Apolipoprotein E-enriched lipoprotein subclasses in normolipidemic subjects

Joyce Corey Gibson, Ardon Rubinstein, Phillip R. Bukberg, and W. Virgil Brown

Division of Arteriosclerosis and Metabolism, Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029

Abstract This study was undertaken to objectively define the lipoprotein association of apoE without ultracentrifugation and represents a description of three distinct apoE-containing lipoprotein (LP) subclasses in normal human plasma. The lipoproteins of whole plasma were fractionated in a continuous manner by molecular sieve chromatography using 4% agarose and the elution profile of apoE was compared to that of apoB, apoA-I, cholesterol, and triglyceride. The data show that all apoE in normal subjects is LP-associated and is confined to three discrete LP subfractions of characteristic size. The largest (fraction I), appears to be a subclass of very low density lipoproteins (VLDL). The other two, however, are distinct in size from the major cholesterol-carrying LP; fraction II is intermediate in size between VLDL and the major apoB-containing lipoprotein, LDL; and fraction III is larger than the major apoA-I-containing lipoproteins, HDL, but smaller than LDL. Parallel chromatography of supernatant and infranatant fractions obtained after ultracentrifugation of serum at each of three different densities, 1.006, 1.019, and 1.063 g/ml confirmed that fraction I is of density less than 1.006 g/ml, but that fractions II and III overlap the conventional density intervals.—**Gibson, J. C., A. Rubinstein, P. R. Bukberg, and W. V. Brown.** Apolipoprotein E-enriched lipoprotein subclasses in normolipidemic subjects. *J. Lipid Res.* 1983. **24:** 886–898.

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Although ultracentrifugation has provided a valuable basis for broad categorization of the major plasma lipoproteins (LP), there is growing evidence of physical, chemical, and metabolic heterogeneity within classes. The traditional density classification is based on the relative proportions of polar lipid, nonpolar lipid, and protein in a LP molecule. No distinction is usually made, however, between LP of similar density but different apolipoprotein composition. The apolipoprotein family concept introduced by Alaupovic (1) is one exception. This proposal remains inadequately tested, however, due to the lack of appropriate techniques that separate lipoproteins based on apoprotein composition. At least 10 specific lipid-binding apolipoproteins have been identified as integral components of LP molecules. In view of the unique structural and functional roles which have been attributed to these apolipoproteins, the apolipoprotein composition of individual LP particles may underlie the significant metabolic heterogeneity demonstrated for LP classes separated by buoyant density.

Recent studies have identified apolipoprotein E (apoE) as a particularly important regulator of LP/cellular interaction. ApoE is a single polypeptide chain of 299 amino acids with a molecular weight of approximately 34,000 daltons, depending on its degree of glycosylation and on the specific isoprotein. Studies in vitro and in situ have suggested that apoE facilitates the hepatic removal of LP's or triglyceride emulsions (2–4). This may be a reflection of the fact that apoE interacts with the LDL receptor (5) and that an additional receptor specific for apoE exists in hepatic tissue (6, 7). Questions remain, however, as to whether the specific association of apoE with other apolipoproteins and lipids may further modify the capacity of a LP to interact with cells.

The concept that LP subfractions relatively enriched in apoE exist in plasma is not new. Heparin-Sepharose chromatography has been utilized previously to isolate apoE-containing subfractions of both VLDL (8, 9) and HDL (10, 11). These studies, however, incorporated an initial ultracentrifugation step which has been shown to result in dissociation of apoE from LP (12). In addition, this affinity technique lacks specificity since apoB also binds to heparin-Sepharose. ApoE-rich LP within HDL (HDL_c) and VLDL (beta VLDL) of cholesterol-fed animals and humans have also been identified by ultracentrifugation and Geon Pevikon electrophoresis (9, 13). However, this approach too has relied on an initial ultracentrifugation that may alter apolipoprotein composition. The potential significance of accurate quantification of these subclasses has been emphasized by the

Abbreviations: apoE, apolipoprotein E; LP, lipoproteins; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; RIA, radioimmunoassay; TMU, tetramethylurea; IEF, isoelectric focusing.

association of beta VLDL levels with atherosclerosis in nonhuman primates (14).

Molecular sieve chromatography using agarose beads provides a method of separating LP on a preparative scale without exposure to ultracentrifugal forces. This procedure has been shown previously to separate the major LP classes from plasma as well as to subfractionate VLDL on the basis of molecular size (15, 16). Three previous studies in humans have attempted to define the association of specific apolipoproteins within this spectrum of plasma LP (17–19). Using 6% agarose, these studies confirmed the known association of apoE with LP having the size of both VLDL and HDL, but this chromatographic medium did not provide sufficient discrimination among LP having the size of VLDL and IDL to more precisely define the apoE content in each of these.

The present study describes the utilization of 4% agarose column chromatography to define the distribution of apolipoproteins and lipids in whole plasma as a function of particle size. This medium is preferable to 6% agarose due to its ability to fractionate molecules in the size range of VLDL and IDL as well as HDL. This separation technique, combined with sensitive and specific radioimmunoassays for apoproteins E, B, and A-I has shown that in normolipidemic subjects apoE is associated with three distinct LP subfractions. The first of these (subfraction I) is coincident with the very low density lipoproteins where apoE has a distribution following that of triglyceride. Fraction II is slightly larger than LDL, and fraction III is intermediate in size between LDL and HDL. Thus, these apoE-containing particles appear to be unique lipoproteins, distinct from the major forms of LDL and HDL.

METHODS

Materials

Sephadex G-150 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Agarose A-15M was purchased from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin (fraction V), ovalbumin, lactoperoxidase, and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol-6000 was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ); sodium decyl sulfate was from Eastman Kodak Co. (Rochester, NY); and aprotinin (Trasylol^{R}) was from Mobay Chemicals (New York, NY). Carrier-free ^{125}I was purchased from ICN Chemicals and Radioisotopes (Irvine, CA). Reagent kits for cholesterol and triglyceride analyses were obtained from Abbott Laboratories (Chicago, IL) and Calbiochem-Behring Corp. (LaJolla, CA), respectively. All other reagents were routinely obtained from Fisher Scientific Co. (Fair Lawn, NJ). Antibodies raised against the primary immunized species were purchased from the Pocono Rabbit Farm, Canadensis, PA.

Lipid analyses

Cholesterol and triglyceride concentrations of plasma and column fractions were measured by specific enzymatic methods using an ABA-100 Analyzer (Abbott Laboratories, Chicago, IL) (20, 21). Lipoprotein cholesterol levels were determined according to Lipid Research Clinic methodology (22), except that dextran sulfate and $MgCl_2$ were used to precipitate the apoB-containing LP before measurement of HDL cholesterol (23).

Isoelectric focusing

Isoelectric focusing was done on VLDL samples prepared by ultracentrifugation at density 1.006 g/ml for 16 hr at 39,000 rpm in a 40.3 rotor in a Beckman L5-65 ultracentrifuge. After delipidation with 20 volumes of acetone followed by 20 volumes of isopropanol, apoVLDL was solubilized in 50 mM ammonium bicarbonate containing 4 mM decyl sulfate and focused on 7.5% polyacrylamide gels containing 8 M urea, as described by Catapano et al. (24) using an ampholine mixture of 60% pH 5–8 and 40% pH 3.5–5. Gels were fixed in two changes of 12.5% trichloroacetic acid and stained with a solution of 0.02% Coomassie G-250 in 3.5% perchloric acid.

