl sulfate, at 10°C. Transfer was carried in two stages: first, at 20 volts for 30 min, then at 45 volts for 60 min. Under these conditions efficient and complete transfer of all proteins was obtained, confirmed by an absence of Coomassie blue-positive material detectable on the agarose gel after electroblotting. The nitrocellulose blots were fixed in 25% v/v isopropanol and 10% acetic acid in distilled water (18) for 15 min, rinsed, then blocked with 10% bovine serum albumin (Sigma) at pH 8.0 for 1 hr at 50°C. Fixation under these conditions did not modify subsequent reactivity of apoA-I, as described below. The blots were then treated with 1% rabbit polyclonal anti-human apoA-1 antiserum in PBS. Reaction was at pH 8.6 for 1 hr at room temperature. The blots were then treated to remove antiserum bound nonspecifically by two successive incubations in PBS containing 0.1% v/v Triton X-100 at 37°C for 30 min. The blots were then rinsed in PBS, and incubated with protein A labeled with ^{125}I (4 mCi in 100 ml of PBS containing 1% bovine serum albumin, pH 7.4) for 1 hr at room temperature. (This reagent was reused for at least 2 months or 10 incubations without change in properties when stored at −70°C under nitrogen between incubations.) Radioactivity bound nonspecifically was removed by incubation in PBS containing 0.1% Triton X-100 for 2 hr at 37°C. Bound radioactivity was localized on air-dried blots by overnight autoradiography on Kodak X-OMAT-AR film. The resulting autoradiogram was aligned with the blot, and the corresponding areas were excised for quantitation of specifically bound radioactivity by gamma scintillation spectrometry.

The total amount of apoA-I in the plasma samples applied to each gel was quantitated by radial immunodiffusion. Briefly, agarose slabs were formed with 1.33% w/v agarose (Bio-Rad, M_r 0.13) containing 3% (w/v) bovine serum albumin–2.67% (w/v) polyethylene glycol in 0.05M Tris-HCl buffer, pH 8.0. Plasma samples and purified apoA-I standards diluted in PBS containing 1% v/v Triton X-100 were applied to 4-mm diameter wells and the plates were incubated at 37°C for 24 hr. Immunoprecipitin ring diameter was determined with a caliper micrometer. The mass of applied apoA-I (up to 0.5 μg/well) was proportional to the square of the diameter of the precipitin ring under the conditions of this assay (c.v. 2.8%).

Isolation of prebeta- and alpha-migrating HDL fractions

Preparative separation of prebeta- and alpha-migrating HDL was carried out by flatbed electrophoresis of plasma along the length of an agarose slab (12.5 × 25 cm) at 400 V. Following electrophoresis, the portions of the gel corresponding to alpha- and prebeta-mobility were excised, and the proteins were electroeluted in a Buchler tube gel apparatus modified to accept three to six gel samples of up to 25 ml each in capacity. The upper buffer chamber, which contained the sample and catholyte, was further modified to accept a cooling coil. The catholyte consisted of 25 mM Tris + 100 mM glycine (pH 8.5), while the anolyte was 150 mM Tris-HCl (pH 8.5) (19). This modification permitted the entire apparatus to be maintained at 10°C throughout the electroelution process. Electroelution was carried out at 5 mA for 20 hr, when a visible schlieren pattern (i.e., the trailing front) passed through the sample vessel and into a collection tube where eluted plasma proteins were retained by a dialysis membrane (19). Recovery of a protein standard (human serum albumin) from the gel under these conditions was >90%.

Determination of lipoprotein and apoprotein molecular weight

The molecular weight of apoA-I-containing lipoproteins was determined by nondenaturing gradient polya-

