Evidence for the distribution of apolipoprotein E between lipoprotein classes in human normocholesterolemic plasma and for the origin of unassociated apolipoprotein E (Lp-E)

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Abstract The distribution of apoE between the major lipoprotein classes of normocholesterolemic plasma has been determined by molecular sieve chromatography, immunoaffinity chromatography, preparative ultracentrifugation, and polyanionic precipitation. Highly comparable values were obtained for the first two procedures (correlation coefficient (r) = 0.958), while the other procedures gave recoveries of apoE in high density lipoprotein that were respectively higher and lower, although total recoveries of apoE were essentially complete in each case. Immunoaffinity chromatography under the conditions described was not accompanied by detectable dissociation or redistribution of apoE. By immunoaffinity chromatography, all the apoE of native plasma was present in the form of complexes also containing either apoA-I or apoB. However, both ultracentrifugation and polyanionic precipitation methods of lipoprotein fractionation dissociated substantial proportions of apoE into both lipid-rich and lipid-poor forms that were unassociated with other apolipoproteins. These forms were derived mainly or exclusively from the dissociation of apoE from lipoproteins containing apoB, while apoE bound to apoA-I was dissociated by neither procedure. When lipoproteins were adsorbed on immobilized antibodies to apoA-I or apoB and dissociated with 3 M NaCNS, the apoE and apoA-I remained associated while the complex of apoB and apoE was substantially dissociated. These results suggest that immunoaffinity chromatography accurately determines apoE distribution in plasma. The results on the in vitro generation of unassociated apoE (Lp-E) are discussed in terms of the "family" concept of lipoprotein structure.—**Castro, G. R., and C. J. Fielding.** Evidence for the distribution of apolipoprotein E between lipoprotein classes in human normocholesterolemic plasma and for the origin of unassociated apolipoprotein E (Lp-E). *J. Lipid Res.* 1984. **25:** 58–67.

Supplementary key words apolipoprotein E distribution • immunoaffinity chromatography

The biological properties of the plasma lipoproteins depend in considerable measure on their apolipoprotein composition. These proteins determine the reactivity of lipoprotein lipids with enzymes, for example (1, 2), or cell surface receptors (3, 4). The apolipoprotein composition of most human lipoprotein complexes is physically stable, as is evident from the consistent data provided by different separation procedures, and is modified only in response to metabolic changes of plasma lipids. However, the distribution of certain apolipoproteins, including some of major metabolic significance, is much more labile. One readily dissociated protein in human plasma is apolipoprotein E. Inconsistent results have been obtained, when any two fractionation procedures have been compared, of the distribution of apoE between the major plasma lipoprotein classes (5–8). An independent estimate of the distribution of apoE could in principle be made by immunoaffinity chromatography. In this procedure antibodies raised against individual apoprotein antigens are immobilized covalently on agarose or a similar insoluble chromatography matrix. This technique has been used to define the native stoichiometry of other apoprotein complexes in normal plasma (9–11). However, validation of the affinity procedure depends on a demonstration that dissociation or transfer of apoprotein does not take place in the course of fractionation. In the present study the known lability of the association of apoE with the major plasma lipoprotein classes was chosen to provide a general and rigorous validation to test the affinity chromatography procedure in its ability to fractionate and define lipoprotein complexes of metabolic consequence from native plasma. At the same time further information has been obtained on the nature and the source of dissociated apoE.

MATERIALS AND METHODS

Blood from laboratory volunteers who had fasted overnight was collected into one-twentieth volume of 0.2 M sodium citrate solution (pH 7.0), cooled in ice water.

Plasma was obtained by centrifugation under these conditions (2000 *g*, 30 min) and then immediately fractionated using one or more of the methods detailed below. Mean plasma cholesterol in this study was 171 ± 35 mg/dl, and mean plasma triglyceride was 53 ± 20 mg/dl (mean ± SD, n = 9).

Ultracentrifugal fractionation of lipoproteins

Plasma was brought to 1 mM disodium EDTA (pH 7.4) and to a density of 1.063 g/ml by addition of NaBr. Centrifugation was carried out for 24 hr at 4–6°C and 38,000 rpm using a 40.3 rotor in a Beckman L3-40 or L3-50 preparative ultracentrifuge (12). The supernatant and infranatant fractions were collected and in some experiments the density of the infranatant was then brought to d 1.21 g/ml with KBr. After recentrifugation under the same conditions for 40 hr, supernatant high density lipoprotein (HDL, d 1.063–1.21 g/ml) was separated from the lipid-poor infranatant solution (d > 1.21 g/ml). The low and very low density lipoproteins (LDL + VLDL) in the d 1.063 g/ml supernatant solution, together with the HDL and lipid-poor fractions, were then dialyzed against 0.15 M NaCl, 1 mM disodium EDTA, pH 7.4, before analysis of their apoE content by the assay described below.

Molecular sieve chromatography

Plasma (1 ml) from citrated blood was passed onto a column (1.2 × 90 cm) of agarose (Biogel 5-M, 200–400 mesh, Bio-Rad, Richmond, CA) (13), equilibrated with 0.15 M NaCl, 1 mM EDTA (pH 7.4). Chromatography was carried out at 4–6°C with a flow rate of 3–4 ml/hr. Fractions (1 ml) were assayed for their content of free and esterified cholesterol with an enzymatic assay (14). The separation of HDL from VLDL + LDL was essentially complete (**Fig. 1**) and the two lipoprotein pools were concentrated by reverse dialysis with Sephadex G-150 (Sigma Chemical Company) and analyzed for apoE content with the assay described below.

