Distribution of apolipoprotein A-IV in human plasma

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Abstract Human apoA-IV was purified from delipidated urinary chylomicrons. Monospecific antibodies were raised in rabbits and used to develop a double antibody radioimmunoassay (RIA). Displacement of ^{125}I-labeled apoA-IV by plasma or purified chylomicron apoA-IV resulted in parallel displacement curves, indicating that apoA-IV from both sources share common antigenic determinants. The apoA-IV level in plasma from normal healthy fasting male subjects (n = 5) was 37.4 ± 4.0 mg/dl, while fat-feeding increased the level to 49.1 ± 7.9 mg/dl ($P < 0.05$) at 4 hr. The apoA-IV level in plasma from abetalipoproteinemic fasting subjects was 13.7 ± 3.1 mg/dl (n = 5). Plasma from a single fasting Tangier subject showed a reduced apoA-IV level of 21.1 mg/dl. The distribution of apoA-IV in fasting and postprandial plasma was determined by 6% agarose gel chromatography. Fifteen to 25% of plasma apoA-IV eluted in the region of plasma high density lipoprotein (HDL), with the remainder eluting in subsequent column fractions. In abetalipoproteinemic plasma this HDL fraction is reduced and lacks apoA-IV, suggesting that at least some of the apoA-IV on these particles is normally derived from triglyceride-rich lipoproteins. Lipemic plasma from a fat-fed subject showed a small rise (3%) in chylomicron-associated apoA-IV. Gel-filtered HDL and subsequent apoA-IV-containing fractions were subjected to 4–30% polyacrylamide gradient gel electrophoresis (4/30 GGE), and apoA-IV was identified by immunolocalization following transfer of proteins to nitrocellulose paper. In normal plasma apoA-IV was localized throughout all HDL fractions. In addition, normal plasma contained apoA-IV localized in a small particle (diameter 7.8–8.0 nm). This particle also contained apoA-I and lipid. A markedly elevated saturated to unsaturated cholesteryl ester ratio was present in gel-filtered plasma fractions containing small HDL, suggesting an intracellular origin of these particles. In abetalipoproteinemic plasma apoA-IV was absent from all HDL fractions except for the small HDL particles, suggesting that they are not derived from the surface of triglyceride-rich particles. All plasmas contained free apoA-IV. In contrast to gel-filtered plasma, lipoprotein subfractions of fasted normal plasma prepared in the ultracentrifuge primarily contained apoA-IV in the d > 1.26 g/ml fraction, suggesting an artifactual redistribution of the apolipoprotein during centrifugation.■ Overall, these data suggest that apoA-IV secretion into plasma is increased with fat feeding, and that apoA-IV normally exists as both a free apolipoprotein and in association with HDL particles. **Bisgaier, C. L., O. P. Sachdev, L. Megna, and R. M. Glickman. Distribution of apolipoprotein A-IV in human plasma.** *J. Lipid Res.* 1985. **26:** 11–25.

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Rat and human apolipoprotein A-IV (apoA-IV) are 46,000 molecular weight proteins found associated with chylomicrons, HDL, and the d > 1.21 g/ml fraction of plasma (1–8). In both species apoA-IV is synthesized with a 20 amino acid signal peptide (9, 10). In the fasting or fat-fed rat, it is estimated that the intestine contributes approximately 60% of the plasma apoA-IV pool (11). Fat feeding in the rat significantly elevates plasma apoA-IV (12) and this has been shown to be associated with increased intestinal apoA-IV mRNA (13). In humans, fat feeding significantly increases the intestinal content of apoA-IV and its secretion on chylomicrons, resulting in the elevation of total plasma apoA-IV (6, 7, 14).

To date, measurements of human apoA-IV have been made by densitometric gel scanning (7) or rocket immunoelectrophoresis (3, 4, 6, 14, 15) usually using apoA-IV purified by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Quantitative densitometric gel scanning is dependent on standardization of proteins based on their relative chromagenicity. Published methods for immunoelectrophoretic measurement of human apoA-IV usually involve prior delipidation and resolubilization of sample protien which may introduce procedural losses (3, 6, 14, 15). In addition, these techniques use large quantities of antiserum and may not possess the necessary sensitivity for certain measurements (i.e., tissue apoprotein levels). We therefore report the development of a sensitive double antibody RIA for apoA-IV. We have employed this assay to measure apoA-IV levels in plasma from fasting and fat-fed subjects as well as to reexamine the distribution of apoA-IV in plasma separated by agarose gel chromatography. Our results indi-

cate that a significant portion of apoA-IV (15–25%) is associated with the major HDL fraction of plasma while the remainder appeared in the lipoprotein free fraction. Further analysis of gel-filtered HDL and lipoprotein free fractions by 4/30 GGE and immuno-localization suggest that in addition, apoA-IV is associated with a small HDL particle. This small HDL particle is poorly resolved from free apoA-IV by column chromatography on 6% agarose.

EXPERIMENTAL PROCEDURES

Purification of apoA-IV

Urinary chylomicrons from previously described subjects with chyluria (7) were isolated at d < 1.006 g/ml by ultracentrifugation at 3×10^6 g-min in an SW-27 rotor in an L8-55 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Fat cakes were removed by tube slicing and chylomicrons were purified by gel filtration on 2% agarose Bio-Gel A-5m, 50–100 mesh (Bio-Rad Laboratories, Richmond, CA), eluted with 0.154 M NaCl, 0.02% EDTA, pH 7.0. Chylomicron-containing fractions were concentrated by ultracentrifugation as described above. Chylomicrons were delipidated in cold ethanol–ether 3:2 (v/v) and ether-washed according to the procedure of Scanu and Edelstein (16). ApoA-IV was purified by gel filtration of chylomicron proteins on Sephadex G-150 SF. Chylomicron apoproteins (20 mg) were solubilized in 50 mM dithiothreitol, 6 M deionized urea, 10 mM Tris-HCl, pH 8.2, and applied to a 2.5×100 cm Sephadex G-150 SF column in a total volume of 2 ml. The column was run in an ascending manner at a flow rate of 3 ml/hr, collecting 1.5-ml fractions. Elution buffer was 6 M deionized urea in 10 mM Tris-HCl, pH 8.2. Fractions enriched in apoA-IV were concentrated in an ultrafiltration cell equipped with a PM-10 membrane (Amicon), made 50 mM with dithiothreitol, and repassed over the column. Purified apoA-IV was dialyzed into water and then lyophilized. Chylomicron apoprotein composition and apoA-IV purity were determined by reduced 5.6% SDS-PAGE (17).

Immunization of rabbits

Antibodies to human chylomicron apoA-IV were raised in rabbits. Animals were initially immunized with 30 μg of apoA-IV intramuscularly and 60 μg of apoA-IV subcutaneously in complete Freund's adjuvent (18). Subsequent boosts of 50 μg were given over multiple intradermal sites along the back in complete Freund's adjuvant at 4, 9, and 12 weeks. Serum prepared from blood taken over 15–20 weeks was utilized in the present study.

Ouchterlony immunodiffusion analysis

Analysis of rabbit anti-human apoA-IV was performed according to the method of Ouchterlony (19) on glass microscope slides coated with 1% agarose in 0.05 M barbital buffer, pH 8.8.

Immunoblot analysis

Immunoblot analysis of apoA-IV or apoA-I was performed on proteins transferred from SDS-polyacrylamide gels, isoelectric focusing (IEF) gels, or 4–30% gradient gels to cellulose nitrate paper. Chylomicron apolipoproteins were separated by 5.6% slab SDS-PAGE. Plasma from fasted or lipemic subjects was gel-filtered over 6% agarose (see below) and fractions (40 μl) were further separated by 4/30 GGE (20–22). Following electrophoresis, proteins from each source were transferred to cellulose nitrate paper at 60 v and 2 hr for 5.6% gels and 16 hr for IEF and 4–30% gradient gels in 25 mM Tris-192 mM glycine 20% methanol (v/v), pH 8.3, and immunoperoxidase-stained according to the method of Towbin, Staehelin, and Gordon (23). Following blocking of nonspecific sites with bovine serum albumin, cellulose nitrate paper strips were incubated at room temperature with rabbit anti-human apoA-IV, rabbit anti-human apoA-I, or non-immune rabbit serum (1:2000) for 2 hr, followed by 1-hr incubations with goat anti-rabbit IgG (1:200) (Cappel Laboratories, West Chester, PA) and then rabbit peroxidase anti-peroxidase (1:1000) (Cappel Laboratories). Between antibody incubations, paper strips were washed six times (5 min each) with 10 ml of 1% horse serum in 50 mM phosphate-buffered saline, pH 7.4. Following a final wash, regions of rabbit peroxidase anti-peroxidase binding were developed with 0.6 mM diaminobenzidine, 3 mM imidazole, 0.01% H_2O_2 and 154 mM NaCl in 10 mM Tris-HCl, pH 7.4.