Subjects

Normolipidemic laboratory personnel and volunteers were utilized for analysis of apoE in serum and LP subfractions. In addition, a series of serum samples (n = 180) representing a male subset of a normal industrial population was stored frozen at −80°C. After lipid analysis, all 108 samples with triglyceride values less than 150 mg/dl were chosen for apolipoprotein analysis. All blood samples were obtained from antecubital veins of subjects who had fasted for at least 10 hr.

Column chromatography

A 2.5 × 100 cm glass column was packed in 4% agarose beads (Biogel A-15M, 200–400 mesh) and equilibrated in 0.01 M sodium barbital, 0.15 M sodium chloride, 0.01% EDTA, 0.02% sodium azide, pH 7, containing 500 KIU Trasylol per ml, in order to inhibit proteolytic activity. For fractionation of LP, 5 ml of plasma (EDTA, 1.0 mg/ml) was applied to the column at 4°C within 24 hr of collection either with or without dinitrothiobenzene (DTNB) and lipoproteins were eluted at 20 ml/hr with the same buffer. Fractions of approximately 10 ml were collected and weighed to

determine exact volume in order to calculate precise elution volumes. The same column was used for all studies reported with no apparent change in flow rate or elution characteristics.

The recoveries of each of the apolipoproteins after gel filtration were determined by comparison of the plasma concentration assayed directly with the summation of the mass in each column fraction divided by the volume of plasma chromatographed. For apoE, these recoveries were 107.9 ± 11.8% (mean ± standard deviation). For apoB, 95.7 ± 13.3% was recovered, and 102.4 ± 8.8% of apoA-I was recovered.

Radioimmunoassay (RIA) of apoprotein E

ApoE purification. ApoE for immunization, standardization, and iodination was purified from VLDL prepared by ultracentrifugation of plasma at d 1.006 g/ml. After a single wash at the same density to remove serum albumin, VLDL was lyophilized and then delipidated by heptane extraction followed by ethanol–ether as previously described (25). The apoVLDL was solubilized in 6 M urea, 0.01 M Tris-HCl, pH 8.2, containing 100 mM sodium decyl sulfate. ApoE was then separated from other VLDL apoproteins by gel filtration over a 2.5 × 100 cm column of Sephadex G-150 equilibrated in 6 M urea, 0.01 M Tris-HCl, 4 mM sodium decyl sulfate, pH 8.2. ApoE thus obtained was slightly contaminated with the apoC peptides and albumin. A further purification was achieved by preparative polyacrylamide gel electrophoresis or by repeated gel filtration. The apoE preparation used for immunization and for standardization was pure as judged by homogeneity on polyacrylamide gel electrophoresis and by failure to react by immunodiffusion analysis against anti-apoB, anti-apoC-III, or anti-human albumin.

Immunization. Antiserum to purified human apoprotein E was prepared by immunization of a goat following the general protocol used for immunization with each apoprotein antigen. Specifically, an initial injection consisting of 100–200 μg of apoE emulsified in complete Freund's adjuvant was followed by two injections of 50–100 μg of apoE emulsified in incomplete Freund's adjuvant at 2-week intervals. All injections were intradermal at multiple sites on the back. The goat antihuman apoE serum so generated was monospecific as judged by Ouchterlony double immunodiffusion. Purified apoA-I, apoC-II, apoC-III, and human serum albumin, as well as an LDL preparation made free of apoE by application to an anti-apoE immunoaffinity column, all failed to displace ^{125}I-labeled apoE in the RIA at protein concentrations at least 2000 times that of the level of detection of apoE.

Iodination. Purified apoprotein E was iodinated by the chloramine T method of Greenwood, Hunter and Glover (26) using 50 μg of chloramine T/5 μg of apoE. A specific activity of 20–35 μCi/μg apoE was usually obtained. Free ^{125}I was removed by dialysis against two 1-liter changes of 0.15 M NaCl, 0.01% EDTA, pH 7.4, followed by dialysis against 1 liter of 5 M guanidine HCl, 0.01 M Tris-HCl, pH 8.0. The ^{125}I-labeled apoE was then repurified by column chromatography on a 1.5 × 100 cm column of Sepharose CL-6B equilibrated in 5 M guanidine HCl, 0.01 M Tris-HCl, 0.1% ovalbumin, pH 8.0. Purified ^{125}I-labeled apoE in guanidine-HCl was then stored directly at −80°C and diluted in RIA buffer (Table 1) immediately prior to assay. This tracer was stable for at least 6 weeks.

Assay. The conditions for the radioimmunoassay of apoE are summarized in **Table 1.** The addition of Tween 20 (0.5%) and of sodium decyl sulfate (2.5 mM) to the basic borate buffer appeared to provide optimal displacement curves and to enhance reproducibility. The use of ovalbumin (0.1%) proved to give greater sensitivity and better precision than the more common use of bovine serum albumin. Glass tubes, 12 × 75 mm, rinsed in 0.01% Tween 20 and then dried, were used in all assays. Pipetting was performed with a Micromedic automatic pipetter diluter. A typical assay consisted of the addition of 100 μl of goat anti-human apoE serum, diluted 1:25,000 in RIA buffer, to each tube followed by the addition of 100 μl of test sample or standard with 300 μl of a solution consisting of ^{125}I-labeled apoE (15,000 cpm) and 0.25 μl of normal goat serum. Each group of assays included a series of standards, five quality control sera (stored frozen at −80°C in small aliquots and thawed once), and the test samples, all in duplicate. In addition, duplicate tubes were included to assess *1*) nonspecific binding by the omission of goat anti-human apoE serum; *2*) maximum binding with excess antibody by the addition of goat anti-human apoE serum at a 100-fold dilution; and *3*) maximum binding at the antibody dilution used for the assay by the omission of unlabeled apoE. After vortexing, assay tubes were incubated for 2 hr at 37°C and then for 40 hr at 4°C. Rabbit anti-goat serum (30 μl) was then added to each tube and incubation was continued for an additional 24 hr. Polyethyleneglycol 6000 (PEG) was then added to a final concentration of 10%. The addition of PEG reduced the amount of second antibody required and did not increase the nonspecific binding. Bound antigen was separated from free antigen by centrifugation at 2500 rpm (1500 *g*) in a Beckman centrifuge for 30 min.

