

Apolipoprotein distribution in human lipoproteins separated by polyacrylamide gradient gel electrophoresis

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Abstract The heterogeneity of serum lipoproteins (excluding very low density (VLDL) and intermediate density (IDL) lipoproteins) and that of lipoproteins secreted by HepG2 cells has been studied by immunoblot analysis of the apolipoprotein composition of the particles separated by polyacrylamide gradient gel electrophoresis (GGE) under nondenaturing conditions. The reactions of antibodies to apoA-I, apoA-II, apoE, apoB, apoD, and apoA-IV have revealed discrete bands of particles which differ widely in size and apolipoprotein composition. GGE of native serum lipoproteins demonstrated that apoA-II is present in lipoproteins of limited size heterogeneity (apparent molecular mass 345,000 to 305,000) and that apoB is present in low density lipoproteins (LDL) and absent from all smaller or denser lipoproteins. In contrast, serum apoA-I, E, D, and A-IV are present in very heterogeneous particles. Serum apoA-I is present mainly in particles of 305 to 130 kDa where it is associated with apoA-II, and in decreasing order of immunoreactivity in particles of 130–90 kDa, 56 kDa, 815–345 kDa, and finally within the size range of LDL, all regions where there is little detectable apoA-II. Serum apoE is present in three defined fractions, one within the size range of LDL, one containing heterogeneous particles between 640 and 345 kDa, and one defined fraction at 96 kDa. Serum apoD is also present in three defined fractions, one comigrating with LDL, one containing heterogeneous particles between 390 and 150 kDa, and one band on the migration front. Most of serum apoA-IV is contained in a band comigrating with albumin. GGE of centrifugally prepared LDL shows the presence of apoB, apoE, and apoD, but not that of apoA-I. However, the particles containing apoA-I, which, in serum, migrated within the LDL size range and as bands of 815 to 345 kDa, were recovered upon centrifugation in the $d > 1.21$ g/ml fraction. GGE of high density lipoproteins (HDL) indicated that most of apoA-I, A-II, and A-IV were present in lipoproteins of the same apparent molecular mass (390–152 kDa). ApoD tended to be associated with large HDL, and this was also significant for HDL apoE, which is present in lipoproteins ranging from 640 to 275 kDa. GGE of very high density lipoproteins (VHDL) presented some striking features, one of which was the occurrence of apolipoproteins in very discrete bands of different molecular mass. ApoA-II was bimodally distributed at 250–175 kDa and 175–136 kDa, the latter fraction also containing apoA-I. The major apoD band was centered at 230 kDa and the single apoE band was centered at 250 kDa; there were other discrete bands for apoA-I at 96 and 56 kDa, for apoD at 130 and 96 kDa, and for apoA-IV at 150 and 68 kDa. A similar size heterogeneity was also seen in the

protein fraction at $d > 1.21$ g/ml. The large particles containing apoE or apoA-IV observed in this fraction and in serum, like those containing apoA-I, may represent apolipoprotein-protein complexes. Lipoproteins, secreted by HepG2 cells, containing apoA-II and apoE were comparable in size to those found in serum, while those containing apoA-I were smaller and not as heterogeneous. Little of the apoA-I, apoA-II, and apoE secreted by the HepG2 cells appears to be associated on the same particles. — Vézina, C. A., R. W. Milne, P. K. Weech, and Y. L. Marcel. Apolipoprotein distribution in human lipoproteins separated by polyacrylamide gradient gel electrophoresis. *J. Lipid Res.* 1988. 29: 573–585.