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crylamide gel electrophoresis. Gels were prepared and run in a Hoefer TE slab gel apparatus (Hoefer Scientific, San Francisco, CA). A density gradient former (Isolabs, Akron, OH) was employed to generate a 4–27% (w/v) linear gradient of acrylamide containing 2.6% bisacrylamide. The gradient was made up in 90 mM Tris + 80 mM boric acid + 3 mM EDTA + 3 mM sodium azide, pH 8.3. Gels were poured at room temperature from the top of the gel cassette inclined at an angle of 30 degrees, and polymerized by the inclusion of 0.05% (w/v) ammonium persulfate and N,N,N'N'-tetramethylethylenediamine. The latter was made to 0.025% in the 4% acrylamide solution and to 0.017% in the 27% acrylamide solution to allow polymerization to occur downwards. Gels were stored at room temperature overnight before use to permit full polymerization. Sample wells were cast from 4% acrylamide solution and polymerized for 30 min. The sample wells were rinsed with electrophoresis buffer and the gels were pre-run at a constant 200 V for 30 min prior to loading the samples. Samples (100 μl of fourfold diluted plasma) adjusted to 5% sucrose (w/v) in electrophoresis buffer were loaded by underlaying into the sample wells and electrophoresed at 80 V until they entered the gel. Electrophoresis was continued at 200 V for a total of 20 hr at 10°C. Upon completion of electrophoresis the separated components were electrophoretically transferred to nitrocellulose membranes in 0.025 M Tris + 0.2 M glycine + 20% methanol + 0.1% w/v sodium dodecyl sulfate (pH 8.3) in two stages: 20 V, 30 min then 45 V, 5 hr at 10–15°C. The blots were analyzed for content of apoA-I as described above. Molecular weight markers (high molecular weight standards, Pharmacia, supplemented with ovalbumin) radioiodinated with $Na^{125}I$ as described above, were included in each gel. This allowed for a direct molecular weight calibration of the apoA-I-containing species from the resulting autoradiogram. Radioiodination did not alter the mobilities of the molecular weight marker proteins when these were compared with the values of untreated standards. Using this method, logarithms of molecular weight were a linear function of mobility to a high degree of accuracy (log M_r vs. R_f, correlation coefficient ($r = -0.997 \pm 0.002$, n = 11) over the molecular weight range of M_r 28–669 kDa.

Other methods

Cross-immunoelectrophoresis was carried out as described by Weeke (20). Plasma total cholesterol and triglyceride levels were determined by autoanalyzer (21, 22). The level of cholesterol in HDL was determined on samples of plasma from which very low and low density lipoproteins had been precipitated with dextran sulfate ($M_r 5 \times 10^5$) (Pharmacia) and $MgCl_2$ (final concentrations 1 mg/ml and 0.1 M, respectively) (23). After the mixture stood for 30 min in ice, the precipitated lipoproteins were removed by centrifugation (1500 g, 30 min) and the total cholesterol content of the supernatant HDL solution was determined enzymatically with cholesterol esterase and cholesterol oxidase, using a fluorimetric assay based on homovanillic acid (23).

RESULTS

Prebeta-migrating HDL in normal human plasma

When native plasma was fractionated by electrophoresis on agarose gel and apoA-I was detected after blotting by solid phase immunoassay, a significant proportion of antigen was detected migrating as a distinct component of slower mobility than the bulk of alpha-migrating HDL (**Fig. 1,** lane 1). This minor apoA-I fraction comigrated with the prebeta (very low density lipoprotein, VLDL) fraction of the major plasma lipoprotein classes. It was detected in the plasma of all donors (n = 34) in this study, both in freshly drawn plasma, and in plasma stored as described under Methods. The presence of prebeta-migrating apoA-I was also demonstrated by cross-immunoelectrophoresis **Fig. 2).** The same pattern was observed when unfractionated plasma was employed.

The following experiments suggest that the prebeta apoA-I was not generated from the major fraction of alpha-migrating apoA-I during the protein-A affinity chromatography. Firstly, prebeta apoA-I was detected when electrophoresis was carried out without preliminary

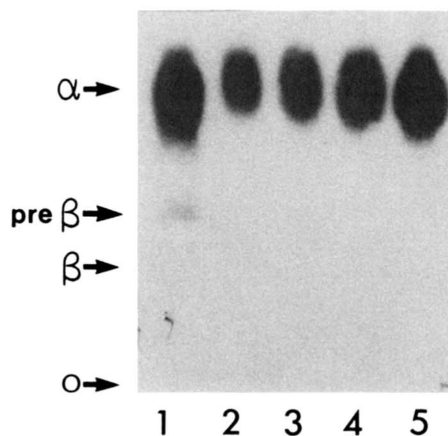

Fig. 1. Effect of electroelution and electrophoresis on HDL. Plasma was electrophoresed in agarose gel as described under Methods. The area of gel corresponding to the alpha-migrating region was excised and electroeluted. This material was electrophoresed again in increasing mass in lanes 2–5 (which contained 0.77, 1.54, 3.08, and 6.15 μg of total apoA-I, respectively). The separated proteins of total plasma (6.0 μg of total apoA-I, lane 1), along with the apoA-I in lanes 2–5, were transferred to nitrocellulose and processed for the detection of apoA-I using anti-apoA-I antiserum and ^{125}I-labeled protein A. The autoradiogram of the processed blot is shown. Alpha-, prebeta-, and beta-electrophoretic positions were determined from gel strips of native plasma electrophoresed concomitantly and stained with Sudan B; O, origin.