Polyanionic precipitation of lipoproteins

Plasma from citrated blood was mixed with 0.05 vol of 1 M $MnCl_2$ and then with the same volume of heparin (2250 IU/ml) (15). The plasma mixture was placed in ice water for 30 min and then the precipitated VLDL and LDL were separated by centrifugation (2000 *g*, 30 min). The supernatant solution was dialyzed against 5% w/v $BaCl_2$ solution (pH 7.0) to precipitate heparin as its insoluble barium salt, then against 0.15 M NaCl, 1 mM EDTA (pH 7.4) before determination of apoE content. In some experiments the precipitated lipoproteins were dissolved in 0.1 M $NaHCO_3$ and then dialyzed in the same way before determination of apoE. The removal of all detectable apoB from the supernatant solution was confirmed by radial immunoassay under conditions where 1.0% contamination would have been detected.

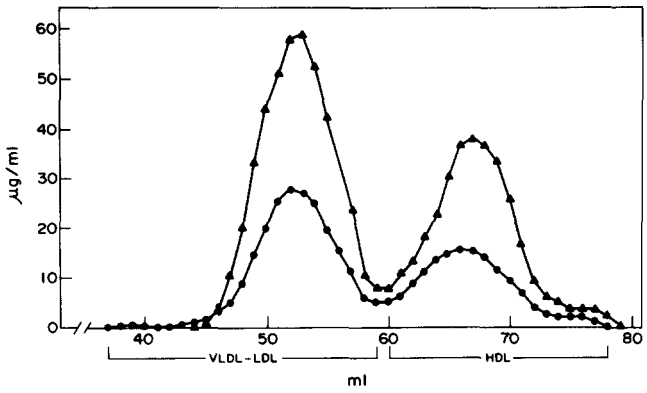

Fig. 1. Molecular sieve chromatography of native plasma. Plasma (1.0 ml) was added to a column of Biogel-5-M agarose in 0.15 M NaCl, 1 mM EDTA (pH 7.0), and the free cholesterol (● —— ●) and esterified cholesterol (▲ —— ▲) contents of the eluted fractions were determined by fluorimetric enzyme assay.

Immunoaffinity chromatography of lipoproteins

Affinity chromatography matrices were prepared with purified antibodies to apolipoproteins A-I or B (the major lipoproteins of HDL or VLDL and LDL, respectively), as previously described (9, 10). Briefly, apoA-I was isolated from delipidated HDL by molecular sieve and DEAE-cellulose chromatography in 6 M urea. The apoprotein so obtained, which showed no detectable level of contaminating proteins by sodium dodecyl sulfate or basic polyacrylamide gel electrophoresis, was injected into rabbits via multiple dermal sites. The antibody raised was purified against authentic apoA-I immobilized covalently on Sepharose 6B (16), and the anti-apoA-I globulin was released with 3 M NaCNS (pH 7.0). The antibody eluted was dialyzed against 0.1 M $NaHCO_3$, and then coupled to CNBr-activated Sepharose 6B (Pharmacia). Columns of antibody matrix used in this study had dimensions of 0.9 × 10 cm and complexed the total apoA-I of 1–1.5 ml of native plasma (1.5–2.5 mg of apoA-I). To determine the distribution of apoE between lipoproteins containing apoA-I and other lipoprotein classes, citrated plasma (1.0 ml) was passed through antibody columns equilibrated with 0.15 M NaCl, 1 mM disodium EDTA (pH 7.4), at a flow rate of 3–6 ml/hr at 4–6°C. All fractions containing detectable protein (17) or cholesterol (14) were pooled, concentrated by reverse dialysis, and the apoE content of the pools was determined immunologically. In some experiments, the column was washed with a further 40 volumes of 0.15 M NaCl, 1 mM disodium EDTA (pH 7.4) and then the bound lipoprotein was released with 3 M NaCNS (pH 7.0). The NaCNS eluate was brought to 1 mg/ml with recrystallized bovine serum albumin (18), dialyzed against 0.15 M NaCl, 1 mM EDTA to remove

NaCNS, then concentrated by reverse dialysis. Its apoE content was then determined immunologically. The albumin (Sigma Chemical Company, St. Louis, MO) had no reactivity with antibody against human apoE.

Antibody to human apoB was obtained with LDL purified by centrifugation between the density limits 1.02–1.04 g/ml. The lipoprotein was further purified from trace non-apoB contaminants by passage through immunoaffinity columns of immobilized antibodies to apolipoproteins A-I, C-III, D, and E (10). It was then injected into rabbits and the antibody raised was purified and covalently complexed to activated Sepharose 6B, as described above. Immunoaffinity chromatography of native plasma on anti-apoB was as described for anti-apoA-I.

Immunoassay of apoE

ApoE was purified from delipidated VLDL by molecular sieve and DEAE-cellulose chromatography, and antibody was raised in rabbits (10). The apoE used as antigen contained no detectable protein contaminants following sodium dodecyl sulfate or basic polyacrylamide gel electrophoresis. Radial immunodiffusion assay (19) was carried out in a medium containing 5% (v/v) antiserum, 0.8% bovine serum albumin (recrystallized, Sigma), 1.0% (w/v) agarose (Bio-Rad), and 1 mM disodium-EDTA in 0.2 M Tris-HCl buffer (pH 7.4). Pure antigen or plasma fractions in 1.0% (v/v) Triton X-100 solution were added to 25-well immunoassay gels prepared on glass plates (10 × 8 cm). The protein content of the primary purified apoE standards was determined by the method of Lowry et al. (17). The well diameter was 4 mm and the plates were incubated in a humidified incubator for 48 hr at 37°C. Under these conditions, more extended incubation produced no further change in the diameter of the precipitin rings, determined with vernier calipers to a reproducibility of ±0.1 mm. Each sample was plated in duplicate at at least two dilutions.