Supplementary key words HepG2 cells

Human plasma lipoproteins are complex multimolecular structures made of one or more molecules of at least one type of apolipoprotein and many molecules of different lipid species. This intrinsic complexity and the relatively weak hydrophobic and ionic bonds that hold these molecules together have always raised much concern about the stability of the lipoproteins and about the introduction of artifacts during their isolation. With most available methods, a choice must be made between resolution and nondenaturing properties. However, the separation of lipoproteins by gradient gel electrophoresis (GGE) which was first introduced by Blanche et al. (1) provides a powerful analytical method that allies an excellent resolution based on size and nondenaturing conditions. To date, most applications of GGE to the characterization of lipoproteins have been limited to the quantitation of particles of different sizes by scanning densitometry using lipid or protein stains (1–4). Therefore, the full potential of this methodology has not been evaluated and more information

Abbreviations: GGE, gradient gel electrophoresis; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; VHDL, very high density lipoproteins.

can be derived from lipid analyses and from the identification and quantitation of apolipoproteins directly in the separated fractions. In the present report, we have characterized the distribution of the major apolipoproteins in lipoproteins of different sizes by immunoblotting of the electrophoretograms. These studies demonstrate that most apolipoproteins are associated with lipoproteins that vary greatly in size and in density and that GGE provides a better definition of the distribution of apolipoproteins in lipoproteins of different sizes than other methods, such as gel filtration. We have also observed that apoA-I and apoE are present in association with large protein complexes that probably do not contain lipids.

MATERIALS AND METHODS

Preparation of serum and serum lipoproteins

Blood drawn from fasted normolipemic subjects by venipuncture into Vacutainer tubes was allowed to clot at 20°C for 1 to 2 hr. The serum was recovered by centrifugation at 1000 *g* for 15 min at 5°C. Disodium ethylene diaminetetraacetate (EDTA), sodium azide, and phenylmethylsulfonyl-fluoride (PMSF) were added to final concentrations of 1 mM, 0.02% (w/v), and 1 mM, respectively.

Lipoprotein fractions from 3 ml of fresh serum were separated by density gradient ultracentrifugation in an SW 41 Ti rotor at 40,000 rpm for 20 hr at 12°C according to the method of Terpstra, Woodward, and Sanchez-Muniz (5) but without prestaining (6). Fractions (0.25 to 0.5 ml) were collected by aspiration at the surface or by tube puncture from the bottom. The fractions corresponding to the classically defined lipoproteins were pooled on the basis of color and their density ranges were estimated from the gradient curve obtained by measurement of a blank gradient centrifuged in parallel with the serum samples: VLDL $d < 1.010$ g/ml; LDL 1.024 g/ml $< d < 1.050$ g/ml; HDL 1.075 g/ml $< d < 1.140$ g/ml; VHDL 1.140 g/ml $< d < 1.20$ g/ml; protein fraction $d > 1.21$ g/ml. The lipoproteins were dialyzed overnight in 10 mM ammonium bicarbonate buffer containing 1 mM EDTA and 0.02% sodium azide. Serum and serum lipoproteins were subjected to gradient gel electrophoresis within 24 hr of preparation.

HepG2 cell culture

The human hepatoblastoma-derived cell line HepG2 was obtained from American Type Culture Collection, Rockville, MD. The cells (initially $3-6 \times 10^6$ per 75-cm² flask) were cultured in 15 ml of medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Burlington, Ontario) in a 5% CO₂/95% air incubator and were subcultured every 6-7 days. The basal medium was Eagle's Minimum Essential Medium (MEM) (Sigma, St. Louis,

MO) with added penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (4 mM) (Sigma). When the cells were almost confluent, 4 to 5 days later, they were washed carefully twice with Dulbecco's phosphate-buffered saline. The cells were then incubated in 15 ml of basal medium supplemented with 0.5 µg/ml insulin and 0.25 µg/ml vitamin K (Sigma). This serum-free medium was replaced in 1 to 2 hr with 15 ml of fresh serum-free medium. After 24 hr, the medium was harvested and pooled (three to six flasks), and the cell debris was pelleted by centrifugation at 1000 *g* for 15 min. EDTA, sodium azide, and PMSF were added to final concentrations of 1 mM, 0.02%, and 1 mM, respectively.

Lipoprotein fractions were isolated from 85 ml of freshly obtained HepG2 medium by sequential ultracentrifugation (7) at densities 1.063 g/ml and 1.21 g/ml in a 70.1 Ti rotor at 48,000 rpm for 20 hr. The fractions were dialyzed overnight against 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, pH 7.4.