Radioimmunoassay procedures

Chylomicron apoA-IV (10 μg of protein) in 80 μl of 0.5 M phosphate buffer, pH 7.5, was iodinated with 1 mCi of carrier-free ^{125}I (New England Nuclear, Boston, MA) for 15 sec by the chloramine-T procedure (24), and routinely resulted in 8–10% ^{125}I incorporation into protein. Free ^{125}I was separated from protein-bound ^{125}I on a column of Sephadex G-25 fine pre-equilibrated with 1% bovine serum albumin and 0.02% NaN_3 in 50 mM phosphate-buffered saline, pH 7.5 (PBSA). The ascending portion of the void peak was 80–90% trichloroacetic acid-precipitable. Gamma counting of gel slices of freshly iodinated ^{125}I-labeled apoA-IV separated by 5.6% SDS-PAGE resulted in a major discrete region of radioactivity at an R_f of 0.50. Antiserum was diluted to bind 70% of immunoreactive apoA-IV and used in RIA. Specific ^{125}I-labeled apoA-IV binding

without competitor antigen present was 20–25% for freshly iodinated tracer.

Plasma was delipidated in chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (25). Following the addition of methanol to a final chloroform–methanol ratio of 1:1 (v/v), a protein pellet was obtained by centrifugation. The protein pellet was washed with diethyl ether and dried under a stream of nitrogen. To determine the suitability of SDS in the RIA procedure, whole or delipidated plasma or chylomicron apoA-IV were preincubated in PBSA in the presence or absence of 1% SDS at 37°C for 30 min.

ApoA-IV (purified from chylomicrons) (0.8–400 ng) preincubated in 1% SDS in PBSA at 37°C for 30 min was used to standardize the assay. Routinely, diluted column fractions (1:5–1:10), unknown diluted plasma (0.1–0.4 μl), and plasma standard (1.88–964 ng apoA-IV equivalence) were preincubated in 1% SDS in PBSA at 37°C for 30 min. In 12 × 75 mm borosilicate glass tubes 100 μl of ^{125}I-labeled apoA-IV (30,000 cpm) in PBSA, 100 μl of non-immune rabbit serum (1:500 stock in PBSA), and 100 μl of rabbit anti-apoA-IV serum (1:400 stock in PBSA) were, respectively, added to 100 μl of preincubated standards or unknown samples. Tubes were incubated for 48 hr at 4°C prior to the addition of 100 μl of excess goat anti-rabbit IgG (1:20 stock in PBSA) (Cappell Laboratories). Incubation continued for an additional 24 hr prior to harvesting immunoprecipitates by centrifugation at 3000 rpm for 30 min at 4°C in a Sorval RC-2 centrifuge. These were washed with 0.5 ml of PBSA and triplicate assay tubes were counted, 1 min each, in an LKB 1270 Rackgamma II gamma counter. ^{125}I-labeled apoA-IV used in RIA protocols was not stored for longer than 2 weeks at 4°C, since loss in immunoreactivity of the antigen was observed upon storage. A plasma standard was calibrated with pure chylomicron apoA-IV and small volumes were stored at −20°C. This standard was used for calibration of all subsequent assays.

Immunoelectrophoresis analysis

Whole or delipidated plasma RIA standards were calibrated by rocket immunoelectrophoretic analysis by the method of Laurell (26). RIA-standardized plasma (78–2500 nl) or apoA-IV (6.6–210 ng) in 5 μl of 4 M urea in 10 mM Tris-HCl, pH 8.2, was applied to electrophoretic plates that were coated with 6% anti-apoA-IV serum in 1% agarose in 0.05 M sodium barbital buffer, pH 8.8. Electrophoresis continued for 2 hr at 50 mA.

Isolation of lipoproteins by ultracentrifugation

Lipoproteins were isolated from fasted plasma by sequential ultracentrifugation in an L8-55 ultracentri-

fuge (Beckman Instruments, Inc.). Triglyceride-rich lipoproteins were isolated in the SW27 rotor by centrifugation at 10^8 g-min and removed by aspiration. Other lipoproteins were isolated in the 50.3 rotor following the addition of KBr. Low density lipoprotein (LDL) was isolated at d 1.006–1.063 g/ml, HDL at d 1.063–1.21 g/ml, very high density lipoprotein (VHDL) at d 1.21–1.26 g/ml, and the lipoprotein free fraction at d > 1.26 g/ml. These were dialyzed into water and subjected to RIA for apoA-IV.

Bio-Gel A-5m chromatography of human plasma

Lipoproteins of human plasma were fractionated by 6% agarose (Bio-Gel A-5m, 200–400 mesh) gel chromatography (Bio-Rad Laboratories). Two ml of plasma was applied to a 100 × 0.9 cm column and eluted with 154 mM NaCl, 0.01% EDTA, 0.01% NaN₃, pH 7.0. Fractions of 1.8–2.0 ml were collected at 30-min intervals and analyzed for lipid and apoproteins as described below. Elution volumes for plasma LDL, HDL, and free protein were characterized with lipoproteins prepared by density flotation fractions (1.020–1.050 g/ml and 1.063–1.21 g/ml) and with bovine serum albumin.

Gradient gel electrophoresis of gel-filtered plasma

Aliquots (40 μl) from specific gel-filtered plasma fractions were removed for GGE 4/30 on PAA 4/30 gradient gels (Pharmacia, Uppsala, Sweden) for 3000 v-hr according to the method of Anderson et al. (21), prior to electrophoretic transfer of proteins to cellulose nitrate paper (23). PAA 4/30 gradient gels can separate particles of diameter of approximately 7–25 nm (20–22). Protein standards (Pharmacia) of thyroglobulin, 660 K, diameter = 13.3 nm; apoferritin, 440 K, diameter = 12.3 nm; catalase, 220 K, diameter = 10.3 nm; lactic dehydrogenase, 140 K, diameter = 8.6 nm; and albumin, 67 K, diameter = 7.4 nm were used to calibrate these gels (20, 21). When isolated lipoproteins are sized by 4–30% gradient gels or negative-stain electron microscopy, similar results are obtained (20, 21).

Fat-feeding studies

Plasma levels of apoA-IV were determined by RIA on blood drawn from normal healthy fasted males between the ages of 20–35 years. Blood was also drawn 4 hr after a meal of ice cream (40 g of fat). In studies where plasma was fractionated by Bio-Gel A-5m column chromatography, lipemic blood was drawn 4 hr after a meal of medium or heavy cream (100 g of fat equivalence).

Lipid analysis

For lipid analysis, aliquots of column fractions or plasma were extracted in 20 volumes of chloroform–methanol 2:1 (v/v) according to the method fo Folch et

al. (25). Prior to separation of the solvent phases of the lipid extract, beta-sitosterol (200 μg) was added as internal standard to correct for procedural losses from the extraction and chromatographic procedures. Aliquots of the lipid extract were removed for phospholipid and cholesterol determinations. Phospholipids were determined according to the method of Bartlett (27), employing dimyristoylphosphatidylcholine as an external standard. No corrections for mass difference between dimyristoylphosphatidylcholine and plasma phospholipids were made. For total cholesterol, aliquots from the lipid extract were taken to dryness and hydrolyzed with 3 ml of 3.3% KOH in ethanol at 75°C for 120 min under N_2. To these, 4 ml of water was added, and sterols were extracted into hexane. For free cholesterol, KOH and heating were omitted from the procedure. Cholesterol was determined on a Jeol JGC-20K gas chromatograph equipped with a flame ionization detector and interfaced to a Hewlett-Packard 3390 A integrator. Cholesterol was resolved from beta-sitosterol under isothermal conditions at 270°C on a 1-m glass column (2 mm inner diameter) packed with 3% OV-17 on gas-Chrom Q (100–120 mesh). Esterified cholesterol was estimated by difference between free and total cholesterol.