Agarose gel electrophoresis

Electrophoresis was carried out in 0.5% (w/v) agarose in barbital buffer (pH 7.4) (20) and the air-dried strips were stained with Sudan Black B to visualize lipoprotein bands.

RESULTS

Immunoassay of apolipoprotein E

The level of apoE in plasma and chromatographic fractions was determined by radial immunodiffusion assay, in which the relationship between the squared diameter of the precipitin ring and the mass of added apoE antigen

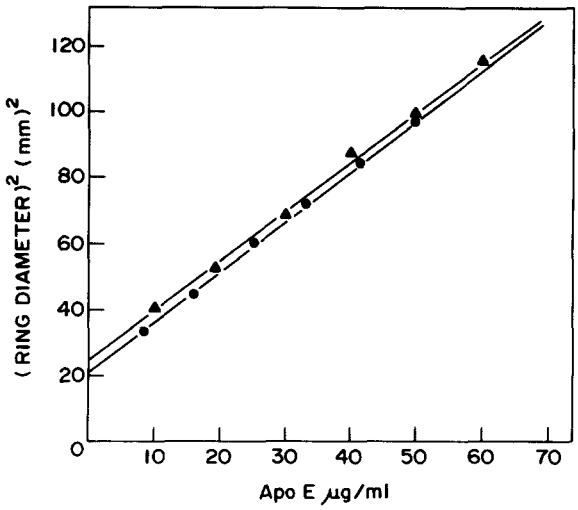

Fig. 2. The dependence of the square of precipitin ring diameter on antigen concentration for pure apoE antigen (▲ —— ▲) or unfractionated plasma (● —— ●). The apoE standard contained 60 μg of protein/ml and the plasma contained 66.6 μg of apoE/ml, determined as described under Methods. Values shown are the means of duplicate determinations at each point which differed by ±1 μg.

was linear over the range 10–125 μg apoE/ml. As little as 0.1 μg of apoE was readily detected in this assay. ApoE levels of individual samples were brought within the linear concentration range by dilution or reverse dialysis as necessary. The immunochemical identity of isolated apoE standard and the reactive antigen in plasma was confirmed from the calculated recovery of 95.5 ± 4.0% of such standard assayed in diluted plasma in the linear range, and by the parallel linear plots of the square of precipitin ring diameter vs. apoE mass in plasma and in pure antigen solution (**Fig. 2**). There was no effect on the assay of apoE when plasma was diluted fourfold with 0.15 M NaCl, with initial apoE concentrations of 27.1–85.0 μg/ml (coefficient of variation 5.3%, n = 24). The mean level of apoE in the plasma of the nine normocholesterolemic subjects in this study was 64.1 ± 12.3 μg/ml (males 56.4 ± 8.4 μg/ml, n = 5; females 73.7 ± 9.4 μg/ml, n = 4). ApoE levels were assayed repeatedly in four of these sub-

TABLE 1. Radial immunoassay of apolipoprotein E in human plasma

	Donor			
	1	2	3	4
X	47.7	84.4	71.3	61.9
SD	±3.6	±3.0	±2.4	±3.2
n	12	11	8	10
CV (%)	7.5	3.5	3.4	3.2

ApoE concentrations are expressed in μg/ml plasma for the mean (X) and standard deviation (SD) of n determinations over a 12-month sampling interval. CV, coefficient of variation.