Polyacrylamide gradient gel electrophoresis (GGE)

Nondenaturing electrophoresis of serum and serum lipoproteins was performed on precast polyacrylamide gradient gels (2-16% or 4-30%, Pharmacia, Uppsala, Sweden) in 14 mM Tris, 110 mM glycine, 0.01% NaN₃, pH 8.3, as described by others (2, 3, 8). The gels were pre-run 30 min at 50 V; the samples (100 to 300 µg of protein of the lipoprotein fractions, 75 to 150 µl of serum or protein fractions) were diluted to 200 µl in electrophoresis buffer containing 6% sucrose and applied across the width of individual gels. Electrophoresis was started at 30 V for 1 hr and continued for 20 hr at 80 V. Gels were stained with Oil Red O (Sigma), Sudan Black (Fisher, Dorval, Quebec) for lipid, or Coomassie Blue G-250 (Bio-Rad, Richmond, CA) for protein. In some cases serum samples were prestained with acetylated Sudan Black B (8). Apparent molecular weights were determined by comparison of electrophoretic mobility with that of protein standards thyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin (Pharmacia HMW Calibration kit, Pharmacia). Unstained gels were used for immunoblotting techniques.

Electrophoresis of freshly obtained HepG2 medium (1 ml) or HepG2 lipoprotein fractions (25-50 µg of protein) was performed as described above. In some cases the medium was concentrated three- to fourfold with collodion bags (average retention 10,000 daltons, Schleicher and Schuell, Keene, NH) before application.

Immunoblotting

After electrophoresis, the separated particles were blotted (9) to nitrocellulose paper (0.45 µm, Schleicher and Schuell) by electrophoretic transfer of the gel in the electrophoretic buffer at 100 mA for up to 72 hr at 4°C.

The papers were cut into 0.8-cm strips and saturated with 3% BSA (Boehringer Mannheim, Dorval, Quebec) in 10 mM Tris, 150 mM NaCl, 0.02% NaN₃, pH 7.4, for 30 min at 37°C in a sealed plastic bag. The strips were then incubated for 90 min at 37°C with appropriate antibody specific for various apolipoproteins diluted 1:3000 in the saturation buffer. After extensive washes with 1% BSA in the Tris buffer, they were then incubated with ¹²⁵I-labeled second antibody (10⁶ cpm/strip) specific for the first antibody for 60 min at 37°C. Excess labeled antibody was thoroughly washed out with 1% BSA buffer. Autoradiographs were made on Kodak XAR-5 film at -70°C with a Cronex Lightning-Plus Intensifying Screen (Du Pont, Wilmington, DE). Exposure times varied in order to optimize visibility of the individual bands. Monoclonal antibodies specific for apoB (10), apoA-I (6), apoE (11), and apoD (12) have been characterized and described previously. Monoclonal antibodies specific for apoA-II and human serum albumin were generous gifts from Linda K. Curtiss (La Jolla, CA); the polyclonal antibody specific for apoA-IV was a kind gift from Karl Weisgraber (San Francisco, CA).

Immunoprecipitation

An aliquot (1 μl) of freshly obtained serum was incubated overnight at 4°C with a 1:250 dilution of monoclonal antibody specific for apoA-I or apoA-II, or both, in PBS containing 1% BSA and 1 mM EDTA pH 7.2. An unrelated antibody was used as control. Final volume was 400 μl. The antigen-antibody complexes were removed by precipitation with Protein A-Sepharose (Pharmacia), armed with rabbit anti-mouse immunoglobulin (Cedarlane, Hornby, Ontario), for 5-6 hr at 4°C. Aliquots (150 μl) of the supernatant solution were separated by nondenaturing electrophoresis on 2.5-27% polyacrylamide gradient gels (Isolab, Akron, OH) and transferred to nitrocellulose paper electrophoretically for 20 to 24 hr at 100 mA. The papers were incubated with antibodies as described for immunoblots and an autoradiograph was made.

Other analyses

Protein was assayed by the method of Lowry et al. (13). Densities were measured with an Anton Paar DMA 40 densitometer (Graz, Austria) at 20°C.