For determination of cholesteryl ester fatty acids, aliquots of gel-filtered plasma fractions were extracted in chloroform–methanol 2:1 (v/v) as described above. Cholesteryl heptadecanoate (50 μg) was added as internal standard. Cholesteryl esters were isolated by thin-layer silicic acid chromatography in hexane–diethyl ether–glacial acetic acid 80:16:4 (v/v/v). Cholesteryl esters were extracted from the silicic acid with diethyl ether–methanol 3:1 (v/v) and transmethylated with 14% BF_3 in methanol (28). Fatty acid methyl esters were quantitated by gas–liquid chromatography under isothermal conditions at 170°C on a 2-m glass column packed with Silar 10c (100–120 mesh).

Triglycerides were determined directly on aliquots of column fractions or plasma using a commercially available enzymatic assay kit (Worthington, Freehold, NJ).

Qualitative apolipoprotein determination

Rabbit anti-human apoA-I antibodies were raised in rabbits as previously described (29). Antibodies against human apoB were obtained commercially (Calbiochem-Behring Corp., La Jolla, CA). Specificity of these antibodies was determined by 1) immunodiffusion analysis against plasma and apoA-I or LDL, and 2) immunoblot analysis against delipidated chylomicrons and LDL. Antibodies to apoB were monospecific by immunodiffusion and immunoblot analysis, and recognized both B-100 and B-48 by immunoblot analysis. Antibodies to apoA-I were monospecific by immunodiffusion and immunoblot analysis. Qualitative detection

of apoA-I and apoB was determined by immunodiffusion analysis of 50 μl of column fractions that were delipidated with ethanol–ether 3:2 (v/v) (16) and resolubilized in 6 M urea, 10 mM Tris-HCl, pH 8.2.

Immunoprecipitation of gel-filtered plasma HDL with rabbit anti-apoA-I serum

Aliquots (100 μl) of gel-filtered fasted plasma HDL were incubated with rabbit anti-human apoA-I (1:100), non-immune rabbit serum (1:100), or PBSA in a total volume of 200 μl for 24 hr at 4°C. Following the addition of 700 μl of goat anti-rabbit immunobeads (Bio-Rad) or PBSA, incubations continued for 24 hr at 4°C. Tubes were centrifuged for 30 min and 3000 rpm and apoA-IV remaining in supernatants was assayed by RIA.

Lipid and protein staining of gradient gels

Adjacent gel-filtered plasma fractions (1 ml) were pooled and concentrated by vacuum dialysis into gradient gel buffer. After addition of sucrose and bromophenol blue, 80% portions of these fractions were subjected to 4/30 GGE and subsequent lipid staining with Sudan Black B according to the method of Pratt and Dangerfield (30). The remaining 20% was subjected to 4/30 GGE and subsequently stained for protein with Coomassie blue (22). The remainder of the gel-filtered plasma fractions was individually analyzed for cholesteryl ester fatty acids (see above).

Isoelectric focusing

Fasted or lipemic plasma (2.5 μl) or gel-filtered fasted plasma fractions (100 μl) were solubilized in 50 μl of 10 mM Tris-HCl, pH 8.2, in 8 M urea containing 2% Ampholine (pH 4–6, LKB) and 10% mercaptoethanol as modified from the method of Menzel, Kladetzky, and Assmann (31) and isoelectrically focused in 2.1-mm-thick slab gels according to the method of Gidez, Swaney, and Murnane (32). Gradient pH was monitored by slicing and extraction of a lane from the gel into distilled water. The remainder of the gel was subjected to immunoblot analysis for apoA-IV.

Protein determination

Protein reported in this study was analyzed by the method of Bradford (33) employing bovine serum albumin as standard. Addition of a 10-fold excess of lipid (Intralipid) to protein mass did not result in any deviation of the standard curve. In addition, for comparative purposes, apoA-IV protein was estimated by the method of Lowry et al. (34) employing bovine serum albumin as standard. For apoA-IV amino acid composition, 30 μg of protein [based on Bradford (33) estimation] was hydrolyzed for 24 hr at 110°C in 6 N HCl in evacuated sealed hydrolysis tubes. To the dried hydrolysate, nor-

leucine in 0.2 N sodium citrate, pH 2.3, was added as internal standard and analyzed on a Beckman 121 MB analyzer. Mass of apoA-IV was determined based on the added internal standard.

RESULTS

Purification of apoA-IV

Purification of apoA-IV from delipidated urinary chylomicrons was accomplished by Sephadex G150 SF column chromatography. A single pass of 20 mg starting material (approximately 10% apoA-IV as determined by densitometry) over the column was not sufficient to result in adequate purification of apoA-IV. ApoA-IV-enriched fractions from two primary runs were pooled, concentrated, and repassed over the column. This procedure resulted in an apoA-IV fraction that appeared greater than 98% pure by densitometric scanning of a Coomassie blue-stained 5.6% SDS-polyacrylamide gel (Fig. 1). This material was injected into rabbits (Experimental Procedures) for antisera production.
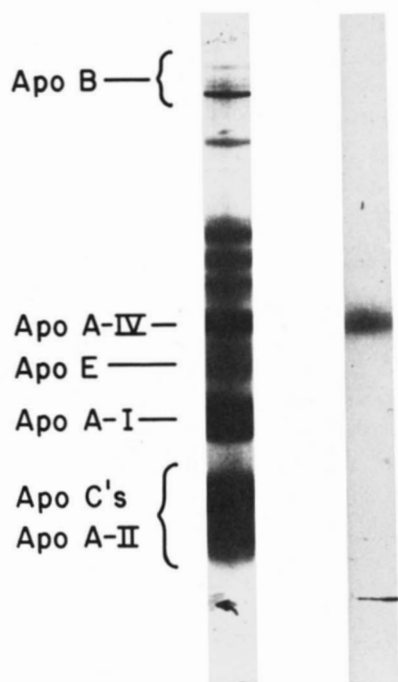

Fig. 1 Purified human apoA-IV from delipidated human urinary chylomicrons. ApoA-IV-enriched fractions from Sephadex G150 SF were pooled, concentrated, and repassed over the same column, and resulted in significant purification of apoA-IV. Details of purification are described in Experimental Procedures. SDS-polyacrylamide (5.6%) gels of chylomicron starting material (100 μg) (left) and purified apoA-IV (5 μg) (right).

Antisera characterization and radioimmunoassay

Antiserum was judged monospecific to apoA-IV by five criteria. *1*) A single precipitant arc of identity was seen when antiserum was tested against human plasma dilutions, delipidated chylomicron apoproteins, and purified apoA-IV (**Fig. 2**). Antiserum did not react against LDL (50 μg), albumin (250 μg), or apoA-I (50 μg). *2*) Displacement of ^{125}I-labeled apoA-IV from anti-apoA-IV with purified chylomicron apoA-IV or plasma gave parallel displacement curves (**Fig. 3**), and suggested the recognition of a single antigenic component of plasma. *3*) When chylomicron apoproteins were electrophoretically transferred from a 5.6% SDS-polyacrylamide slab gel to cellulose nitrate paper and visualized by immunoperoxidase staining, a single specific line was apparent on paper strips initially incubated with human apoA-IV antiserum. *4*) This antiserum immunoprecipitated radiolabeled apoA-IV specifically from a mixture of the primary translation products of intestinal and hepatic (Hep G2 cells) mRNA (10). *5*) In addition, lipoprotein subfractions (prepared in the ultracentrifuge) were used in the RIA. Parallel displacement occurred with these fractions inasmuch as they appeared to contain minute amounts of apoA-IV. Thus, the protein mass necessary for 50% displacement of ^{125}I-labeled apoA-IV by triglyceride-rich lipoproteins occurred at 10 μg, by HDL at 10.5 μg, and by the d > 1.26 g/ml fraction at 9.5 μg; for apoA-IV this value was 70 ng. With LDL, only 10% displacement occurred at 10 μg. The predominant proteins in ultracentrifugally prepared triglyceride-rich lipoproteins are apoB, apoE, and apoC's; in LDL, apoB; in HDL, apoA-I and apoA-II; and in the d > 1.26 fraction, albumin and immunoglobulins.