Initially, purified apoE was used as standard for this assay. The protein content of the apoE standard was determined by the method of Lowry et al. (27) using bovine serum albumin as standard. A more reproducible and stable standard curve was obtained, however, when a serum sample was calibrated against this purified

TABLE 1. Summary of apolipoprotein RIA conditions

	ApoA-I	ApoB	ApoE
Buffer	0.1 M borate, 3% BSA, 0.5% Tween 20, pH 8.5	0.1 M borate, 3% BSA, 0.5% Tween 20, 5 mM decyl SO_4, pH 8.5	0.1 M borate, 0.1% Ovalbumin, 0.5% Tween 20, 2.5 mM decyl SO_4, pH 8.5
Nonspecific binding	3%	1%	3%
Maximum binding	88–92%	82–86%	78–85%
Working range	2–100 ng/ml	0.2–30 μg/ml	1–100 ng/ml
Coefficients of variation:			
Within assay	4.9%	4.8%	8.1%
Between assay	9.0%	8.7%	12.1%
Normal values			
Males:	1.34 ± 0.25 mg/ml (n = 108)	1.14 ± 0.30 mg/ml (n = 108)	46.7 ± 16.3 μg/ml (n = 108)

apoE primary standard and used routinely as secondary standard. Specifically, the serum to be calibrated was assayed against the purified apoE standard in duplicate in ten different assays and the average apoE concentration determined (42.0 ± 6.7 μg/ml) was used as the true apoE concentration of the secondary standard. This serum was stored at −80°C and used in multiple dilutions as standard for all assays.

Coefficients of variation were determined by repeated analyses of the five quality control sera. The within assay coefficient of variation (8.1%) was calculated as the average of the coefficient of variation (standard deviation/mean × 100) of five replicates of five quality control sera measured in a single assay. The between assay coefficient of variation (12.1%) represented the average of the coefficients of variation for each of the five quality control sera assayed in 14 different assays.

Validation of the RIA for apoE. A necessary condition for the validation of any radioimmunoassay is the demonstration that measured values are independent of dilution over the working range. In **Fig. 1,** the logit-log graph of the displacement of ^{125}I-labeled apoE by multiple dilutions of standard serum, VLDL, and HDL is graphed. The parallelism of these curves confirms this condition for the assay of apoE. In a separate assay, this secondary serum standard was shown to displace in parallel with purified apoE (slopes of logit/log plots were −1.044 and −1.039, respectively).

Since accurate measurement of apolipoproteins is frequently confounded by the apparent masking of antigenic sites by associated lipid, each apolipoprotein RIA was tested to verify that each apoprotein antigen was fully exposed under the usual assay conditions. For apoE, this antigenic expression was tested in two ways. In the first place, three serum samples, nine VLDL samples from hypertriglyceridemic and normal subjects, and six HDL preparations were delipidated using acetone–isopropanol. The apoE concentration before and after delipidation was determined. After correction for an 8% loss of apoE that occurred during delipidation (assessed by inclusion of ^{125}I-labeled apoE), the average ratio of the apoE value before delipidation to the value after delipidation was 1.10 ± 0.24 (n = 18), indicating that even in the presence of high triglyceride levels, all apoE was measured.

In addition, a known quantity of purified apoE was added in varying proportions to one normolipidemic and one hypertriglyceridemic (TG = 379 mg/dl) plasma.

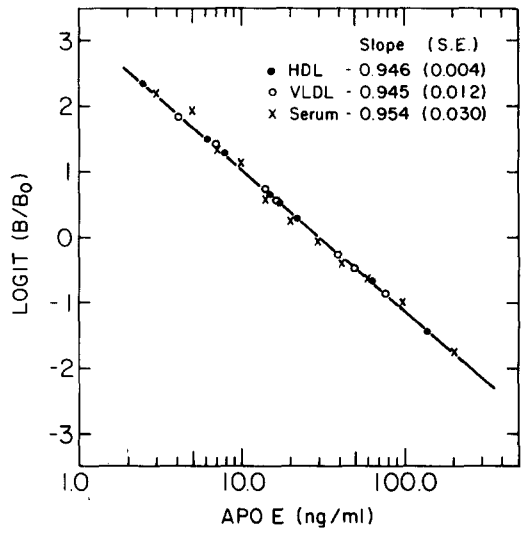

Fig. 1. Logit-log plot representing the displacement of ^{125}I-labeled apoE by serial dilutions of human serum, VLDL, and HDL. The identity of the slopes of the two LP-associated forms of apoE with the serum standard demonstrate that measurement of apoE is independent of dilution.

The recoveries—assessed as the observed/expected × 100—of eight different combinations ranged from 92.0–135%, (x = 113.5 ± 16.8%). We have also measured the serum apoE levels in 108 males selected for triglyceride levels less than 150 mg/dl. The average serum apoE was 46.7 ± 16.3 μg/ml. In this total population of males in this latter sample, the plasma apoE level increased with plasma TG, and the average of the plasma apoE in the 108 males with TG less than 150 mg/dl was significantly less than that in 70 males with TG greater than 150 mg/dl. The five quality control sera used in our assay have been assayed independently by Dr. C. Blum by a similar RIA procedure (17). The average of the ratio of the total plasma apoE levels measured by the present assay to that measured by Dr. Blum in a single assay was 0.979 ± 0.149 with a range of 0.801–1.214.

Radioimmunoassay of apoprotein A-I

Apoprotein A-I purification. ApoA-I for immunization, standardization, and iodination was purified from HDL prepared from normal human plasma by a combination of precipitation and gel filtration. Specifically, apoB-containing lipoproteins were removed from plasma by precipitation with dextran sulfate and $MgCl_2$ (23). The supernatant was then exhaustively dialyzed against 0.01 M Tris, 0.01% EDTA, adjusted to density, 1.195 g/ml with solid KBr, and ultracentrifuged to separate the HDL from other serum proteins. After a single wash at this same density, HDL was lyophilized, delipidated (25), and solubilized in 6 M urea, 0.01 M Tris, 50 mM dithiothreitol (DTT), pH 8.2. ApoA-I was separated from other HDL apoproteins by gel filtration over a 2.5 × 100 cm column of G-150 equilibrated in 6 M urea, 0.01 M Tris HCl, and 4 mM DTT, pH 8.2. The major peak, apoA-I, was further fractionated on DEAE Sephacel in 6 M urea using a linear gradient of Tris-HCl (pH 8.2) between 0.01 and 0.1 M Tris-HCl. This apoA-I was pure as determined by 10% polyacrylamide gel electrophoresis in SDS (28).

Immunization. Antiserum to purified apoA-I was raised in New Zealand white rabbits following the same immunization protocol described for apoE. Monospecificity was established by Ouchterlony double immunodiffusion and by RIA analysis.

Iodination. Purified apoA-I was iodinated using the lactoperoxidase method (29); a specific activity of 20–35 μCi ^{125}I/μg apoA-I was usually obtained. After dialysis against 0.15 M sodium chloride, 0.01% EDTA, pH 7.4 to remove free ^{125}I, the ^{125}I-labeled apoA-I was dialyzed against 6 M urea, 0.01 M Tris, pH 8.2, and purified by gel filtration over a 2.5 × 50 cm column of Sephadex G-150 equilibrated in 6 M urea, 0.01 M Tris-HCl, and 0.1% BSA, pH 8.2. Over 99% of the radioactivity eluted in a single peak. The peak fractions were immediately dialyzed against 5 mM ammonium bicarbonate, 0.05% Tween 20, and then diluted in 0.1 M sodium borate, 0.05% Tween 20, and 0.2% BSA. They were stored in aliquots of appropriate size at −80°C. This tracer was stable for 6–8 weeks.