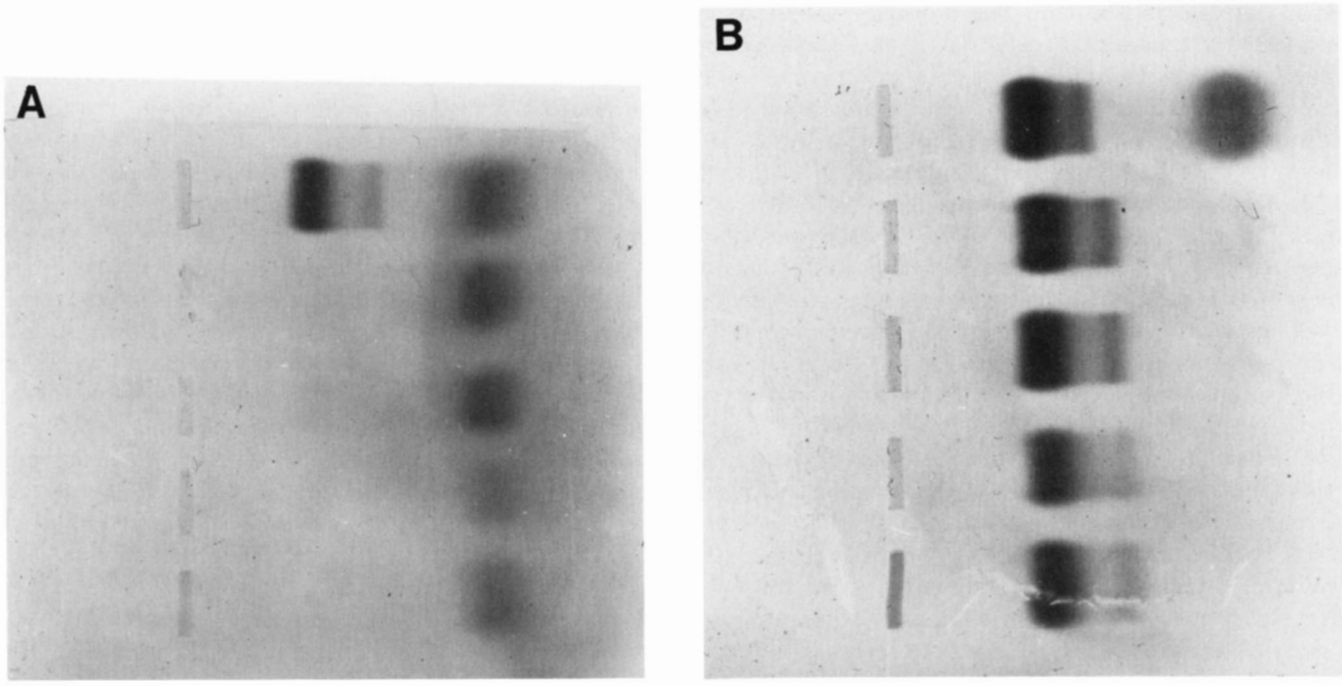

Fig. 3. A. Agarose strip gel electrophoresis. Top, unfractionated plasma and, in order from top to bottom, HDL separated by polyanionic precipitation, ultracentrifugation, molecular sieve chromatography, and affinity chromatography. The lipoprotein bands are visualized with Sudan Black after fixation in ethanol–acetic acid–water 75:5:20 (v/v/v). B. Agarose strip gel electrophorograms of (top) unfractionated plasma and (in order from top to bottom) VLDL + LDL separated by polyanionic precipitation, ultracentrifugation, molecular sieve chromatography, and affinity chromatography. The lipoprotein bands were fixed and stained as above.

jects over a 12-month interval (**Table 1**) and highly consistent values were obtained.

Plasma lipoprotein electrophoresis

The separation of HDL from VLDL and LDL during centrifugation, polyanion precipitation, molecular sieve chromatography, and immunoaffinity chromatography was compared (**Fig. 3**). There was no difference in the migration rates of the lipoprotein bands obtained by the different procedures. Thus, lipoproteins recovered by the different procedures were complexed in similar lipid-rich forms; and while HDL was specifically removed from native plasma by immunoaffinity chromatography immobilized anti-apoA-I, both VLDL and LDL were removed by immunoaffinity chromatography on anti-apoB. Sequential chromatography with these two immunoadsorbents generated a plasma fraction containing no lipoprotein material detectable by agarose gel electrophoresis (**Fig. 4**).

Effects of fractionation on the distribution of lipoprotein apoE

Molecular sieve chromatography was previously used to fractionate lipoprotein species containing apoE (5, 6,

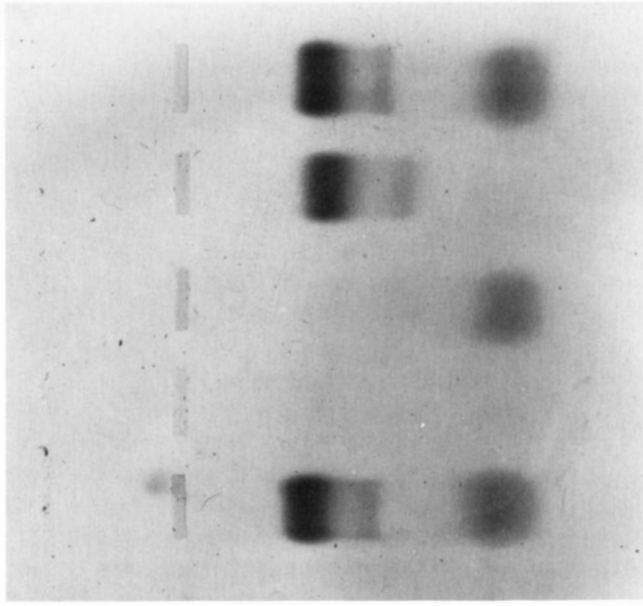

Fig. 4. Agarose strip gel electrophoresis. Top and bottom, unfractionated plasma and, in order from top to bottom, plasma from which HDL had been removed by immunoaffinity chromatography on immobilized anti-apoA-I; from which VLDL and LDL had been removed by immunoaffinity chromatography on immobilized anti-aopB; and from which both fractions had been removed by chromatography sequentially on both affinity supports.