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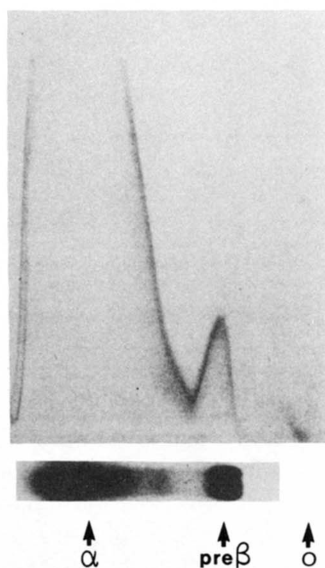

Fig. 2. Crossed immunoelectrophoresis of normolipidemic plasma depleted of IgG as described under Methods, reacted with anti-apoA-I antiserum. The results of this technique are compared with those of single-dimension agarose gel electrophoresis (below) in which the gel strip was blotted and processed with anti-apoA-I antiserum and ^{125}I-labeled protein A, as described in the legend to Fig. 1. Electrophoresis was conducted from right to left.

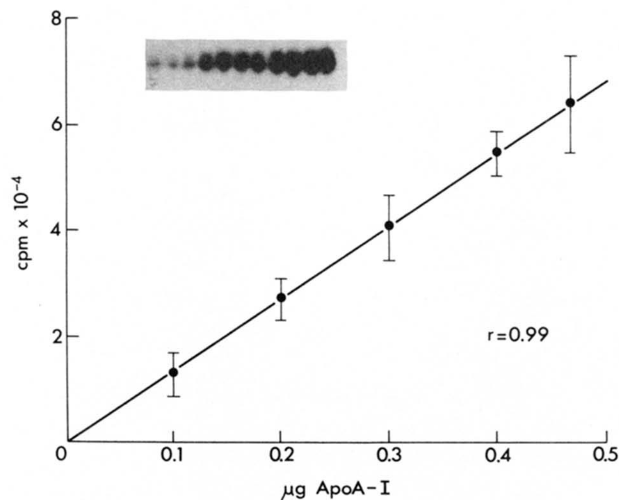

Fig. 3. Calibration curve for the solid-phase radioimmunoassay of prebeta-migrating apoA-I. Isolated apoA-I was diluted in amounts ranging up to 0.5 µg in a medium of plasma depleted of both IgG and apoA-I, electrophoresed, and quantitated from the prebeta migration band as described under Methods. The data are averaged from the results of four separate assays. Error bars are means ± one standard deviation. A representative autoradiogram of the areas quantitated, corresponding to 0.1, 0.1, 0.1, 0.2, 0.3, 0.3, 0.3, 0.4, 0.5, 0.5, and 0.5 µg of apoA-I (left to right), is illustrated (inset).

removal of endogenous plasma IgG (data not shown). Secondly, when the alpha-migrating fraction of apoA-I was refractionated by a second electrophoresis under the same conditions, there was no further generation of prebeta apoA-I that could be detected upon subsequent assay (Fig. 1, lanes 2–5). This result, while not ruling out changes caused by electrophoresis in a small, particularly unstable fraction of apoA-I, appears to rule out generation by dissociation from the major fraction of HDL in plasma. Conversely, eletrophoresis of prebeta apoA-I did not generate any detectable alpha-migrating material. There was no change in the level of prebeta apoA-I when the electric field was varied between 15 and 30 volts/cm during electrophoresis, suggesting that this variable was not a factor in the generation of prebeta apoA-I. Finally, when plasma was fractionated by Sephadex G-100 molecular sieve chromatography, a "small HDL" fraction of prebeta mobility was obtained which coeluted with albumin, as has been previously reported (5). Since molecular sieve chromatography has been demonstrated to cause little or no detectable dissociation of apolipoproteins from lipoproteins, even of labile complexes (24), this result also suggests that the prebeta apoA-I observed by electrophoresis was a component of native plasma, and was not generated during fractionation.