Assay. The general conditions for the RIA of apoA-I are summarized in Table 1. Optimal conditions for full expression of apoA-I included the addition of 0.5% Tween 20 and 3% BSA to 0.1 M borate buffer. For a typical assay, 100 μl of rabbit anti-human apoA-I diluted 1:12,000 in RIA buffer was added to each tube. This was followed by the addition of 100 μl of each test or standard solution of apoA-I with 300 μl of ^{125}I-labeled apoA-I (15,000 cpm) and 0.25 μl of normal rabbit serum. In analogy with the apoE RIA, quality control sera and appropriate mixtures to measure nonspecific and maximum binding were included in each assay as described. Goat anti-rabbit serum and PEG were used to separate bound and free antigen as described for the apoE RIA.

Between and within assay coefficients of variation are shown in Table 1.

Validation of the apoA-I RIA. The condition of parallel displacement of ^{125}I-labeled apoA-I by purified apoA-I, serum, and HDL over the working range of the assay was confirmed as shown in **Fig. 2.** As initially shown by Mao and Kottke (30), the inclusion of Tween 20 in the RIA buffer resulted in complete antigenic expression of lipoprotein bound apoA-I. This condition was verified in three ways. First, seven sera and five HDL preparations were delipidated with acetone–isopropanol. The average apoA-I measured in the intact sera and

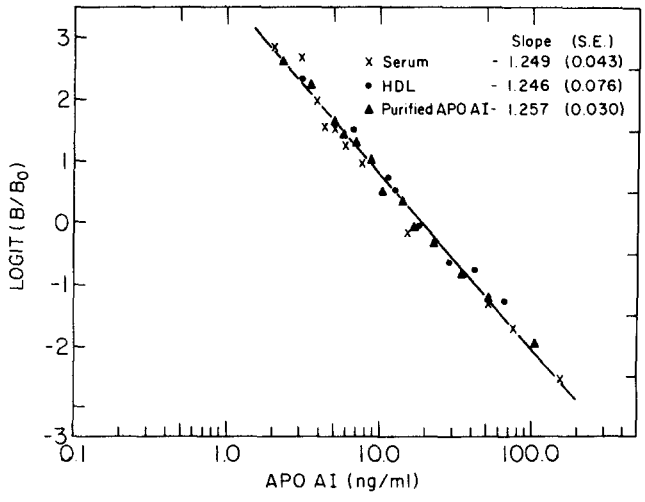

Fig. 2. Logit-log plot representing the displacement of ^{125}I-labeled apoA-I by unlabeled apoA-I and by serial dilutions of human serum and HDL. The identity of the slopes indicates that measurement of apoA-I in serum and in HDL is independent of dilution.

HDL was 1.58 ± 0.43 mg/ml and 2.33 ± 0.83, respectively. After delipidation the average values were 1.43 ± 0.48 and 2.38 ± 0.70, respectively, indicating that no unmasking of antigenic sites in serum or in HDL had occurred with delipidation. Secondly, purified apoA-I was added to a series of serum samples and the apparent recoveries were evaluated. In six recovery studies, the expected/observed apoA-I values ranged from 91–116%, (average = 98.2 ± 9.5%). Thirdly, 35 HDL samples were prepared by sequential ultracentrifugation (31) and the measured apoA-I levels were compared to the protein mass determined by the method of Lowry et al. (27), using BSA as a standard. The average of the measured apoA-I concentration in these HDL taken as a percentage of the total protein was 63.7 ± 8.3%. This is consistent with previous reports on the apoA-I content of HDL (32, 33). Furthermore, the average plasma apoA-I level of 108 normolipidemic males was 1.34 ± 0.25 mg/ml, a value very similar to that reported by others (31, 32).

Radioimmunoassay of apoprotein B

Purification. Apoprotein B was purified for immunization, but apoB in intact LDL was used both as standard and as tracer. LDL was obtained by ultracentrifugation of plasma within the density range 1.02–1.05 g/ml. To prepare apoB, LDL was delipidated as described for VLDL and HDL, and LDL apoB was solubilized in 6 M urea, 0.01 M Tris-HCl, 200 mM decyl sulfate, pH 8.2. ApoB was then purified by gel filtration over a 2.5 × 100 cm column of Sephadex G-100 equilibrated in 6 M urea, 0.01 M Tris-HCl, 50 mM decyl sulfate. The apoB eluting in the void volume was free of other apolipoproteins as judged by polyacrylamide gel electrophoresis.

Immunization. Antiserum to purified apoB was raised by immunization of a sheep, according to the protocol described for apoE. This anti-human apoB serum was monospecific as judged by both Ouchterlony double immunodiffusion and by failure of apoA-I, apoC-III_2, or of human serum albumin to show displacement in the RIA. Sheep anti-human and apoB IgG was prepared from whole antiserum by sodium sulfate precipitation (34).

Iodination. ^{125}I-labeled LDL apoB was obtained by iodination of LDL (1.02–1.05 g/ml) according to a modification (35) of the ICl method of McFarlane (36). After iodination, ^{125}I-labeled LDL was dialyzed first against 0.15 M NaCl, 0.01% EDTA, pH 7.4, and then against 0.1 M glycine, 2.5 mM sodium decyl sulfate, 0.05% Tween 20, pH 10, in preparation for gel filtration on 6% agarose (Biorad A5-M) in the same buffer containing 0.1% BSA. A specific activity of 0.15–0.20 μCi ^{125}I/μg of LDL protein was usually obtained. This purified tracer was stored at 4°C and was stable for approximately 4 weeks.

Assay. General conditions for the RIA of apoB are summarized in Table 1. As for apoE and for apoA-I, the inclusion of detergents (in this case, 0.5% Tween 20 and 5 mM decyl sulfate) provided optimal assay performance. A typical assay tube included 100 μl of sheep anti-human apoB IgG (25 mg/ml), diluted 1:3500 in assay buffer, 100 μl test or standard solution, and 500 μl of ^{125}I-labeled LDL (15,000 cpm) with 0.25 μl of normal sheep IgG. Quality control sera and blanks were included as described for apoE. After incubation at 4°C for 48 hr, 50 μl of rabbit anti-sheep serum was added and incubation was continued for 24 hr. Bound apoB was pelleted and washed as described for apoE.

As standard, multiple dilutions of a calibrated serum standard proved to be more stable than purified LDL. For calibration of this secondary standard, several discrete LDL fractions were prepared by repeated ultracentrifugation within the density interval 1.02–1.05 g/ml. Each LDL preparation was dialyzed against 0.1 M glycine, 0.05% EDTA, pH 10, stored at 4°C, and used within 1 week for calibration of a standard serum pool. The Lowry protein value of the LDL was used as a measure of LDL apoB since no other apoproteins could be detected by Ouchterlony double diffusion analysis against anti-apoE, anti-apoC-III, anti-apoA-I, and anti-human serum albumin. The average apoB value of the standard serum was then determined and used as the true apoB value of the secondary standard for subsequent assays. At approximately 6-week intervals, the serum was restandardized against freshly prepared LDL. Calibrated against four different LDL preparations over 3 months, this serum pool in ten dilutions read 0.767 ± 0.063 mg/ml, 0.720 ± 0.062 mg/ml, 0.723 ± 0.058 mg/ml, and 0.722 ± 0.062 mg/ml. Between and within assay coefficients of variation were determined as described for apoproteins E and A-I and are summarized in Table 1.