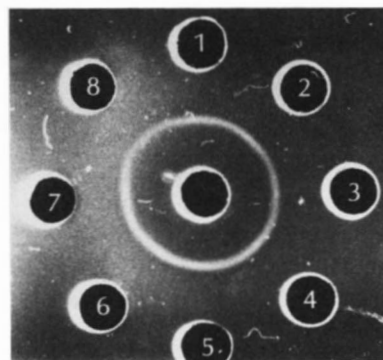RIA samples (plasma, column fractions, or purified apoA-IV) were routinely preincubated in 1% SDS-PBSA

Fig. 2 Monospecificity of apoA-IV antisera by Ouchterlony (19) immunodiffusion. Each well contained 10 μl of: wells 1–6, serial plasma dilutions in 50 mM phosphate-buffered saline, pH 7.5, 1:2–1:64, respectively; well 7, delipidated urinary chylomicrons in 6 M urea, 10 mM Tris-HCl, pH 8.2, 50 μg; well 8, apoA-IV in 6 M urea, 10 mM Tris-HCl, pH 8.2, 2 μg; center, rabbit antiserum.
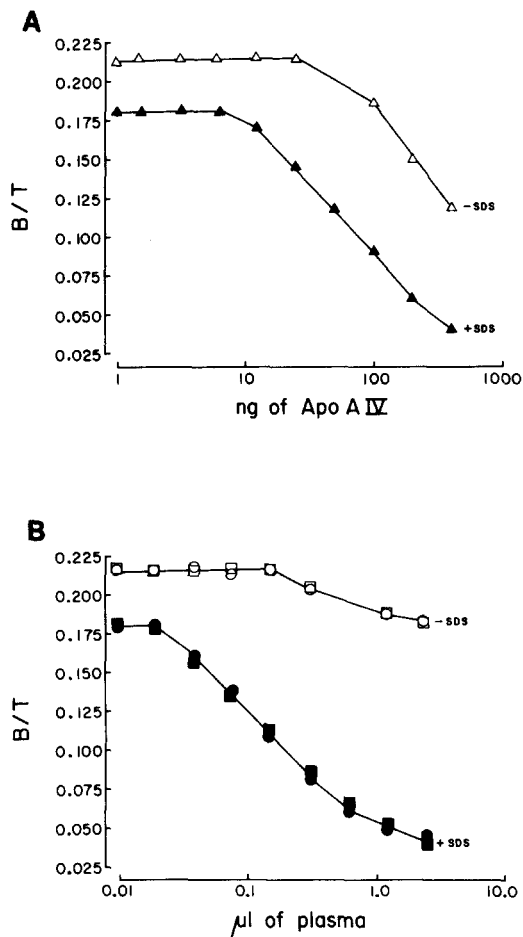
A

B

Fig. 3 Radioimmunoassay of human apoA-IV. Purified chylomicron apoA-IV (A) or whole or delipidated plasma (B) was subjected to RIA in the absence (open symbols) or presence (closed symbols) of SDS as described in Experimental Procedures. Whole plasma (●, ○); delipidated plasma (■, □); chylomicron apoA-IV (▲, △).

for 30 min at 37°C prior to addition to assay as described in Experimental Procedures. Under the conditions of this assay, linear displacement of apoA-IV occurs between 6.25 and 400 ng of apoA-IV (Fig. 3). No additional apoA-IV was detected when plasma was delipidated prior to assay and resolubilized with 1% SDS-PBSA, when compared to intact plasma solubilized with 1% SDS-PBSA (Fig. 3). However, as with purified apoA-IV, the addition of SDS to either delipidated or intact plasma resulted in recognition of additional apoA-IV (Fig. 3). Coefficient of variation within an assay was 12.4% (n = 9) and between assays was 13.9% (n = 4).

Rocket immunoelectrophoresis of whole or delipidated plasma resulted in identical values. When RIA plasma standard (38.6 mg/dl) was calibrated with apoA-IV by rocket immunoelectrophoresis, a value of 38.2 mg/dl was obtained. Protein estimation of apoA-IV by the method of Bradford (33), by the method of Lowry et al. (34), or by amino acid composition gave

values (relative to the Bradford determined value) of 100%, 102.5%, and 84.1%, respectively. Mass determined by amino acid composition is slightly underestimated since tryptophan mass is not included. Amino acid composition of apoA-IV agreed closely with published compositions (2, 4, 5, 7).

Effect of fat feeding on plasma apoA-IV levels

Blood was taken from five normal healthy male subjects who had fasted for 12 hr. Following ingestion of a fat load (40 g of fat), blood was drawn at 4 hr. As shown in **Table 1**, with fat feeding, plasma levels of apoA-IV increased from 37.4 ± 4.0 mg/dl to 49.1 ± 7.9 mg/dl ($P < 0.05$). ApoA-IV levels measured in fasted abetalipoproteinemics were significantly decreased (37% of fasted normal level) as shown in Table 1. In a single patient with Tangier disease, plasma apoA-IV was 56.4% of fasted normal levels (Table 1).

Plasma distribution of apoA-IV

In a separate experiment, blood was drawn from a subject who had fasted for 12 hr and again 4 hr after the ingestion of 100 g of fat (medium cream). With fat feeding, plasma triglycerides rose from 145 mg/dl to 236 mg/dl and apoA-IV levels were elevated by 25%. Plasma was subjected to gel filtration on 6% agarose (Bio-Gel A-5m). Fractions were monitored for absorbance at 280 nm and analyzed for phospholipid, cholesterol, triglycerides, apoA-I, apoB, apoA-IV, and protein as described in Experimental Procedures. Under these conditions, apoB-containing chylomicrons, VLDL, and intermediate density lipoprotein (triglyceride-rich fractions) were separated from apoB-containing LDL (cholesteryl ester-rich particles), and from apoA-I-containing HDL (phospholipid enriched) particles (**Fig. 4**). Routinely we found quantitative recovery of apoA-

TABLE 1. ApoA-IV levels in plasma from normal, abetalipoproteinemic, and Tangier disease subjects measured by RIA

	ApoA-IV (mg/dl ± SEM)	
Subject	Fasted	4-hr Fat-Fed (40 g of fat)
1	38.5 ± 4.0	35.8 ± 4.9
2	38.3 ± 3.2	51.1 ± 3.1
3	22.8 ± 2.0	27.0 ± 3.1
4	47.4 ± 3.2	66.9 ± 2.5
5	40.0 ± 3.6	65.7 ± 7.7
Mean[a]	37.4 ± 4.0	49.1 ± 7.9
Abeta 1	24.9 ± 2.0	
Abeta 2	8.5 ± 0.9	
Abeta 3	11.0 ± 0.6	
Abeta 4	8.0 ± 0.4	
Abeta 5	16.1 ± 0.4	
Mean	13.7 ± 3.1	
Tangier 1	21.1 ± 1.6	

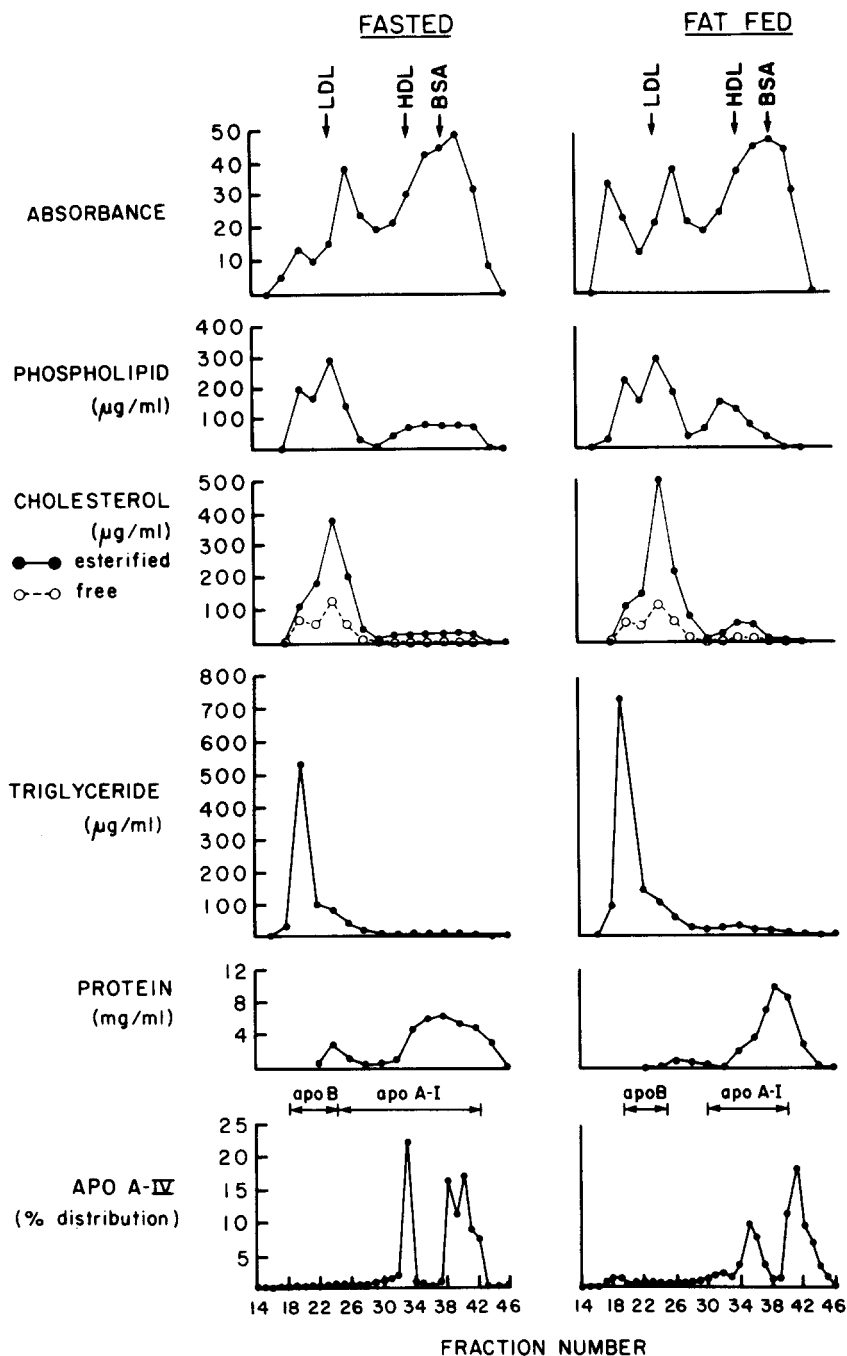[a]t-Test for paired difference ($P < 0.05$).