Quantitation of prebeta apoA-I in normal plasma

As shown in **Fig. 3,** the radioactivity of ^{125}I-labeled protein A bound by the solid-phase immunoassay was linear with the volume of plasma and mass of apoA-I present over the range 0–500 ng of apoA-I. To determine whether the presence of other proteins of plasma modified the values obtained, isolated purified apoA-I was added to the same plasma and the increment of total radioactivity in the assay was determined. As shown in **Fig. 4,** the

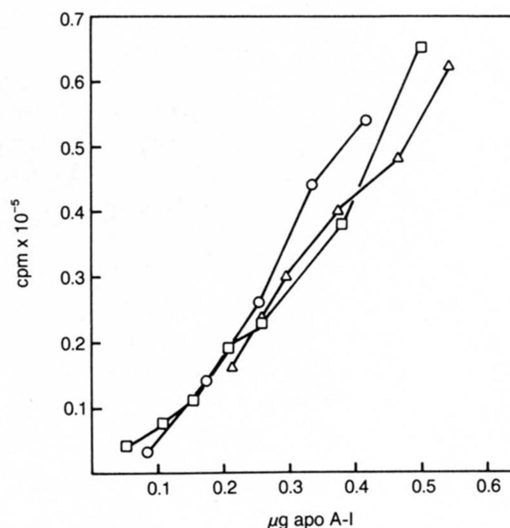

Fig. 4. Comparison of the reactivity of prebeta-migrating apoA-I in plasma in the presence and absence of exogenous isolated apoA-I. Prebeta A-I from 5–25 µl of plasma, depleted of IgG, and containing 5.5–27.5 µg of total apoA-I, was quantitated by blotting (○—○). Separate samples were prepared to contain identical amounts of plasma to which 0.25 µg of purified apoA-I was added (△—△). The combined (native + exogeneous) prebeta apoA-I was quantitated similarly. Finally, purified apoA-I was added in the amounts indicated to plasma from which native apoA-I had been previously removed by immunoaffinity chromatography (□—□).

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slopes of the plots for the prebeta-migrating component of plasma alone, of plasma plus exogenously added apoA-I, and apoA-I alone were very similar (slopes 1.37, 1.43, and 1.35×10^5 cpm/μg, $r = 0.98$–0.99). This suggests that native plasma prebeta-migrating apoA-I and isolated apoA-I react similarly in the assay.

The mass of prebeta-migrating apoA-I in normal human plasma measured from such calibration curves was 61 ± 26 μg • ml^{-1} plasma (Table 1). The proportion of apoA-I migrating in the prebeta fraction was determined by comparison of this mass with that of total apoA-I determined in the same plasma samples by radial immunoassay. This proportion was $4.2 \pm 1.8\%$ of total apoA-I for normolipidemic fasting plasma (n = 16 in each case).

The effects of freezing and storage on this fraction are shown in **Fig. 5.** When plasma was quickly frozen at $-70°$C and immediately rethawed, there was a slight but insignificant rise in the level of prebeta apoA-I. There was a slight decrease in concentration over the next 4 weeks under the same conditions, but the mean concentration in the thawed samples overall ($4.0 \pm 0.4\%$ of total plasma apoA-I) was not significantly different from that assayed in unfrozen plasma. This finding indicates that prebeta apoA-I represents a stable minor component of normal plasma.

Molecular properties of prebeta apoA-I

The molecular size of prebeta apoA-I was determined by two-dimensional electrophoresis. These experiments indicated that prebeta apoA-I was present mainly or exclusively in two species of molecular weight 67–75×10^3 **(Fig. 6 a, b)** of which the larger was slightly less acidic (migrating more slowly in the first dimension). Since these species did not overlap those of the major species of normal plasma apoA-I (molecular weights 160–250×10^3),

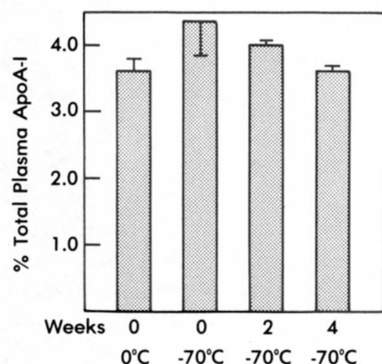

Fig. 5. Effect of storage of whole plasma at $-70°$C on the quantitation of prebeta-migrating apoA-I. Normolipidemic plasma was assayed by the solid-phase immunological technique in ice-cooled unfrozen plasma (0°C) and in plasma thawed after storage from 0–4 weeks. Error bars are one standard deviation (n = 4).