Validation of apoB RIA. **Fig. 3** represents the displacement of ^{125}I-labeled LDL by multiple dilutions of LDL, serum, and VLDL. The parallel curves generated by the logit-log analyses confirm that the apoB value of these samples is independent of dilution.

Evaluating the antigenic expression of lipoprotein-associated apoB is confounded by the insolubility of delipidated apoB in aqueous buffers. We used two different approaches to this problem. In one study, two VLDL preparations and seven sera were evaluated by RIA before and after incubation with lipoprotein lipase purified from bovine milk (37). This treatment served to hydrolyze 80–100% (mean ± 96.8%) of the triglyceride present. The ratio of apoB measured directly to apoB measured in samples treated with lipoprotein li-

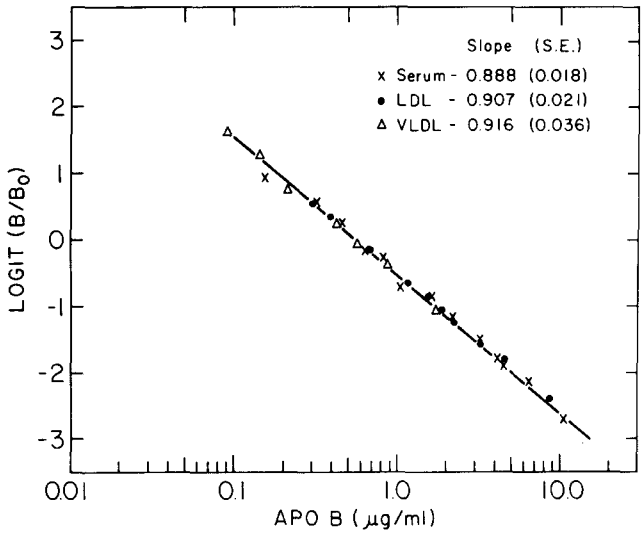

Fig. 3. Logit-log plot representing the displacement of ^{125}I-labeled LDL by unlabeled human LDL and serial dilutions of human serum and VLDL. The parallelism of the displacement curves is shown by the identity of the slopes and verifies that apoB measurement is independent of dilution in both serum and VLDL.

pase was 1.00 ± 0.11. In addition, an estimate of the apoB content of five VLDL samples was made using the TMU procedure of Kane (38) and this value was compared to the apoB concentration determined by RIA. The ratio of RIA/TMU values ranged from 0.81–1.51 with a mean ± SD = 1.16 ± 0.30, indicating that the RIA was not underestimating apoB in the presence of lipid.

RESULTS

Study subjects

In this initial study, the apolipoprotein distribution in 11 normolipidemic subjects, 6 males and 5 females, was examined. **Table 2** summarizes the clinical characteristics, and plasma lipid and apolipoprotein levels of these subjects. Ten of the 11 individuals studied had total cholesterol, triglyceride, LDL, and HDL cholesterol levels within the normal range for age reported by the Lipid Research Clinics (39), and plasma apolipoprotein levels that were similar to published normal values (17, 18, 30, 32, 33, 40) and similar to those determined by us for a control population of adult males (Table 1). Subject 1 had very low plasma LDL cholesterol and correspondingly low apoB levels on repeated analysis. ApoE is known to exist as several isoforms of varying charge. These may be distinguished by both one- and two-dimensional isoelectric focusing pattern (IEF). The one-dimensional IEF pattern of these subjects has been examined. These data have been interpreted in Table 2 in terms of homo- or heterozygosity for the three major apoE isoforms apoE-2, apoE-3, and apoE-4. Unequivocal assignment of these isoform patterns is only possible with two-dimensional electrophoresis or from IEF before and after cysteamine treatment.

Column chromatography

Application of whole fasting plasma from these normal subjects to 4% agarose columns resulted in the complete separation of the three major lipoproteins, VLDL, LDL, and HDL, as identified by the cholesterol and triglyceride profiles of the subject shown in **Fig. 4.** The effectiveness of this separation was confirmed by analysis of apoB, the major apolipoprotein of LDL and of apoA-I, the dominant apolipoprotein of HDL (Fig. 4). In all studies thus far, apoB chromatographed in a peak coincident with the major cholesterol peak. ApoA-I was coincident with the region of the second cholesterol peak, although the ratio of cholesterol to A-I content was usually higher in the earlier fractions of this peak. In contrast, the pattern of elution of apoE was complex. In the 11 normolipidemic fasting subjects studied thus far, apoE was distributed among three LP regions.

TABLE 2. Clinical characteristics and lipid and apolipoprotein values

Subject	Sex	Cholesterol	Triglyceride	HDL-Chol	ApoB	ApoA-I	ApoE	ApoE Isoform Pattern
		mg/dl	*mg/dl*	*mg/dl*	*mg/ml*	*mg/ml*	*mg/ml*	*μg/ml*
1	M	137	30	68	0.346	1.75	32.7	E4
2	F	222	38	83	0.810	1.92	32.8	E3/4
3	M	167	52	68	0.520	1.51	31.9	E4
4	F	181	81	55	0.626	1.45	80.7	E3/4
5	M	182	54	44	0.928	1.65	49.8	E2/4
6	F	164	53	32	0.738	1.52	17.7	E3/4
7	M	206	58	40	0.800	1.29	42.5	E3
8	F	185	93	37	0.929	1.21	38.9	E3
9	M	165	118	36	0.781	1.40	35.2	E2/3
10	F	186	99	36	0.989	1.42	51.8	E3
11	M	210	54	65	0.921	1.79	67.9	E3

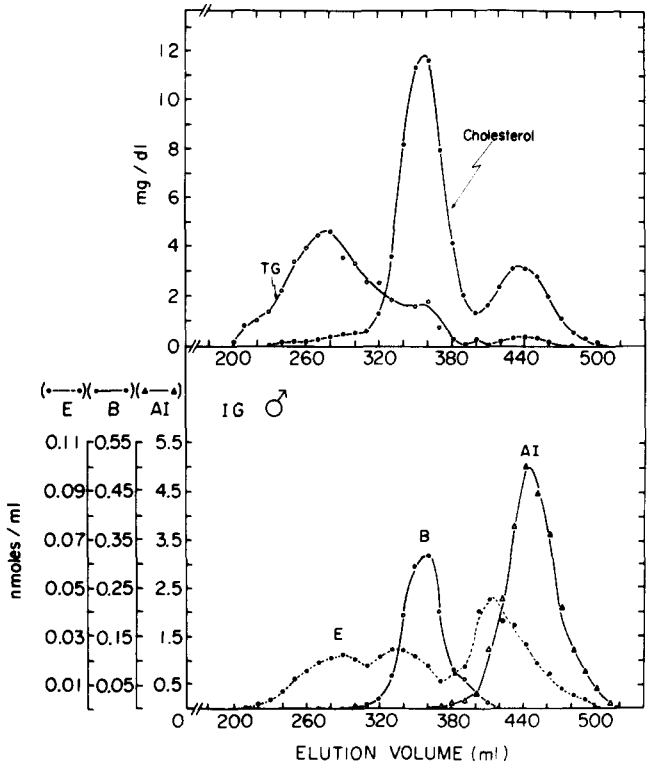

Fig. 4. Lipid and apolipoprotein profiles of normal human plasma after 4% agarose column chromatography. ApoB and apoA-I cochromatograph with LDL and HDL cholesterol, respectively, but apoE elutes in three peaks. Peak I is coincident with VLDL, peak II is intermediate in size between VLDL and LDL and peak III is slightly larger than the major HDL fraction.