Fig. 4 Bio-Gel A-5m gel filtration profile of normal plasma. Fasted or 4-hr lipemic plasma was gel-filtered over Bio-Gel A-5m. Column fractions were monitored at 280 nm. Phospholipid, cholesterol, cholesteryl ester, triglyceride, protein, and apoA-IV were quantitated from aliquots of column fractions as described in Experimental Procedures. Qualitative presence of apoB and apoA-I in column fractions was monitored by Ouchterlony (19) immunodiffusion analysis as described in Experimental Procedures. Indicated by arrows are the elution volumes of human LDL (d 1.020–1.050 g/ml), HDL (d 1.063–1.21 g/ml), and bovine serum albumin (BSA).

IV applied to columns (>95%). Gel filtration of ^{125}I-labeled apoA-IV (50,000 cpm) and BSA (20 mg) resulted in comigration of these proteins, with no radioactive peak in the region of HDL. Two major protein peaks were eluted, the latter being composed predominantly of albumin. Under the conditions of separation, there appeared to be partial resolution of HDL from the major protein peak. In fasted plasma, apoA-IV eluted as two major peaks. The earlier peak, representing 24% of the apoA-IV, coeluted with HDL. The later

peak (76% of the apoA-IV) eluted with the descending portion of the free protein peak. In lipemic plasma, apoA-IV eluted in three peaks, a minor peak (3%) associated with the triglyceride-rich lipoproteins, a peak co-eluting with HDL (26%), and a peak (71%) associated with the descending portion of the free protein region. In a second subject, plasma was obtained after a 12-hr fast, and 4 hr after the ingestion of heavy cream (100 g of fat). Plasmas were separately gel-filtered over 6% agarose and fractions were monitored for apoA-IV by RIA (Fig. 5, lower panel). Apparent HDL and subsequent agarose column fractions which contained apoA-IV were subjected to 4/30 GGE and immunolocalization of apoA-I, and apoA-IV was determined (Fig. 5,

upper panel). Immunolocalization of apoA-I appears over the entire HDL region encompassing various particle diameters, and confirms the known presence of this apoprotein in many HDL subclasses (Fig. 5). Immunolocalized apoA-IV appears to be present in the same regions, but is more heavily localized in a region corresponding to a particle of 7.8–8.0 nm diameter. In addition, free apoA-IV is prominent in late column fractions (fractions 42–45). In both fasted and lipemic plasma, apoA-IV present in the major HDL fractions (fractions 34–40) appeared as free apoA-IV following GGE 4/30. This may be due to displacement of apoA-IV from these HDL particles during electrophoresis. In order to show that apoA-IV is indeed present on HDL
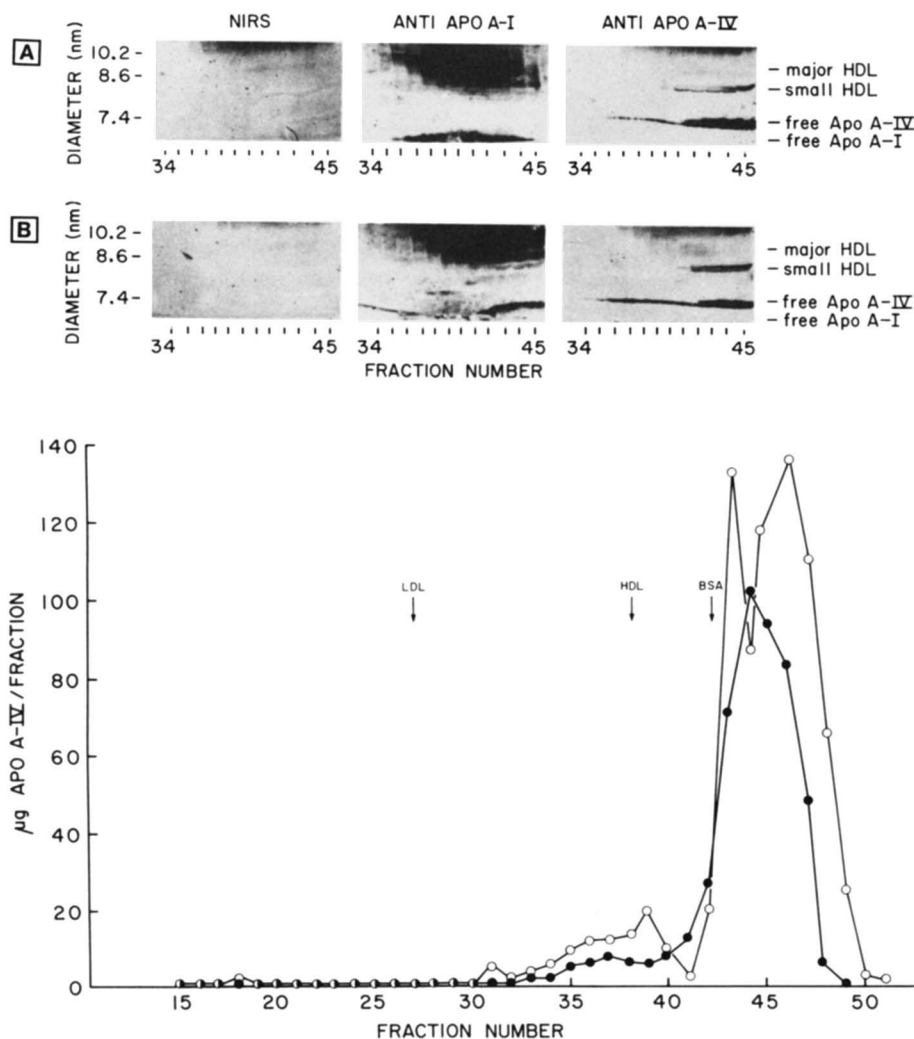

Fig. 5 ApoA-I and apoA-IV immunolocalization of Bio-Gel A-5m gel-filtered plasma. Aliquots of Bio-Gel A-5m gel filtered fasted (closed circles) and lipemic (open circles) plasma were analyzed for apoA-IV mass by RIA (lower panel). Aliquots (40 µl) of fractionated fasted (A) or lipemic (B) plasma containing apoA-IV (fractions 34–45) were resolved by 4/30 GGE and transferred to cellulose nitrate paper and immunolocalized for apoA-I, apoA-IV, and nonspecific staining with non-immune rabbit serum (upper panel). Mean migration distances of major plasma HDL and small spherical HDL were calibrated from high molecular weight standards applied to duplicate gels (20, 21). Indicated by arrows are the elution volumes of human LDL (d 1.020–1.050 g/ml), HDL (d 1.063–1.21 g/ml), and bovine serum albumin (BSA).

particles in these column fractions, antiserum to apoA-I was added to column fractions (pooled fractions 35–38 from fasting plasma, Fig. 5) and the amount of apoA-IV remaining in solution was determined after apoA-I immunoprecipitation. As shown in **Table 2**, 78% of the apoA-IV in these fractions was immunoprecipitated with anti-apoA-I serum. Thus the major proportion of apoA-IV appears associated with HDL particles and becomes dissociated during electrophoresis. ApoA-I seems less easily dissociated from HDL fractions during electrophoresis since immunolocalization shows that it is associated mainly with HDL particles, although some is free in these fractions.