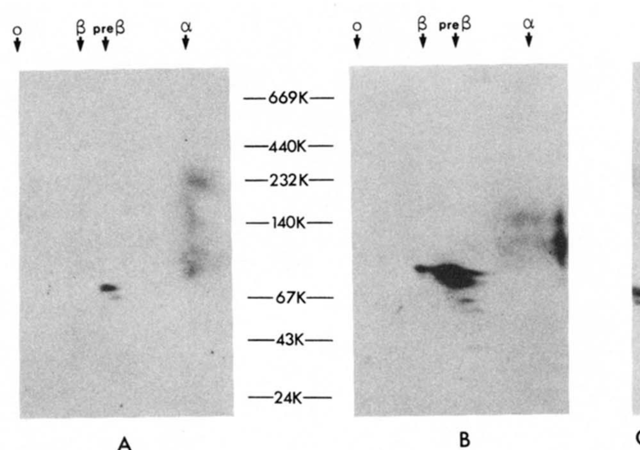

Fig. 6. Two-dimensional electrophoretic analysis of apoA-I in plasma by agarose electrophoresis and by nondenaturing polyacrylamide gel electrophoresis. Plasma from normolipidemic plasma (panel A) or hypertriglyceridemic plasma from a patient with Type II diabetes (Table 1, patient #15) (panel B) was electrophoresed into agarose, and then in a second dimension into a linear nondenaturing 4–27% polyacrylamide gradient gel, and immunoblotted to reveal apoA-I components by autoradiography. Molecular weights indicated were determined with labeled standards as described in Methods. Panel C is an autoradiogram depicting apoA-I components electroeluted from the prebeta region of an agarose gel and analyzed by gradient gel electrophoresis.

the molecular weight of prebeta apoA-I could also be accurately determined by one-dimensional nondenaturing polyacrylamide gel electrophoresis. The prebeta region of preparative agarose gels of whole normolipidemic plasma was electroeluted and applied directly to the polyacrylamide gradient gel. A representative gel is shown in Fig. 6c. The apparent molecular weight (M_r) values for the smaller and larger species of prebeta apoA-I in normolipemic plasma were $67{,}000 \pm 3{,}000$ and $75{,}000 \pm 4{,}000$, respectively (n = 11).

The isoprotein composition of these prebeta apoA-I components in whole plasma was analyzed two-dimensionally. The first dimensional separation by agarose electrophoresis was followed by a second dimensional separation by electrofocusing into agarose containing 8 M urea. The apoA-I isoprotein patterns were compared with those defined from isolated apoA-I from delipidated bulk HDL. Comparison of the spectra obtained indicated that the slower and faster components of prebeta apoA-I contained the isoproteins A-I$_{(4)}$ and A-I$_{(5)}$, respectively (25).

Prebeta apoA-I in hyperlipidemic plasma

Several studies have reported the presence of increased concentrations of small HDL in hyperlipidemia, including essential hypertriglyceridemia (5), hypercholesterolemia (6), and congenital LCAT deficiency (7). As shown in **Fig. 7,** when plasma of patients from each of these categories was fractionated by agarose gel electrophoresis, an increased concentration of apoA-I in the prebeta frac-

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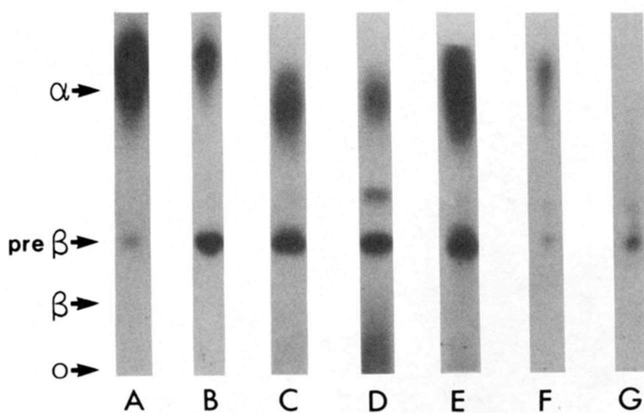

Fig. 7. ApoA-I distribution in normolipidemic and hyperlipidemic plasma, as determined by agarose electrophoresis followed by immunoblotting. The arrow indicates the prebeta migration position quantitated. Lane A: normolipidemic plasma; lane B, hypertriglyceridemia secondary to noninsulin-dependent diabetes; lane C, hyperchylomicronemia secondary to lipoprotein lipase deficiency; lane D, combined hyperlipidemia; lane E, familial hypercholesterolemia; lane F, hypertriglyceridemia secondary to apoC-II deficiency; lane G, congenital lecithin:cholesterol acyltransferase deficiency.

tion was evident by autoradiograph. This was confirmed by quantitative solid phase immunoassay (Table 1). Significantly increased proportions and concentrations of apoA-I were present in each of these groups of patients.