The first peak of apoE was coincident with the peak triglyceride in fasting subjects, indicating that this fraction of the plasma apoE was a component of VLDL (fraction Iv). In nonfasting subjects, peak I actually comprised two separate fractions, one coincident with chylomicrons in the void column (Ic) and one eluting with VLDL (Iv). Two to 30% of the plasma apoE was associated with VLDL in these fasting samples. The two dominant peaks of apoE in normolipidemic subjects, however, were distinctly different in size compared to the major LP. The first of these was intermediate in size between VLDL and LDL (fraction II) and the other was slightly larger than HDL (fraction III). The distribution of apoE in the plasma of individual subjects was not affected by the immediate addition of the inhibitor of lecithin cholesterol acyl transferase, DTNB (1.4 mM) to fresh plasma. It is noteworthy that in freshly chromatographed plasma, all apoE was associated with LP fractions. By contrast, when a plasma sample was rechromatographed after storage at 4°C for 1 month, 12.5% of the apoE eluted later, near the salt volume of the column, suggesting that dissociation had occurred during storage.

In general, three patterns for the distribution of plasma apoE among these chromatographically distinguished subfractions emerged from the normolipidemic subjects studied. These patterns are diagrammed in **Fig. 5.** The particular pattern observed was unrelated to sex or to total plasma apoE level. Also, in this small sample of normotriglyceridemic subjects, plasma triglyceride levels were not independently related to the apoE distribution. In contrast, the apoE distribution was related to the plasma concentration of apoA-I. These data are summarized in Table 2 and **Table 3.** Specifically, the tendency for apoE to be associated with fraction III was positively correlated ($r = 0.79$) with plasma apoA-I levels. Partial correlation analysis confirmed that this relationship was independent of plasma triglyceride levels. The first type of pattern observed was a dominant peak of apoE in fraction III (top, Fig. 5). Four of the seven subjects with this pattern also had HDL cholesterol levels that were significantly greater than the LRC norms for age and sex (38) and had very low plasma triglyceride concentrations. The second pattern was a more uniform distribution of apoE between fractions II and III, but with very little associated with the VLDL fraction Iv (middle, Fig. 5). The two subjects demonstrating this pattern were similar in having low triglyceride levels and low to normal HDL levels. The third pattern demonstrated a significant amount of apoE distributed among each of the three apoE-containing fractions (bottom, Fig. 5). The two subjects with this pattern both had low HDL cholesterol levels and plasma triglyceride levels that were higher than the other subjects, though well within normal limits.

In order to compare the distributions of apoE among LP separated by column chromatography and by ultracentrifugation, separate plasma samples from nine of the subjects shown in Table 3 were submitted to sequential ultracentrifugation and the apolipoproteins were analyzed in the $d < 1.006$, 1.006–1.019, 1.019–1.063, 1.063–1.21, and $d > 1.21$ g/ml fractions. These data are tabulated in **Table 4.** Whereas chromatographic separation documented an association of apoE with VLDL and with two other discrete LP subfractions, ultracentrifugation described a more uniform distribution of apoE between VLDL, IDL, LDL, HDL, and a form with $d > 1.21$ g/ml. The substantial quantities of apoE (27.4–45.5%) in the $d > 1.21$ g/ml fraction, confirmed reports of others (12, 17) and raised important questions as to the proportionality of these losses from the different LP classes.

A second limitation of using ultracentrifugation for separation of apoE-containing lipoproteins, is that the flotation densities of the lipoprotein subfractions isolated by column chromatography clearly overlap the conventional density ranges used for lipoprotein sepa-

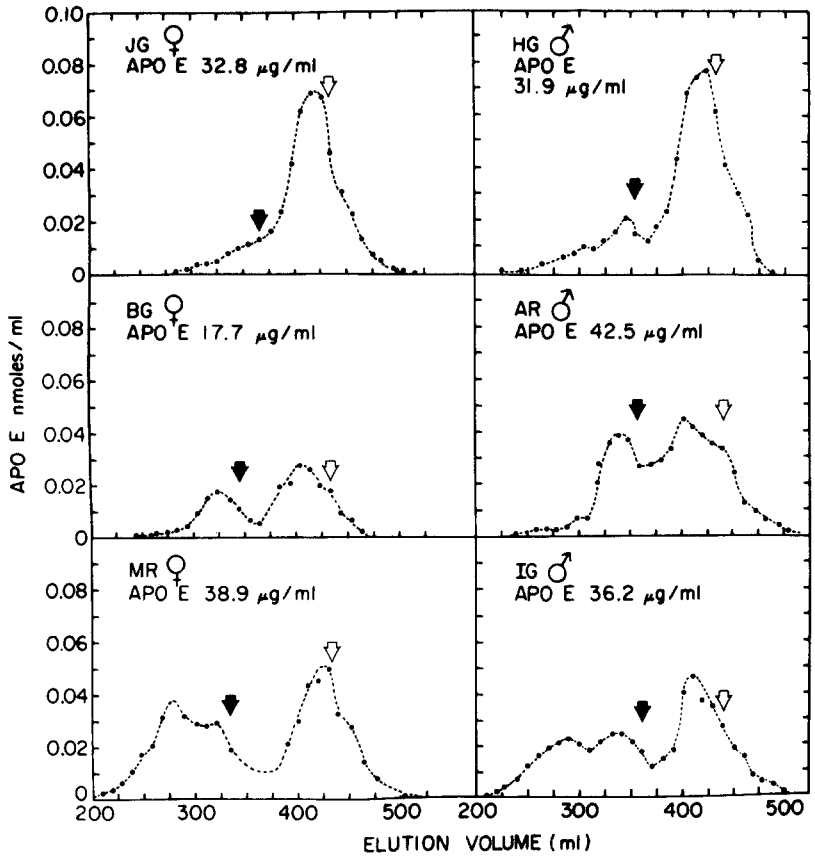

Fig. 5. Summary of apoE profiles from normolipidemic subjects after 4% agarose chromatography. Total plasma apoE concentration is at upper left. Open arrows denote the peaks of HDL and apoA-I and closed arrows denote LDL and apoB.

ration. This was shown in two studies. In the first, replicate aliquots of fractions Iv, II, and III after agarose chromatography of whole plasma from one subject were submitted to ultracentrifugation at the fixed densities of 1.006, 1.019, 1.063, 1.125, and 1.21 g/ml in a Beckman 40.3 fixed angle rotor at 39,000 rpm for 20 hr (d 1.006, 1.019, 1.063 g/ml) or 40 hr (d 1.125, 1.21 g/ml). After reaching equilibrium, the apoE of the upper and lower halves of each tube was measured and the apoE content of each density interval could then be calculated by difference. As expected, greater than 85% of the apoE in fraction Iv, floated at d 1.006 g/ml and more than 94% floated at d 1.019 g/ml. ApoE fractions II and III, however, could not be clearly assigned to any of the density intervals used. After centrifugation,