Several changes appear to result from fat feeding. As seen in Fig. 5, the mass of apoA-IV appears elevated in all fractions corresponding to apoA-IV in the HDL region (fractions 35–40) and in the major protein fraction of plasma (fractions 40–50). Polyacrylamide electrophoresis (4/30 GGE) and immunolocalization of apoA-IV on these fractions shows more prominent staining in all fractions. This is particularly true of the smaller HDL fractions (fractions 42–45) which also exhibits staining for apoA-I.

We found four apoA-IV isoproteins (pI 5.28–5.40) by immunoblot analysis of isoelectrically focused fasting or lipemic plasma (**Fig. 6**). We did not detect elevation (increased staining intensity) of any specific isoform in lipemic plasma. It was of interest to determine whether any of the apoA-IV isoforms selectively bound HDL. Therefore, gel-filtered plasma fractions were subjected to IEF and subsequent immunoblot analysis for apoA-IV (**Fig. 7**). We found that major HDL-containing fractions (32–38) predominantly contained the two more acidic isoproteins, while the unresolved region of small HDL and free apoA-IV (fractions 39–44) contained all four isoforms. The descending portion of this unresolved region (fractions 43–44) which is enriched in free apoA-IV appeared to contain a greater abundance of the two more basic isoproteins.
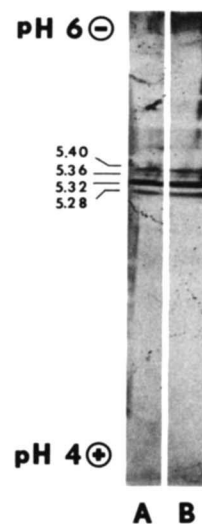

Fig. 6 Immunolocalization of apoA-IV isoproteins in normal plasma. Fasted (A) or 4-hr lipemic (B) plasma (2.5 μl) were isoelectrically focused (pH 4–6) and transferred to cellulose nitrate paper and immunolocalized for apoA-IV as described in Experimental Procedures.

In order to assess the effects of ultracentrifugation on the plasma distribution of apoA-IV, a duplicate sample of fasting plasma (Fig. 5) was submitted to ultracentrifugation. ApoA-IV distributed as follows: triglyceride-rich lipoproteins, 5.6%; LDL, 0.1%; HDL, 0.7%; VHDL, 0.8%; and lipoprotein-free fraction, 92.9%. This distribution does not parallel that obtained when lipoproteins are fractionated over agarose (Figs. 4 and 5), and probably represents an artifactual redistribution of apoA-IV during centrifugation.

Since apoA-IV is thought to enter the plasma as a chylomicron component, it was of interest to study the plasma distribution of apoA-IV in abetalipoproteinemia, a condition in which chylomicrons are not secreted (35). As shown in Table 1, apoA-IV levels are reduced. ApoA-IV was absent in the major HDL region of gel-filtered fractions and eluted as a single subsequent peak (**Fig. 8**). GGE 4/30 and apoA-I immunolocalization over the major HDL region and subsequent apoA-IV-containing peak revealed markedly reduced apoA-I immunoperoxidase staining, suggesting a significant reduction of the major HDL fraction (Fig. 8, insert). ApoA-IV immunolocalization (Fig. 8, insert) revealed the presence of an apoA-IV-associated particle which had coincident apoA-I staining (fractions 38–44). This particle appeared slightly smaller (diameter 7.5 nm) than the particles seen in normal plasma (Fig. 5, upper panel). Free apoA-IV was also present in these fractions.

Since it was of interest to determine whether or not the small HDL with apoA-I and apoA-IV contained lipid, gel-filtered plasma fractions were subjected to 4/30 GGE and stained for protein (**Fig. 9A**) or lipid

TABLE 2. Immunoprecipitation of HDL apoA-IV with anti-apoA-I serum

Conditions[a]	Percent ApoA-IV Remaining in Supernatant[b]
Fractions alone	100
Fractions + anti-apoA-I serum + immunobeads	22
Fractions + non-immune serum + immunobeads	97
Fractions + immunobeads	98

[a]Major HDL (fractions 35–38, Fig. 5) from gel-filtered fasting plasma was incubated in the presence of non-immune rabbit serum (1:100), rabbit anti-human apoA-I (1:100), or PBSA overnight at 4°C. Incubation continued for an additional 24 hr in the absence or presence of excess goat anti-rabbit immunoglobulin-linked immunobeads.
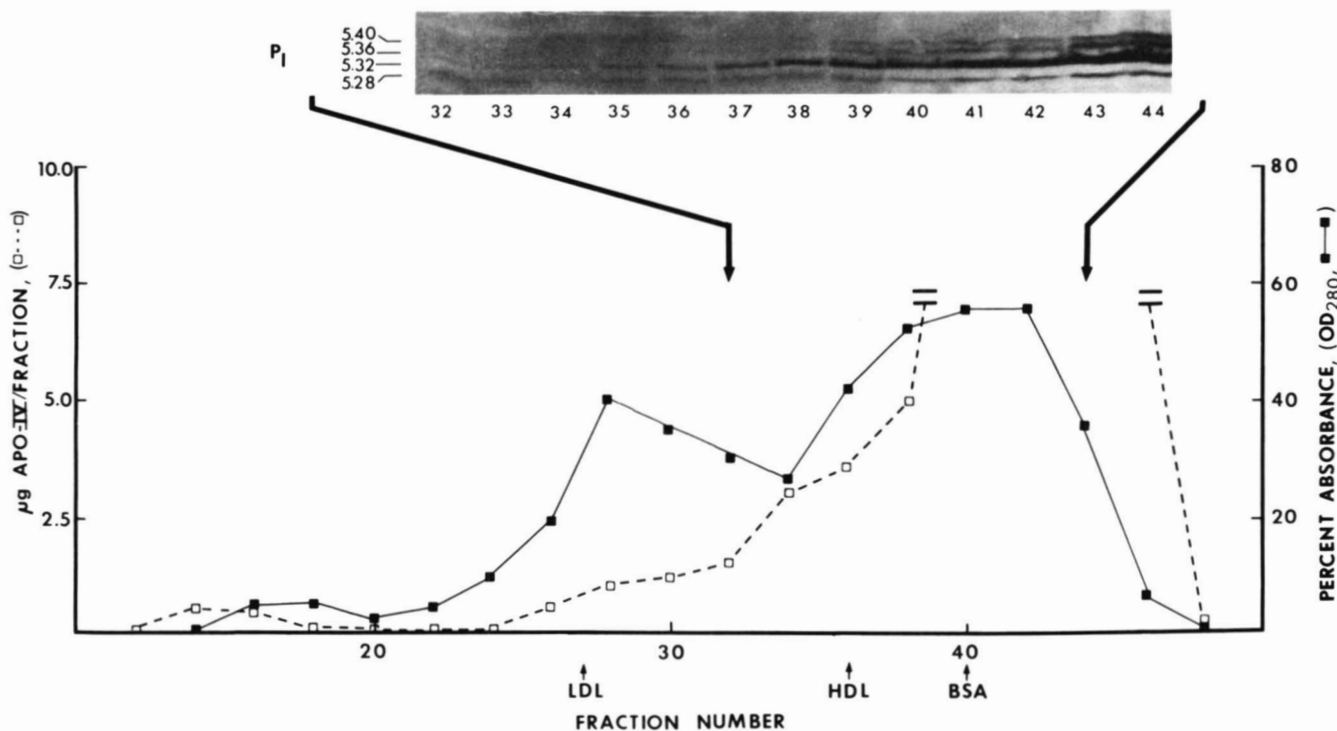[b]Supernatants from these were removed and analyzed for apoA-IV by RIA.