RESULTS

The results presented here are typical of the data obtained with the sera of four normocholesterolemic, normotriglyceridemic, healthy donors. The chemical composition of the serum lipoproteins isolated from two of these donors by density gradient centrifugation is presented in **Table 1**. These lipoproteins present no unusual characteristics but it should be noted that the density values used for the isolation of some of the lipoprotein classes differ from those classically applied in sequential ultracentrifugation. HDL obtained here between 1.075 and 1.140 g/ml are poor in HDL₃ and VHDL obtained between 1.140 and 1.20 g/ml are enriched in dense HDL₃. Density gradient centrifugation was selected over sequential centrifugation in order to minimize apolipoprotein losses to the protein fraction. The apolipoprotein compositions of these density fractions as judged by SDS polyacrylamide gel electrophoresis were analogous to those reported in the literature (not illustrated).

Size heterogeneity of serum lipoproteins

Upon electrophoresis of serum on GGE of 4-30%, we have identified five main classes of Lp A-I particles which are labeled as regions 1 to 5 (left of **Fig. 1**) based on the following considerations. Region 1 contains traces of Lp A-I within the size range of LDL which is identified by the strong apoB signal. This region also includes very strong signals for apoE and apoD and minor signals for apoA-IV and albumin. Upon close examination, region 1 has doublet of apoA-I bands which may coincide with a doublet of apoE bands, and an apoD band that coincides with apoB.

TABLE 1. Relative composition of the serum lipoproteins isolated by density gradient centrifugation

Composition	Density (g/ml)			
	VLDL < 1.010	LDL 1.024-1.050	HDL 1.075-1.140	VHDL 1.140-1.20
	%			
Protein	10.9-8.6*	21.7-21.0	36.3-37.7	51.0-66.2
Cholesterol	3.5-4.1	9.9-7.8	4.1-3.4	2.5-1.9
Cholesteryl esters	11.7-10.1	45.2-45.8	27.5-27.6	23.6-17.2
Triglycerides	56.3-61.2	3.2-4.9	3.0-3.8	2.1-0
Phospholipids	17.7-15.9	19.9-20.5	29.1-27.5	20.8-14.7

*Percent of mass in the lipoproteins of two representative normolipemic donors.

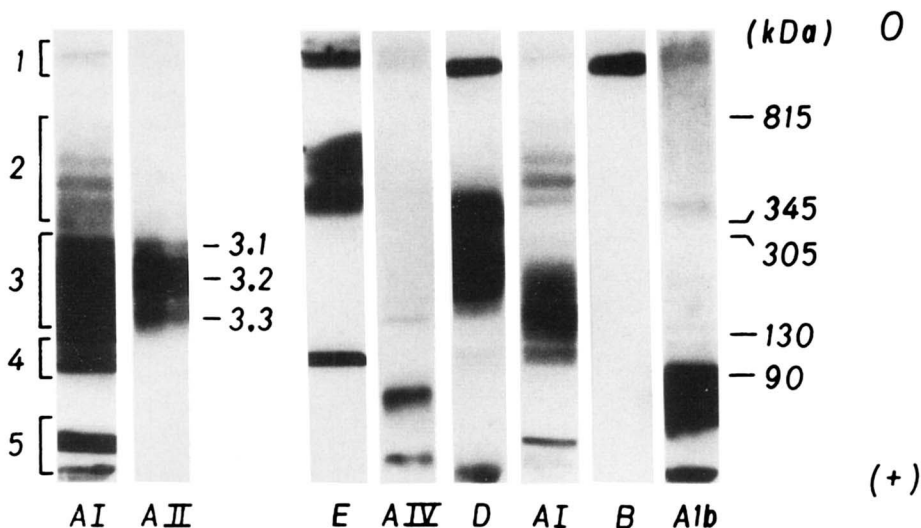


Fig. 1. Autoradiograph of an immunoblot of serum electrophoresed on 4–30% polyacrylamide gradient gels. Replicas were incubated with antibody specific for the indicated apolipoprotein. Two different exposures of the immunoblot with anti-apoA-I are included for identification of the major and minor bands. Molecular weights were estimated from calibration of the gel with high molecular weight protein standards.