8, 21). In the present studies the distribution of apoE between HDL and LDL + VLDL was determined simultaneously by immunoaffinity chromatography, using immobilized antibody to apoB, and by molecular sieve chromatography on agarose. In the case of molecular sieve chromatography, it has been shown (6, 21) that HDL containing apoE elutes with higher apparent molecular weight than does bulk HDL. Possible overlap of HDL containing apoE into the VLDL + LDL fraction (Fig. 1) was determined by assaying that proportion of apoE in plasma from which apoB had been removed by affinity fractionation, which eluted in the VLDL + LDL fraction molecular sieve chromatography. This was found to represent < 2% (1.4%; 1.5%; two experiments) of apoE. This finding indicates that the separation of HDL containing apoE from VLDL + LDL by molecular sieve chromatography under the conditions described in this study was essentially complete. As shown in **Table 2,** there was a high degree of correlation and good quantitative agreement in the estimates of HDL-associated apoE obtained by these two procedures. The mean difference between estimates was 1.0 ± 3.3 μg/ml of plasma and the correlation coefficient (r) was 0.958. Approximately one-half of total plasma apoE was removed by anti-apoB or was recovered in the VLDL + LDL fraction of plasma recovered after molecular sieve chromatography. Plasma that had been passed in succession through columns of antibodies to apoB and apoA-I contained no detectable apoE under conditions where <2% of plasma concentration would have been detected (**Fig. 5**). Therefore, these results, and the electrophoretic measurements preceding (Figs. 3 and 4) suggest that little change in the composition of HDL occurs during immunoaffinity chromatography; further evidence is given below. On the other hand, significantly different, and more variable, recoveries of apoE with HDL were obtained with the ultracentrifugal and polyanion precipitation fractionation methods (**Table 3**). In comparison with the affinity procedure, the recovery of apoE with HDL by polyanion precipitation was on average 20% lower and more variable (r = 0.638 vs. the immunoaffinity procedure). Centrifugal separation of HDL, even when as the result of the single centrifugal step studied here, resulted in a significantly greater recovery of apoE with HDL than with any of the other methods, and a correlation coefficient (r) of 0.655 vs. the immunoaffinity procedure for the same plasma samples. The difference in the level of apoE recovered with HDL by these procedures was not due to an incomplete separation of HDL from other lipoproteins, since in all experiments less than 2% of apoA-I was recovered with VLDL + LDL, and less than 3% of plasma apoB was recovered in the HDL fraction by radial immunodiffusion assay (19). Neither was it due to differences in recovery, which was similar in the different procedures (90 ± 6% of plasma apoE for molecular sieve chromatography; 94 ± 6% for immunoaffinity chromatography; 95 ± 6% for polyanionic precipitation; and 97 ± 6% for ultracentrifugation). Accordingly, the modified recoveries of apoE with HDL found after ultracentrifugation or polyanion precipitation represent in vitro modifications of native lipoprotein composition.

Further validation of the immunoaffinity chromatography of apo E

The experiment described above (Fig. 5) indicated that in normolipidemic plasma there was no significant level of apoE unassociated with either apoA-I or apoB, i.e., that Lp-E was not present. Other experiments confirmed that immunoaffinity chromatography did not modify apoE

TABLE 2. Determination of HDL apoE levels by affinity chromatography and molecular sieve column chromatography

	Plasma ApoE	I HDL ApoE (Affinity)	II HDL ApoE (Molecular Sieve)	II-I
		μg apoE per ml plasma		
	46.0	22.8 (0.50)[a]	23.3 (0.51)	+0.5
	48.7	21.7 (0.44)	23.0 (0.47)	+1.3
	57.6	36.1 (0.40)	30.8 (0.52)	−5.7
	58.3	30.2 (0.52)	34.7 (0.59)	+4.5
	66.5	32.4 (0.49)	34.4 (0.51)	+2.0
	73.2	33.3 (0.45)	35.4 (0.48)	+2.1
	87.1	54.5 (0.65)	57.2 (0.66)	+2.7
Mean ± SD	62.5 ± 14.4	33.0 ± 10.9 (0.49 ± 0.08)	34.1 ± 11.4 (0.53 ± 0.06)	+1.0 ± 3.3

[a] Values in parentheses represent the proportion of apoE recovered in the HDL fraction.

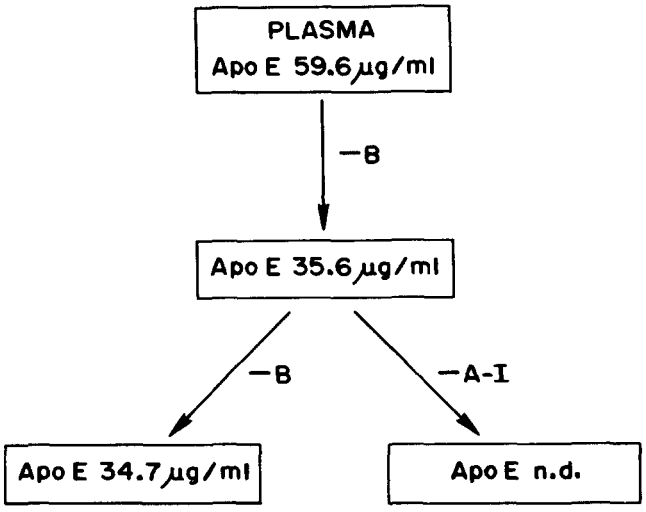

Fig. 5. Effects of sequential chromatography on affinity adsorbents against apoA-I and apoB. Plasma was chromatographed first against anti-apoB, then the eluate of nonadsorbed proteins was rechromatographed on either anti-apoA-I or anti-apoB, as described under Methods. Values indicated represent apoE concentration corrected to plasma protein concentration. The initial plasma contained 1.43 mg/ml apoA-I and 0.55 mg/ml apoB. The eluate of nonadsorbed proteins after initial anti-apoB chromatography contained 1.40 mg/ml apoA-I (relative to original plasma protein concentration) and 0.01 mg/ml apoB. After a second chromatography on anti-apoB, the plasma contained 1.42 mg/ml apoA-I and no detectable apoB (<0.005 mg/ml). After chromatography against apoA-I, the nonadsorbed protein contained no detectable apoA-I (<0.01 mg/ml) and 0.006 mg/ml apoB (n.d., apoE concentration of <1.0 μg/ml).