Fig. 7 Immunolocalization of apoA-IV isoproteins of Bio-Gel A-5m filtered fasted plasma. Aliquots of column fractions were analyzed for apoA-IV mass by RIA (open squares) and absorbance at 280 nm (closed squares). Aliquots (100 μl) of fractions 32–44 were subjected to IEF on pH 4–6 gels and transferred to cellulose nitrate paper and immunolocalized for apoA-IV (insert). Indicated by arrows are the elution volume of LDL (d 1.020–1.050 g/ml), HDL (d 1.063–1.21 g/ml), and bovine serum albumin (BSA).
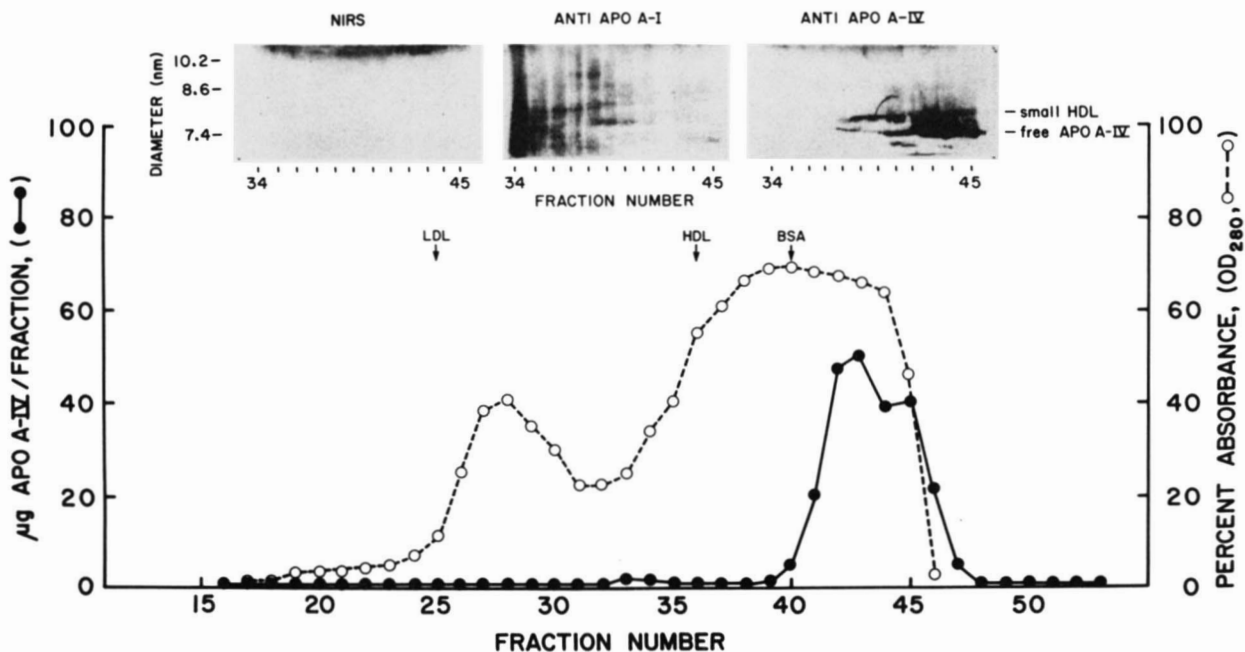

Fig. 8 Bio-Gel A-5m gel filtration of fasted abetalipoproteinemic plasma. Aliquots of column fractions were analyzed for apoA-IV (closed circles) mass by RIA and optical density by absorbance at 280 nm (open circles). Aliquots (40 μl) of column fractions 34–45 were resolved by 4/30 GGE and transferred to cellulose nitrate paper and immunolocalized for apoA-I, apoA-IV, and nonspecific staining with non-immune rabbit serum (inserts). Mean migration distances of major plasma HDL and small spherical HDL were calibrated from high molecular weight standards applied to duplicate gels (20, 21). Indicated by arrows are the elution volumes of LDL (d 1.020–1.050 g/ml), HDL (d 1.063–1.21 g/ml), and bovine serum albumin (BSA).

(Fig. 9B). As shown in Fig. 9B a large number of lipid-staining particles were present in fractions 32–41. The trailing of LDL into fractions 32–35 was apparent by staining near the top of these lanes, while the staining of many HDL subclasses was apparent in fractions 34–41 (diameter 7.8–13.3 nm). Light blue (not black) staining in the region of albumin (diameter 7.4 nm) may represent lipid bound to albumin or minor impurities in Sudan Black B bound to albumin (30). Of interest was the lipid staining of 7.8–8.0 nm diameter particles most apparent in fractions 40–41. These particles also contain apoA-I and apoA-IV (Fig. 5).

We have previously shown an enriched population of small HDL particles in rat mesenteric lymph (20). These particles contained predominantly saturated cholesteryl esters and were shown to be of secretory origin. Therefore it was of interest to examine the ratio of saturated to unsaturated cholesteryl esters of gel-
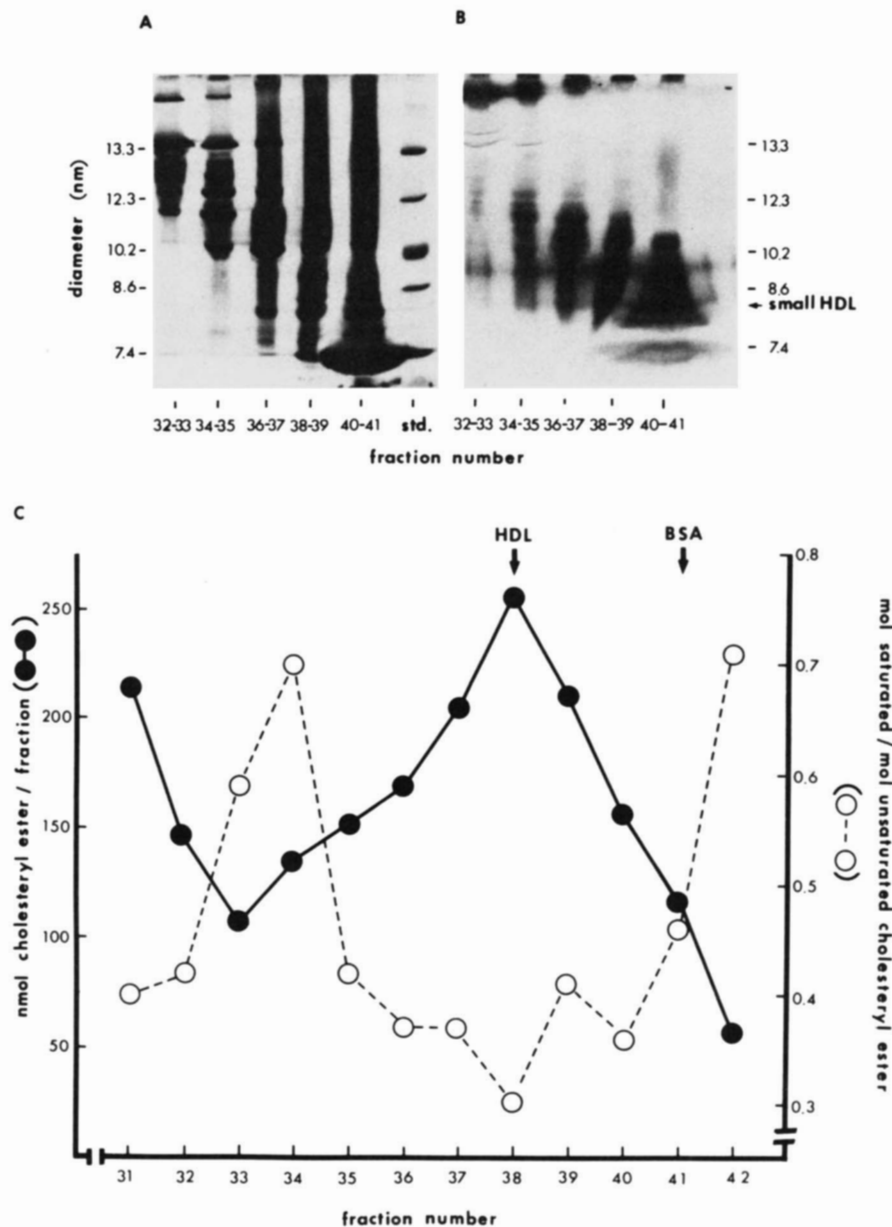

Fig. 9   Bio-Gel A-5m gel filtration of fasted normal plasma: evaluation of lipid containing particles and saturated cholesteryl esters. Two ml of normal plasma was fractionated by Bio-Gel A-5m chromatography. Aliquots of pooled fractions were concentrated and subjected to 4/30 GGE and stained for protein (A) or lipid (B). Aliquots of individual fractions were extracted and analyzed for cholesteryl ester mass (—●—) and degree of saturation (– –○– –) (C) as described in Experimental Procedures.

filtered plasma. Fig. 9C shows both the cholesteryl ester mass and saturated-to-unsaturated ratio over the HDL region of gel-filtered fasted plasma. Two apparent regions of increased saturated-to-unsaturated cholesteryl ester ratio were noted. Both regions had reduced cholesteryl ester mass. The first region (fractions 33–34) preceded the main peak of major HDL (fractions 35–40), while the second region (fractions 41–42) eluted in fractions that followed major HDL. The first region (fractions 33–34) eluted with fractions where we normally observe elution of discoidal HDL in LCAT-deficient plasma.[2] The second region had coincident migration with gel-filtered apoA-I–apoA-IV small HDL (Fig. 5).