Region 2, which includes components of 815 to 345 kDa, is characterized by the presence of five discrete bands of lipoproteins containing apoA-I and by the major signals for apoE bands, some of which may comigrate with the apoA-I-containing lipoproteins. In the zone of the smallest of these lipoproteins we also observe the presence of apoD.

Region 3, which includes components of 305 to 130 kDa, contains the majority of apoA-I and is characterized by the presence of all the apoA-II signal: three main subclasses can be observed which probably correspond to Lp A-I/A-II populations of discrete sizes which are labeled 3.1, 3.2, and 3.3. The presence of major signals for apoD and of traces of apoA-IV is also noted.

Region 4 which includes components of 130 to 90 kDa, is characterized by two well-defined bands of apoA-I-containing lipoproteins which do not contain apoA-II. However the apoA-I band corresponding to the smaller particle comigrates exactly with a sharply defined and strong signal for apoE and with a weak signal for apoD. These particles may therefore represent species of Lp A-I/E, some of which may also contain apoD.

Between regions 4 and 5, there is a large portion of the gel that contains absolutely no apoA-I. This phenomenon is probably related to the exclusion effect exerted by the very high concentration of albumin molecules that migrate to this position (see albumin signal in the right lane of Fig. 1). Consequently it is possible that the lipoprotein bands identified in region 4 would have migrated farther in the absence of albumin. However, it should be noted that the presence of albumin did not prevent the migra-

tion of lipoprotein particles of small molecular weight that are characterized by the exclusive presence of apoA-IV. Region 5, which includes a strong apoA-I band at 56 kDa, extends to the migration front and contains all the free apolipoproteins.

When total serum was electrophoresed on 2–16% GGE, we observed a better resolution of the lipoproteins identified above in regions 1, 2, and 3; however, lipoproteins that migrated to regions 3.3, 4, and 5 had migrated out of the gel and were not seen (Fig. 2). In region 1, it is clear that lipoproteins containing apoA-I are different from those containing apoB since they migrate to different positions. However, apoD and apoB migrated to the same position indicating that they are probably on the same lipoproteins. In region 2, we observed that some of the particles containing apoA-I and apoE migrated together at positions 2.3, 2.4, and 2.5 and that apoE was also present in a smaller particle that contains little or no apoA-I. In region 3, there is a good concordance of the signals for apoA-I and apoA-II which represent the Lp A-I/A-II. Interestingly, apoD is present between positions 3.1 and 3.2 where little apoA-I and apoA-II is found.

LDL size heterogeneity

When LDL were isolated by centrifugation and electrophoresed on GGE of 2–16% (Fig. 3A), we observed no definite signals for apoA-I or apoA-II. In serum as in isolated LDL, apoB and apoD were present on particles of the same size and therefore may reside on the same particles; apoE was present as a diffuse signal, probably reflect-

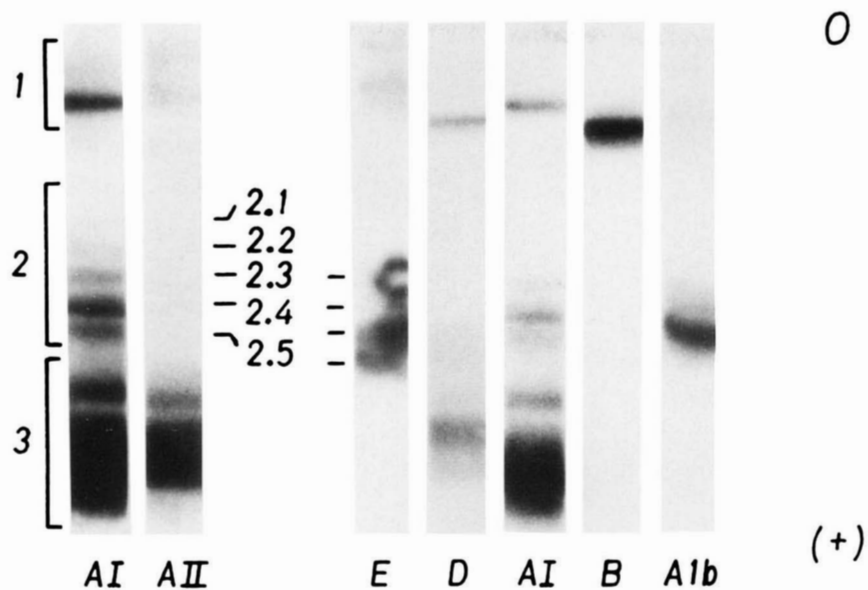


Fig. 2. Autoradiograph of an immunoblot of serum electrophoresed on 2-16% polyacrylamide gradient gels. Replicas were incubated with antibody specific for the indicated apolipoprotein.