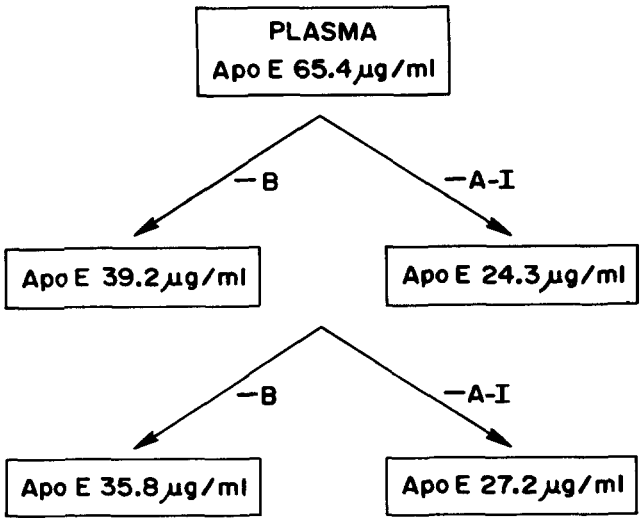

Fig. 6. Effects of the remixing of affinity chromatography eluates on the distribution of apoE. Plasma was initially chromatographed on either anti-apoA-I or anti-apoB as shown. The nonadsorbed proteins were then mixed in the proportions 0.5:1 relative to plasma and rechromatographed on anti-apoA-I or anti-apoB. Values shown indicate apoE concentrations relative to the protein concentration of the original plasma and are the means of duplicate or triplicate determinations reproducible within <2%. The initial plasma contained 1.52 mg/ml apoA-I and 0.62 mg/ml apoB; after initial chromatography on anti-apoA-I, residual apoA-I in the nonadsorbed eluted protein was 0.03 mg/ml (relative to plasma); while after chromatography on anti-apoB, apoB in the nonadsorbed proteins was undetectable (<0.005 mg/ml). After remixing and anti-apoA-I chromatography, residual apoA-I was 0.01 mg/ml; while after anti-apoB the nonadsorbed apoB was undetectable.

distribution in plasma. Passage of the eluate from the anti-apoB affinity step through a second column of the same matrix resulted in the loss of little, if any, further mass of apoE. This finding indicated that there was no measurable dissociation of apoE during fractionation. When plasma fractions, from which either apoB or apoA-I had been removed, were remixed in different proportions (**Fig. 6**), there was <10% difference on average in the mass of apoE recovered with these lipoproteins when they were subsequently reseparated under the same conditions. This finding indicates that there was little re-equilibration of apoE between apoA-I and apoB-containing lipoproteins during chromatography under the con-

TABLE 3. Determination of HDL apoE levels by immunoaffinity chromatography, polyanionic precipitation, and ultracentrifugation

	Plasma	HDL ApoE (Affinity) I	HDL ApoE (Precipitation) II	HDL ApoE (Ultracentrifugation) III	II-I	III-I
			μg apoE per ml plasma			
	40.7	23.5 (0.58)[a]	16.4 (0.40)		−7.1	
	48.0	27.2 (0.57)	25.9 (0.54)	34.5 (0.72)	−1.3	+7.3
	54.1	31.2 (0.58)	24.1 (0.45)	39.2 (0.72)	−7.1	+8.0
	59.1	40.8 (0.69)	25.4 (0.43)	51.5 (0.87)	−15.4	+10.7
	65.3	33.6 (0.51)	26.2 (0.40)	44.7 (0.68)	−7.4	+11.1
	68.5	30.6 (0.45)	31.4 (0.46)	56.8 (0.83)	+0.8	+26.2
	72.2	39.5 (0.55)	36.9 (0.51)	58.6 (0.81)	−2.8	+19.1
Mean ± SD	58.3 ± 11.4	32.3 ± 6.3 (0.56 ± 0.07)	26.6 ± 6.3 (0.46 ± 0.05)	47.5 ± 9.7 (0.77 ± 0.07)	−5.7 ± 5.3	+13.7 ± 7.4

[a] Values in parentheses represent the proportion of apoE recovered in the HDL fraction.

ditions described. These findings, taken together with the preceding, strongly suggest that the distribution of apoE between VLDL and LDL on the one hand, and HDL on the other, determined by immunoaffinity chromatography, accurately represents that present in the unfractionated native plasma.

The nature and origin of unassociated apoE (Lp-E) generated by the fractionation of plasma

The results above suggested that, as a result of ultracentrifugation or polyanionic precipitation, either there was net redistribution of apoE bound to HDL or LDL + VLDL, or else that apoE unassociated with either apoA-I or apoB was generated during fractionation and was co-purified with one or another fraction to give rise to a modification of the native apoE content of the lipoprotein that was only apparent. These alternatives were investigated by further immunoaffinity chromatography experiments. Because in a number of earlier studies (22–24) lipoprotein "families" had been defined on the evidence of lipoprotein structures dissociated from immunoaffinity columns with 3 M NaCNS, the effects of this procedure were studied both for any possible effects on the association of apoE with the HDL or with the VLDL + LDL lipoprotein fractions. As shown above, when plasma was centrifuged at d 1.063 g/ml, apoE was distributed between supernatant (VLDL + LDL) and infranatant (HDL) lipoprotein classes. However, when both fractions were passed through affinity matrices binding apoA-I or apoB, significant levels of apoE were found in both nonadsorbed fractions. These results indicate the presence of dissociated apoE after centrifugation, contrary to findings in unfractionated native plasma. As shown in **Table 4,** about one-third of apoE in VLDL + LDL after centrifugation at d 1.063 g/ml was not bound to apoB. As shown in **Table 5,** when similar experiments were carried out with the d > 1.063 g/ml fraction, about 20% of apoE was unassociated with apoA-I. However, comparison of estimates of the apoA-I-bound apoE obtained by direct affinity chromatography and by centrifugation followed by affinity chromatography show that these differed by only 1.6 ± 2.1 μg/ml. This finding indicates that the apoE dissociated by centrifugation is derived mainly or completely from the apoE bound to apoB in native plasma, while apoE bound to apoA-I remains in complex form.