DISCUSSION

In the present study, we describe a method for the purification of urinary chylomicron apoA-IV by gel filtration on Sephadex G-150 SF. It was essential to add a reducing agent and to repass apoA-IV-enriched fractions in an ascending manner in order to achieve sufficient purification to obtain material suitable for production of monospecific antibodies. Starting with 40 mg of chylomicron protein (approximately 4 mg of apoA-IV), 700 μg of apoA-IV was obtained.

An RIA to quantitate apoA-IV was developed. Under conditions of the assay, linear displacement of ^{125}I-labeled apoA-IV occurred between 6.25–400 ng of apoA-IV. To optimize recognition of antigen in this RIA, as reported for other apolipoproteins (36–41), a detergent (SDS) was added to expose additional apoA-IV antigenic determinants. Thus measurement of apoA-IV in this RIA made use of an external serum standard calibrated with purified apoA-IV. Unlike reported (3, 4, 6, 15) immunoelectrophoretic assays, in the present RIA both standard and unknown samples undergo identical manipulation prior to assay. In immunoelectrophoretic quantitative apoA-IV assays some samples require delipidation (6), prior to resolubilization and spotting of small aliquots (2–10 μl) to plates, and may lead to procedural losses.

In the present study, the ingestion of 40 g of fat by normal males resulted in a 31% elevation in plasma apoA-IV levels. These data are relatively similar to those previously reported when apoA-IV was measured by rocket immunoelectrophoresis; however, the values for apoA-IV when measured in these studies are 2–3 times greater and appear to represent absolute plasma values for this apolipoprotein. This apparent discrepancy is unclear; however, unlike all other reported purifications, SDS was not utilized in the purification of chylomicron apoA-IV. We confirmed these plasma A-IV levels by two independent methods (quantitative immunoelectrophoresis and RIA).

The localization of apoA-IV to plasma HDL subfractions extends previous data from our laboratory (6, 7, 20, 42, 43). Studies employing ultracentrifugal preparation of human lipoproteins have failed to demonstrate apoA-IV as an HDL component. As shown in the present study, apoA-IV is dissociated from plasma lipoproteins during ultracentrifugation. Green et al. (6) showed that 23% of plasma apoA-IV was associated with plasma HDL when lipoproteins were separated by gel filtration. These results were confirmed in the present study (Figs. 4 and 5). We have extended these results by further localizing apoA-IV to subfractions of HDL. Our studies indicate that apoA-IV is broadly distributed throughout the HDL fraction when this is further analyzed by 4/30 GGE or by immunoprecipitation of HDL particles with apoA-I antiserum (Table 2). Of the four plasma apoA-IV isoforms we find that major HDL contains the two more acidic forms. Preliminary data suggest that free apoA-IV is enriched in the two more basic forms. The apoA-IV isoform composition of small HDL awaits isolation of these particles. Thus, apoA-IV lipid binding, at least in the case of major HDL, appears to reside in specific isoprotein structure.

Of particular interest was the finding that apoA-IV levels were markedly reduced in abetalipoproteinemia (Table 1), a condition marked by absent triglyceride-rich lipoprotein secretion from liver and intestine (35). Localization of apoA-IV to HDL subfractions in abetalipoproteinemia showed an absence of apoA-IV in most HDL subfractions, suggesting that a portion of these HDL particles (and apoA-IV) is derived from triglyceride-rich lipoproteins. It is well established that one source of plasma HDL derives from a redistribution of apoproteins and phospholipid from the surface of triglyceride-rich lipoproteins subsequent to their lipolysis (44–49). The present studies suggest that apoA-IV is also transferred to HDL during this process. This is also supported by the increased apoA-IV levels after lipid feeding, a portion of which is associated with the plasma HDL fraction (Fig. 5). While the intestine is a major source of plasma apoA-IV it is possible that this apoprotein is also secreted by liver. Human hepatoma G 2 cells (50) secrete apoA-IV (10), although the lipoprotein localization of hepatic apoA-IV is unknown. Thus it is possible that a portion of the apoA-IV associated with plasma HDL may also derive from the liver, but direct proof of this is lacking.

A major finding in the present study was the localization of apoA-IV to a small HDL particle with an apparent diameter of 7.8–8.0 nm as assessed by 4/30 GGE.

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[2]Sachdev, O. P., C. L. Bisgaier, and R. M. Glickman. Unpublished results.

Fat feeding appeared to increase the concentration of these particles as assessed by the intensity of staining for apoA-IV in this HDL subfraction (Fig. 5), suggesting an intestinal origin for at least some of these particles. Other evidence supporting the secretory origin of these particles was the finding that gel-filtered fractions containing small HDL had an elevated saturated-to-unsaturated cholesteryl ester ratio (Fig. 9C). This finding suggests that the cholesteryl esters of these particles are predominantly derived through the action of intracellular acyl:cholesterol acyl transferase. The finding that this fraction was prominent in abetalipoproteinemic plasma also strongly argues for an independent secretion of these particles that is not dependent on triglyceride-rich lipoprotein secretion. In abetalipoproteinemia, these particles appeared slightly smaller than particles seen in normal plasma, and may represent a more nascent form of secretory HDL. Similar particles have been described by Decklebaum et al. (51) in abetalipoproteinemia and by others (21, 22) in normal plasma. The failure to find apoA-IV associated with these particles was undoubtedly due to losses resulting from ultracentrifugation. Strong evidence for direct secretion by the intestine of small spherical HDL particles is provided by studies of rat mesenteric lymph HDL. Forester et al. (20) isolated small spherical HDL particles from mesenteric lymph and demonstrated active secretion of intestinally derived cholesteryl ester in these particles. These particles also had elevated saturated cholesteryl ester and contained both apoA-I and apoA-IV. More recently, Magun, Brasitus, and Glickman (42) isolated similar particles from rat intestinal Golgi, further supporting the nascent origin for these particles. Similar spherical HDL particles have been isolated from rat liver Golgi (52).

The metabolism of similar small spherical particles in human plasma is suggested by their prominence in various states of lecithin:cholesterol acyltransferase (LCAT) deficiency, i.e., familial LCAT deficiency (53, 54) and acquired LCAT deficiency in alcoholic liver disease (43).[3] In these conditions small spherical particles are a prominent HDL component and appear to increase in number during fat feeding (43, 54). It is presumed that these particles acquire additional core cholesteryl ester through LCAT action and are transformed into larger HDL forms.

Although there is no known metabolic function for apoA-IV, recent studies suggest that apoA-IV may play a role in LCAT activation. Steinmetz and Utermann (55) demonstrated that apoA-I or apoA-IV containing proteoliposomes are suitable activating substrates for LCAT. Studies from our laboratory[4] in two apoA-I- and apoC-III-deficient subjects demonstrated a normal plasma percentage and fatty acid composition of esterified cholesterol, suggesting formation by LCAT

although they lack apoA-I, a known activator of this enzyme (56, 57). Finally, recent studies by DeLamatre et al. (58) clearly showed marked alterations in apoA-IV distribution correlating with LCAT activity. The precise interrelationship of apoA-IV and LCAT activity requires further study.

Overall, the present studies demonstrate that plasma apoA-IV is increased with fat feeding. Furthermore, it appears that, in addition to chylomicron and free apoA-IV, a portion of this protein is associated with subfractions of plasma HDL. At least some of the apoA-IV associated with the major HDL fraction appears to be derived from triglyceride-rich lipoproteins, while the small HDL particle appears to be of secretory origin. Whether or not small HDL serves as precursor to the major HDL of normal plasma is unknown. The significance of this apoprotein on HDL in relation to lipoprotein metabolism warrants further investigation.

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