ing its association with heterogeneous lipoproteins, and it was absent from the major lipoproteins which contained apoB and/or apoD. Essentially, similar results were obtained with GGE of 4-30% (Fig. 3B). In either gel system, the appearance of the apoE signal as a doublet probably reflects the presence of a large amount of LDL of a well-defined size that do not contain apoE.

HDL size heterogeneity

HDL were electrophoresed on GGE of 4-30% which achieved a good resolution of these lipoproteins by size (Fig. 4, top panel). The majority of apoA-I, apoA-II, and apoA-IV were present in lipoproteins of the same size (390-152 kDa) but a bimodal size distribution was mostly

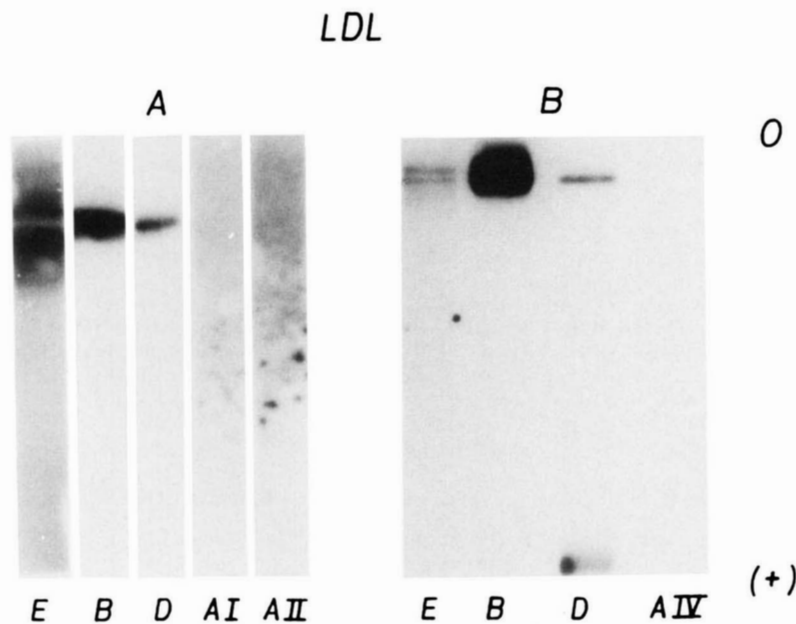


Fig. 3. Autoradiograph of immunoblots of LDL electrophoresed on polyacrylamide gradient gels. Replicas were incubated with antibody specific for the indicated apolipoprotein. A: A blot of serum LDL (100 µg of protein) separated on 2-16% gels; B: a blot of serum LDL (300 µg of protein) separated on 4-30% gels.