TABLE 4. Dissociation of apoE and its recovery in the d 1.063 g/ml supernatant fraction

Experiment	Plasma ApoE	Total Supernatant ApoE I	Unassociated Supernatant ApoE II	II/I
		μg apoE per ml plasma		
1	61.1	17.3 (0.28)	4.0 (0.06)	0.23
2	63.2	22.8 (0.36)	7.0 (0.11)	0.30
3	65.8	18.1 (0.27)	7.1 (0.11)	0.39
4	66.1	19.0 (0.29)	7.7 (0.12)	0.40

Plasma was centrifuged at d 1.063 g/ml, and after dialysis against 0.15 M NaCl, the supernatant fraction (VLDL + LDL) was passed through an affinity column containing immobilized anti-apoB. The apoE fraction not retained by the affinity column (II) was compared to the apoE content of the total d 1.063 g/ml supernatant.

Similar analysis was made of the associations of apoE after polyanionic precipitation of VLDL and LDL. As shown in **Fig. 7,** apoE dissociated by fractionation was recovered in precipitated, resolubilized lipoprotein. Subsequent immunoaffinity chromatography with antibodies against apoA-I and apoB indicated that the mass of apoE associated with apoA-I was unchanged while that complexed to apoB had been reduced, indicating that, as was the case during centrifugation, it was the apoB-associated fraction of apoE that had been preferentially dissociated.

The association state of apoE was also determined in those lipoproteins that could be recovered by 3 M NaCNS from immunoaffinity chromatography. Plasma was passed through immobilized antibody to apoA-I, and the adsorbed lipoproteins containing apoA-I were dissociated with 3 M NaCNS, dialyzed against 0.15 M NaCl, 1 mM EDTA, then passed again through the same adsorbent, and the mass of unassociated apoE in the nonadsorbed eluate was determined. Similar experiments were carried out with immobilized antibodies to apoB. As shown in **Table 6,** apoE complexed in native plasma to apoA-I was undissociated by 3 M NaCNS. On the other hand, when the same experiment was carried out with the fraction of apoE complexed in native plasma to apoB, substantial dissociation was found to have occurred (46 ± 11% of that found in this fraction in the original plasma).

Taken together, these experiments indicate that it is primarily or exclusively that apoE complexed in native plasma to apoB which is dissociated by centrifugation, $MnCl_2$-heparin, or 3 M NaCNS to generate the unassociated apoE (Lp-E) present after fractionation. Under the same conditions, apoE complexed with apoA-I remains undissociated.

DISCUSSION

This research represents the first detailed validation of immunoaffinity chromatography for the fractionation of the labile complexes containing apoE that are present in human plasma.

This study firstly provides very strong evidence that both molecular sieve chromatography and immunoaffinity chromatography permit a determination of the lipopro-

TABLE 5. Comparison of the levels of apoA-I-bound apoE in plasma determined by ultracentrifugal centrifugation and by direct immunoaffinity chromatography

Plasma ApoE	Total d > 1.063 ApoE (A)[a]	Unassociated d > 1.063 ApoE (B)[b]	ApoA-I-bound d > 1.063 ApoE (A − B)	B/A	ApoA-I-bound ApoE (direct affinity chromatography) (C)[c]	C − (A − B)
	μg apoE/ml plasma				*μg apoE/ml plasma*	
52.5	24.3	5.5	18.8	0.22	23.6	+4.8
57.4	42.3	9.6	32.7	0.22	32.6	−0.1
58.8	40.1	10.0	30.1	0.25	32.4	+2.3
63.2	32.6	5.0	27.6	0.15	29.2	+1.6
64.2	35.0	6.6	28.4	0.19	27.9	−0.5

[a] Determined as the infranatant fraction of a single centrifugation at d 1.063 g/ml.
[b] Determined as the concentration of nonadsorbed apoE after chromatography of the d 1.063 g/ml infranatant solution, after dialysis, on anti-apoA-I antibody.
[c] Determined as the nonadsorbed fraction of plasma from affinity chromatography on anti-apoB antibody.

tein distribution of apoE that reflects the distribution present in native plasma. The advantages of the immunoaffinity chromatography method lie in its speed and convenience, the analysis of a single pooled eluate, and the potential of the affinity method to separate, by sequential chromatography on adsorbants specific for different lipoprotein antigens, each of the lipoprotein species of plasma under mild conditions. Both ultracentrifugation and polyanionic precipitation resulted in a substantial redistribution of apoE between the major lipoprotein classes recovered by these procedures when compared to the other two methods. On the other hand, immunoaffinity chromatography under the conditions described here, was not associated with any substantial dissociation or redistribution of apoE. In particular, this study indicates that, in normocholesterolemic plasma, there is no detectable level of apoE unassociated with either apoA-I or apoB.