TABLE 3. ApoE distribution among lipoproteins separated by column chromatography

Subject	E-I	E-II	E-III	Plasma ApoE
		%		μg/ml
1	4.0	8.8	87.3	32.7
2	1.7	9.0	89.2	32.8
3	8.2	16.2	75.6	31.9
4	3.8	11.2	85.0	80.7
5	2.3	17.4	80.4	49.8
6	9.5	29.1	61.4	17.7
7	3.7	37.2	59.2	42.5
8	30.5	23.1	46.3	38.9
9	26.6	23.4	49.9	35.2
10	2.4	18.6	80.9	51.8
11	1.5	9.6	88.9	67.9

TABLE 4. ApoE distribution of lipoproteins separated by ultracentrifugation

Subject	<1.006	1.006–1.019	1.019–1.063	1.063–1.21	>1.21
			%		
2	0	3.4	46.9	22.4	27.4
3	13.4	4.7	21.4	27.2	33.3
4	12.1	3.4	20.7	33.3	30.6
5	29.9	3.4	11.4	25.5	29.8
6	25.8	6.4	15.0	13.0	39.8
7	12.4	1.9	26.3	31.6	27.8
8	25.8	3.4	14.9	15.9	40.0
9	22.3	4.6	17.1	10.6	45.5
10	34.6	2.7	19.0	14.6	29.2

63% of the apoE in fraction II was of density less than 1.006 g/ml and approximately 25% was of density 1.006–1.019 g/ml. Most of the apoE in fraction III (48%) was within the density interval 1.063–1.125 g/ml (HDL_2) and 37% was included between densities 1.125–1.21 g/ml (HDL_3). The remainder was found in the d > 1.21 g/ml infranatant. Individual subjects may vary slightly in the proportions of apoE found in each density interval depending on the degree of lipidation of the LP. The general pattern described, however, was confirmed in one other subject studied.

In a separate series of experiments the lipoproteins were first isolated by ultracentrifugation and then subjected to column chromatography. For this, replicate aliquots of fresh EDTA plasma were submitted simultaneously in separate tubes to ultracentrifugation at densities 1.006, 1.019, and 1.063 g/ml in a Beckman 40.3 rotor at 39,000 rpm for 20 hr. The upper 3 ml and the lower 3 ml were each diluted to 5 ml and subjected to agarose column chromatography as described previously for plasma. The results of this study are shown in **Fig. 6.** The plasma distribution of apoE (Fig. 6a), demonstrated the anticipated three regions of the apoE immunoreactivity, fraction II representing a shoulder of fraction I in this subject (320 to 370 ml).

All of the apoE in fraction I (between 200 and 320 ml) appeared to be included in the d < 1.006 g/ml fraction and all of that in fraction III (between 370 and 500 ml) was of density > 1.006 g/ml (Fig. 6b). Fraction II (between 320 and 370 ml), on the other hand, was included in both d < 1.006 and >1.006 g/ml fractions. Centrifugation at 1.019 g/ml showed that all of apoE in fraction I and nearly all of that in fraction II was of density less than 1.019 g/ml and that fraction III was of density greater than 1.019 g/ml (Fig. 6c). After centrifugation at 1.063 g/ml (Fig. 6d), most but not all, of apoE in fraction III was in the 1.063 bottom fraction, indicating that this fraction overlapped the 1.063 g/ml density cut. These data thus confirm that apoE fractions II and III are not discretely defined by conventional density intervals. Two other subjects were also studied after a single ultracentrifugation at 1.019 g/ml. The resulting chromatographic profiles confirmed that fraction I was all of density less than 1.019 g/ml and fraction III was of density greater than 1.019 g/ml. Fraction II, however, though primarily of density less than 1.019 g/ml, was also identified in the LP fraction of density greater than 1.019 g/ml.

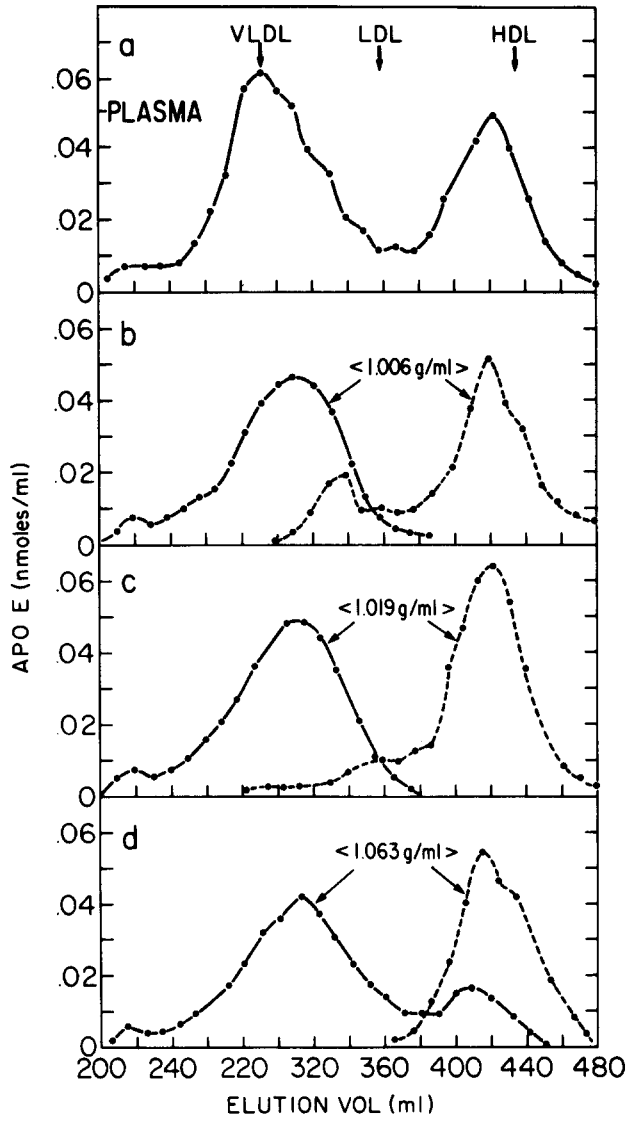

Fig. 6. Distribution of apoE after 4% agarose chromatography of whole plasma and of LP fractions obtained after ultracentrifugation at d 1.006, 1.019, and 1.063 g/ml. After centrifugation for 20 hr the top and bottom halves of the centrifuge tubes were chromatographed. The solid line represents the elution profile of the supernatant fraction and the broken line represents that of the infranatant after ultracentrifugation. In whole plasma, fraction I elutes between 200 and 320 ml, fraction II between 320 and 370 ml, and fraction III between 370 and 500 ml. The corresponding elution volumes were used to characterize these fractions after ultracentrifugation.

DISCUSSION

The major objective of the present study was to provide a more complete description of the distribution of apoE in normal human plasma. Several previous studies have evaluated total plasma apoE levels in normal and hyperlipoproteinemic subjects (17–19). Although total plasma levels of apoE do appear to characterize certain types of hyperlipoproteinemia, particularly dysbetalipoproteinemia (Type III) (17, 18), total apoE levels have proved to be quite insensitive to dietary and other perturbations associated with changes in plasma lipids (41–43). On the other hand, subtle changes in apoE content

of specific LP fractions may be characteristic of physiologically important metabolic processes.