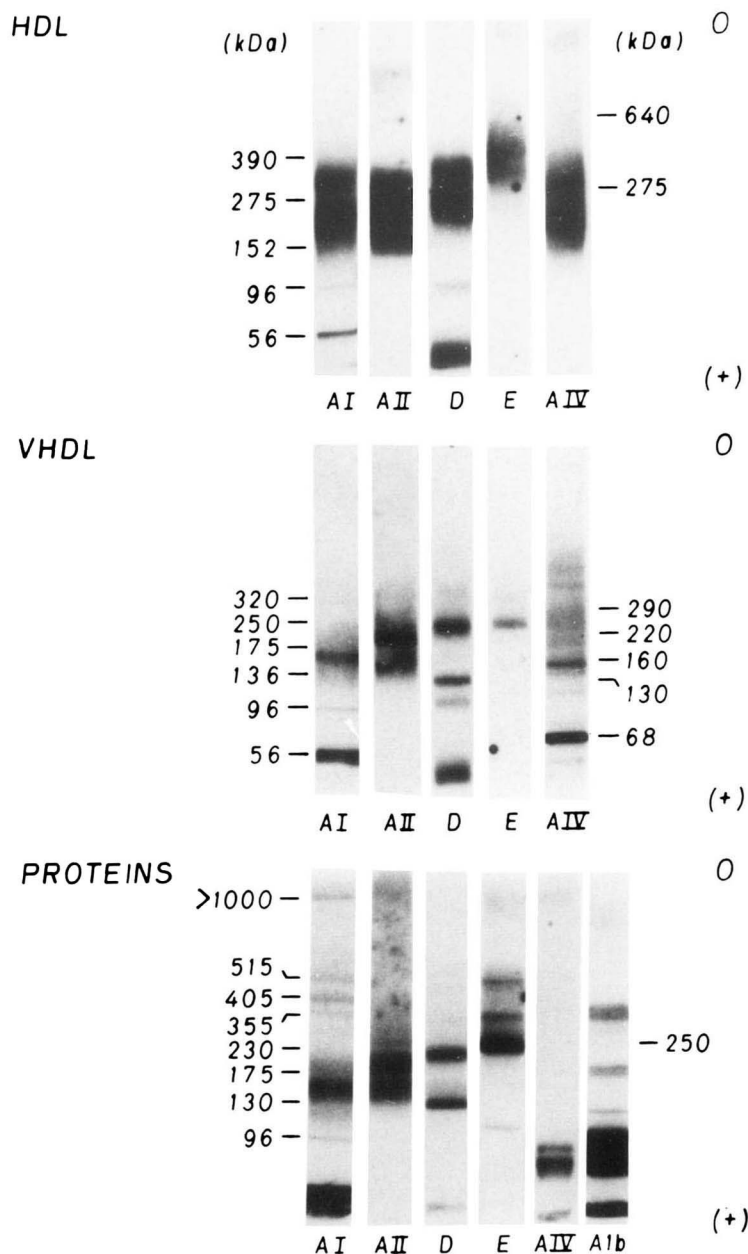


Fig. 4. Autoradiograph of immunoblots of serum lipoprotein and protein fractions electrophoresed on 4–30% polyacrylamide gradient gels. Replicas were incubated with antibody specific for the given apolipoprotein as indicated in each lane. To obtain the appropriate signal in the immunoblot, different amounts of lipoproteins were electrophoresed. Top: Serum HDL, 100 μ g of HDL protein for A-I and A-II or 300 μ g of HDL protein for D, E, and A-IV was applied to the gel. Middle: Serum VHDL, 100 μ g of VHDL protein for A-I, A-II, and D or 300 μ g of VHDL protein for E and A-IV was applied. Bottom: Serum protein fraction, 75 μ l of sample for A-I and A-II or 200 μ l for D, E, A-IV, and Alb was applied.

observed for apoA-I. ApoD and apoE tended to be associated with the larger lipoproteins. This trend was more pronounced for apoE which was associated only with the largest lipoproteins of 640 to 275 kDa. A defined lipoprotein band was identified at 96 kDa which contained apoA-I and apoD but no apoA-II, apoA-IV, or apoE. Finally, there was evidence of free apoA-I (mostly present

as apoA-I dimer at 56 kDa) and of free apoD on the migration front.

VHDL size heterogeneity

VHDL electrophoresed on GGE of 4–30% exhibited great size heterogeneity in lipoprotein species (Fig. 4, middle panel). The distribution of apoA-II-containing