In contrast to the results described above of the two-dimensional electrophoretic pattern for normal plasma, similar analysis of plasma from hyperlipidemic patients revealed noticeable differences (e.g., Fig. 6b, patient #15). The molecular weight distribution of apoA-I lipoproteins was clearly altered. Most noticeably, the increase in total prebeta-migrating apoA-I noted above was found to be associated with an increase in the number of distinct prebeta-migrating apoA-I species. At least seven of the latter were observed of apparent molecular weight ranging from 43,000 upwards to about 100,000. The molecular weights of the major prebeta-migrating species of hyperlipidemic plasma were characteristically higher than those in normal plasma. On the other hand, the alpha-migrating apoA-I species were of lower molecular weight on average.

DISCUSSION

The purpose of the present study was to develop a quantitative solid phase immunoassay that could be used to determine the concentrations of molecular species of HDL in plasma fractionated by isocratic electrophoresis or isoelectric focusing. The techniques involved in electrophoretic transfer are usually not quantitative, particularly for higher molecular weight (>10,000) proteins, and in the absence of methanol and sodium dodecyl sulfate (17, 26, 27). This appears to be the first description of a detergent-free transfer technique for lipoproteins. In this study it was used to quantitate the concentration of a subfraction of HDL[1] in normal and pathological plasma samples. The data obtained indicate that normal plasma invariably contains a small proportion of prebeta-migrating HDL in the form of two reproducible, low molecular weight species; while in several classes of hyperlipidemia, a higher proportion of HDL with these properties is present in a more complex mixture of species.

While the major part of human HDL consists of complexes containing both apoA-I and apoA-II, with molecular weights in the $1.8-2.5 \times 10^5$ range and alpha-mobility by electrophoresis, it has also long been recognized that a smaller proportion of HDL in human plasma has quite different properties. By immunoaffinity chromatography, lipoprotein particles containing ApoA-I but not apoA-II were found in both centrifugally isolated HDL (28) and in native plasma (3, 8, 12, 29). While some of these lipoproteins contain apoA-I in association with other apolipoproteins such as apoD and ApoE (8, 29), in others only apoA-I was detectable, unassociated with other apolipoproteins. Low molecular weight particles containing apoA-I were found in plasma fractionated by molecular sieve chromatography, where dissociation of apolipoproteins from native particles is unlikely to be a factor (23). Higher proportions of low molecular weight HDL were found in congenital LCAT deficiency (7). Prebeta-migrating HDL was found in plasma but in a greatly increased proportion (relative to alpha-migrating HDL) in the HDL fraction of arterial intima (30, 31). While none of the studies above was designated to provide direct quantitation of these minor HDL subfractions in plasma, the proportion of HDL containing only apoA-I, of low molecular weight HDL, and of prebeta-migrating HDL in normal plasma, was quite similar in each case (about 5% of total apoA-I) (3, 5, 30). In the present study, two-dimensional gradient gel electrophoresis indicated that the major components of prebeta-migrating HDL had a molecular weight ($67-75 \times 10^3$) closely similar to that previously described for the small HDL isolated from plasma by molecular sieve chromatography (about 50×10^3) (5). These data, taken together, suggest that prebeta HDL represents the minor low molecular weight fraction of HDL previously reported.

Further studies will be required to determine the origin and fate of prebeta HDL and, in particular, the basis for the multiple electrophoretic forms of prebeta-migrating HDL in some hyperlipidemic plasma samples. The present study, as a first step, describes a straightforward assay of this lipoprotein species in native plasma, that should be

[1]In view of the likelihood that apoA-I in the d > 1.21 g/ml fraction of plasma consists, in large part at least, of HDL protein stripped from native HDL by ultracentrifugation (9), the term HDL is used here to include all those lipoproteins in fasting plasma that contain apoA-I.

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suitable for following its metabolism in plasma and determining its significance in the enzymatic and transfer reactions of plasma in which HDL plays a part. The method described should also be suitable for the quantitation of other apolipoproteins separation by any other of the fractionation systems which use one- or two-dimensional combinations of electrophoresis, isoelectric focusing, or diffusion to separate lipoproteins on the basis of charge or molecular weight. ■

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