The second major finding of this study is that unassociated apoE in both lipid-rich and lipid-poor forms is readily generated by using certain other fractionation procedures. Thus, a single centrifugation at d 1.063 g/ml dissociated about one-fifth of total apoE from its native lipoprotein complexes; and of the apoE recovered in the

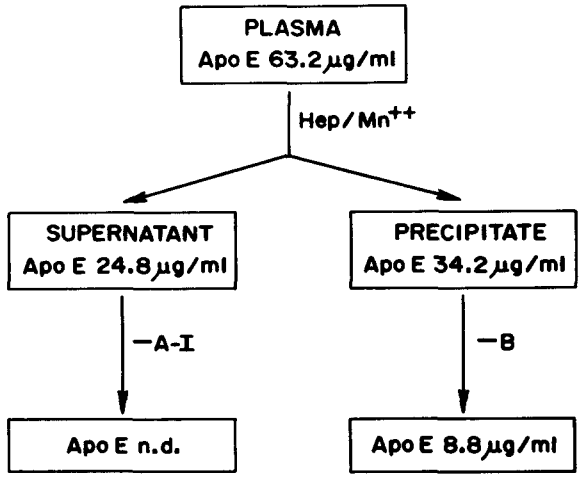

Fig. 7. Effects of polyanionic precipitation with heparin-$MnCl_2$ on the distribution of apoE in plasma. Plasma was mixed with $MnCl_2$ and heparin, as described under Methods, and the supernatant and infranatant fractions (the latter after resolubilization in 0.1 M $NaHCO_3$) were dialyzed first against 5% w/v $BaCl_2$ (pH 7.0) and then against 0.15 M NaCl, 1 mM disodium EDTA (pH 7.4). Total apoE in each fraction was determined, and the apoE not retained by affinity columns containing antibodies against apoA-I or apoB was also determined. The values shown represent apoE concentrations (as μg/ml, corrected to original plasma volume). The original plasma contained 1.07 mg/ml apoA-I and 0.71 mg/ml apoB. ApoB was not detectable in the heparin-$MnCl_2$ supernatant fraction (concentration <0.005 mg/ml) while after anti-apoA-I affinity chromatography, apoA-I concentration was 0.02 mg/ml. The precipitated lipoproteins (VLDL + LDL) contained an apoA-I concentration of 0.01 mg/ml, while after anti-apoB chromatography, the nonadsorbed apoB concentration (relative to plasma) was 0.01 mg/ml.

TABLE 6. Dissociation of apoE from lipoprotein complexes by 3 M NaCNS

Experiment	Plasma ApoE	Anti-apoB Immunoaffinity Chromatography I	Anti-apoB Immunoaffinity Chromatography II	Anti-apoB Immunoaffinity Chromatography II/I	Anti-apoA-I Immunoaffinity Chromatography I	Anti-apoA-I Immunoaffinity Chromatography II
	μg apoE per ml plasma					
1	55.8	33.0	11.0	0.33	29.2	n.d.
2	58.3	25.3	14.4	0.57	36.6	n.d.
3	73.2	37.5	14.9	0.40	23.8	n.d.
4	87.1	23.8	12.6	0.53	65.6	n.d.

Values are expressed in terms of apoE concentration (μg per ml plasma) as apoE recovered in the 3 M NaCNS eluate after affinity chromatography after dialysis (I), and as the apoE nonadsorbed on subsequent rechromatography (II) on the same immunoaffinity adsorbent. The ratio II/I for anti-apoB chromatography represents the proportion of apoB-bound apoE in native plasma recovered in unassociated form after elution with 3 M NaCNS. Unassociated apoE from anti-apoA-I chromatography was not detectable (n.d., < 1.0 μg/ml in these experiments).

lipid-rich supernatant under these conditions, one-third represented dissociated protein. It is possible that part of the reaction of some VLDL fractions with apoB, E lipoprotein receptors involve this dissociated apoE fraction (25). On the other hand, the binding of apoE to complexes containing apoA-I was stable to the same procedures. This may indicate fundamental differences in the molecular basis of apoE binding to the high and very low density lipoprotein classes in human plasma.

A final point relates to the lipoprotein "families" of unassociated individual apoproteins complexed with lipids that have been defined for many of the plasma apolipoproteins on the basis either of the complexes present in centrifugally isolated lipoprotein fractions (as for Lp-E and Lp-C) (26, 27), complexes dissociated from immunoadsorbers with 3 M NaCNS (Lp-F) (24), or after both procedures (Lp-D) (9). The present study indicates that at least in the case of apoE, these procedures are exactly those that generate lipoprotein species that have the properties of those families but which were not present in native plasma. It may also be relevant that in the case of apoD, direct immunoaffinity chromatography of native plasma in another study revealed the presence of no unassociated lipoprotein apoD (Lp-D) (9), in contrast to the finding made after centrifugation and treatment with 3 M NaCNS (23). It must also be emphasized, however, that the results of all these studies, and our own, refer to normocholesterolemic plasma, and that the presence of unusual associations of lipids and apoproteins in pathological plasma samples is in no way ruled out.

In summary, this study suggests that immunoaffinity chromatography can accurately define the molecular association of even labile apoproteins, such as apoE, in native plasma, even when these associations are unstable to classical fractionation techniques.

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