Most previous evaluations of the distribution of apoE among the human plasma LP have utilized the technique of preparative ultracentrifugation either alone or as a preliminary to subfractionation. This technique has at least two disadvantages: *1*) dissociation of apoE from LP occurs; and *2*) arbitrary discontinuities within the spectrum of apoE-containing LP are created. Previous studies have consistently documented the association of apoE with all lipoprotein density classes as well as with a nonlipoprotein fraction of d > 1.21 g/ml when LP fractions were prepared by ultracentrifugation (12, 17, 18). Blum, Aaron and Sciacca (17) however, utilizing 6% agarose column chromatography to fractionate LP in whole plasma, concluded that when plasma was not exposed to ultracentrifugation, all apoE was associated with lipoproteins. Our data confirm this conclusion and strongly suggest that the process of ultracentrifugation leads to a dissociation of apoE from one or more LP fractions. A recent study examining the specific effects of several components of the ultracentrifugal process on the association of apoA-I with HDL, concluded that the most likely cause of the known apoA-I dissociation from HDL was the high pressure generated by ultracentrifugal force (44). Significantly, this study showed that not all apoA-I was capable of dissociating and suggested that more than one pool of LP-associated apoA-I may exist. If an analogous situation exists for apoE, then ultracentrifugal losses may be nonuniform from the various density classes.

Equilibrium ultracentrifugation is a technique that effects a discontinuous separation of lipoprotein classes on the basis of buoyant density. This classification was historically created to maximize separations of LP on the basis of lipid content and is well suited to the fractionation of certain apolipoproteins, such as B and A-I. However, quantitatively minor lipoprotein subfractions that may not possess density characteristics of the major lipid-carrying lipoproteins may overlap several density classes. That this is the case is strongly suggested by our own ultracentrifugal data and those of others which have documented an association of apoE with all major density classes. By contrast, our column chromatographic separation of LP showed that apoE is found in three major size distributions, two of which do not correspond in terms of size or density to the major lipoproteins. These apoE-containing subfractions are quantitatively minor in terms of the lipids and apolipoproteins associated with the major cholesterol-transporting LP, but may be highly significant in a metabolic context.

Fainaru et al. (9) utilized 4% agarose chromatography to specifically characterize beta-migrating VLDL from cholesterol-fed dogs and from human subjects with Type III Hyperlipoproteinemia. Although these investigators did not describe the apoE distribution of these LP, they did identify two LP fractions, on the basis of cholesterol profile, that were rich in apoE. One of these eluted in the void volume and probably represented chylomicrons. The second fraction was smaller than VLDL in size, and may correspond to the fraction II we describe. We have studied the apoE distribution among chromatographically separated LP of one subject with a type III phenotype and have documented a peak of cholesterol that was coincident with the apoE fraction II and was clearly distinct from VLDL and from LDL as identified by the apoB profile. Thus, the apoE fraction II that we describe in normal human plasma may be the normal counterpart of the beta-VLDL accumulating in type III hyperlipoproteinemia and in cholesterol-fed animals. In view of the capacity of beta-VLDL to promote cholesterol esterification and accumulation in mouse peritoneal macrophages (9), the identification and quantification of this apoE fraction may be an important addition to the assessment of cardiovascular disease risk.

HDL has similarly been shown to be heterogeneous in terms of size and apoprotein composition. HDL has traditionally been divided, according to ultracentrifugal criteria, into HDL_2 and HDL_3 (45). Anderson et al. (46), utilizing density gradient ultracentrifugation, further subdivided HDL_2 into two subfractions, the larger and less dense HDL_{2b} (1.063–1.10 g/ml) and HDL_{2a} (1.10–1.125 g/ml) (46). More recently, Blanche et al. (47) have identified HDL subfractions after gradient gel electrophoresis (47). Blum et al. (17) were the first to report a separation by size of a discrete apoE-containing HDL subfraction in humans, but the existence of this subfraction was inferred in a number of earlier studies. Using Geon Pevikon preparative block electrophoresis, Mahley, Weisgraber, and Innerarity (48) isolated HDL_2 subfractions from hypercholesterolemic animals (dogs and swine) that differed with respect to apoE content. The most cholesteryl ester- and apoE-rich was termed HDL_c (48). Both Weisgraber and Mahley (10) and Marcel et al. (11) subsequently utilized heparin-Sepharose affinity chromatography to isolate apoE-enriched HDL subspecies which may be counterparts to HDL_c in human plasma. The apoE fraction III we describe may also be analogous to HDL_c, but due to the fact that the first step in HDL_c isolation is centrifugation at d 1.063–1.125 g/ml, HDL_c may represent only part of fraction III.

Our data go further in suggesting that even within these subclasses, heterogeneity may exist with respect to the association of apoE with other apolipoproteins and lipids. Specifically, in our studies, normal and hyperlipidemic subjects were similar in consistently demonstrating a shoulder on the downslope of the apoE profile which described fraction III (Fig. 5). In view of

the changing relative concentrations of apoE and apoA-I in this region, particles of slightly different size with varying ratios of apoE and apoA-I may exist.

A subject not addressed in this study is the stability of the association of apoE with other apolipoproteins and lipids in these subfractions. The distribution observed within the plasma LP spectrum of any given subject may reflect a steady state mass equilibrium with continuing exchange of apoE molecules. Alternatively, the structures may be relatively stable requiring specific metabolic processes to cause apoE transfer, such as the action of lipoprotein lipase. In either case, it is to be expected that perturbations such as the induction of alimentary lipemia would alter this mass equilibrium, and thus the apoE profile, in parallel with chylomicron flux and transfer of its components to higher density LP (49). We[1] and others (50) have observed that heparin administration in vivo results in a decrement in apoE associated with fraction I that is matched by a rise in apoE in fraction III. In the steady state, however, the stability of the LP association of apoE is not known. Studies are underway to specifically isolate these apoE-containing subclasses and to examine the kinetics of apoE transfer in vivo and in vitro.

In summary, this study has documented the association of apoE with discrete lipoprotein subfractions. The distribution of plasma apoE among each subfraction cannot be inferred from total plasma apoE levels. On the basis of the size and ultracentrifugal characteristics of these subfractions, fractions II and III may represent normal counterparts or precursors of LP subspecies described previously under abnormal conditions of cholesterol feeding (43). In view of the possibility that these abnormal subfractions, (beta-VLDL and HDL_c) have opposing roles in cholesteryl ester accumulation, description of the distribution of apoE among these subfractions and definition of factors determining this distribution may be significant in terms of assessing atherosclerotic disease risk. Gel filtration appears to be the only technique available to fractionate significant quantities of these LP subfractions in a continuous fashion without exposure to ultracentrifugation. For the present study, the combination of gel filtration and RIA analysis has been sufficient for the identification and quantification of these subfractions. More investigative studies, however, will require preparative techniques such as immunoaffinity chromatography (51) to specifically isolate the apoE-containing lipoprotein particles.

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