lipoproteins is clearly bimodal between 250–175 kDa and 175–136 kDa whereas the distribution of apoA-I particles presents only one maximum between 175 and 136 kDa. Therefore, lipoproteins containing both apoA-I and apoA-II may only occur in the latter size range in VLDL which, as noted above, also contains the dense HDL₃. The larger apoA-II-containing lipoproteins are partially aligned with a large band of apoD centered at 230 kDa, with the only band of apoE centered at 250 kDa and with minor signals for apoA-IV. This electrophoretic migration could be compatible with the existence of lipoproteins containing apoA-II, D, E, and traces of A-IV in the size range of 250–175 kDa, but not of lipoproteins containing apoA-I, D, and E. However, lipoproteins containing apoA-I, A-II, and A-IV could occur in the size range of 160–130 kDa. It should be noted that the particle size distribution of apoA-IV was very heterogeneous ranging from 600 to 68 kDa. The band at 68 kDa was the most reactive and may represent an apoA-IV monomer associated with some lipids since no other apolipoprotein was seen at that position. The apoD signal was also seen at three discrete positions at 130 kDa, 100 kDa, and on the migration front.

Apolipoprotein distribution by size in the protein fraction

The protein fraction of serum defined as $d > 1.21$ g/ml surprisingly exhibited a large size heterogeneity of particles containing apolipoproteins (Fig. 4, bottom panel). The major apoA-I- and apoA-II-containing particles resembled those observed in VLDL. Again, there is a bimodality of apoA-II distribution with the two populations of particles having molecular weights at 230 to 175 kDa and 175 to 130 kDa, respectively. As in VLDL, the ratio of apoA-I to apoA-II is greater in the smaller particles of 175 to 130 kDa than in those of 230 to 175 kDa. Also similar to the VLDL, the major band of apoA-I was present as monomeric apoA-I on the migration front. It is noteworthy that this fraction does not contain free apoA-II or apoE but some free apoD and apoA-IV.

The major apoE band is sharply defined and centered at 250 kDa as noted above in VLDL. Likewise, the two major apoD bands are well defined and, respectively, centered at 230 and 130 kDa. However, none of the major bands for apoD or apoE present in the protein fraction of serum appear to be associated with each other or with the major bands for apoA-I and apoA-II. This segregation of the apolipoproteins is more clearly observed in the protein fraction than in VLDL. A faint apoE band is also seen which is lined up with the apoA-I band at 96 kDa and which probably corresponds to the same band seen in GGE of serum (Fig. 1). The migration of apoE to that position may be due to the presence of some lipids as well as to the exclusion effect exerted by albumin.

The most surprising observation was the presence of apolipoproteins such as A-I, A-IV, and E in high molecular

weight regions (i.e., above 350 kDa). Since the fraction electrophoresed is the protein fraction of $d > 1.21$ g/ml, only small amounts of lipids such as cholesterol, phospholipids, and free fatty acids may be present in association with apolipoproteins. Therefore, large and lipid-rich lipoproteins are not expected to be found in this fraction. Nevertheless, we have consistently observed the presence of apoA-I in well-defined bands in the LDL size range and in the 515, 405, and 355 kDa range. Well-defined bands of apoE were also seen at 515 and 355 kDa.

Comparison of apolipoprotein distribution by size amongst serum density subfractions

Having noted above the heterogeneity of apolipoprotein associations in particles of different sizes, it is informative to compare the size distribution of particles containing a given apolipoprotein across the spectrum of lipoproteins separated by ultracentrifugation. The comparison made for apoA-I distribution (Fig. 5) shows that only traces of apoA-I are present in the LDL fraction and that most of the apoA-I, presumably initially associated with LDL, migrate to a band of 56 kDa. In HDL, the distribution of the majority of apoA-I-containing lipoproteins is bimodal between 390–275 kDa and 275–130 kDa. HDL also contain well-defined bands of apoA-I at 96 and 56 kDa. In VLDL, the bimodality of apoA-I particles is lost and the signals for apoA-I are weaker and centered at 170 kDa. The bulk of VLDL apoA-I is present as a wide band (possibly a doublet) centered at 56 kDa, while the second most important band is sharp and well-defined at 96 kDa. In the protein fraction of serum, most of apoA-I is present as expected as free monomeric A-I which migrates at the front. Another important apoA-I band is seen at 100 kDa which may be the same as the band migrating to 96 kDa in the other lipoprotein fractions and which is retarded by the high concentration of albumin. In addition, the protein fraction also contains some apoA-I associated with particles of 275–152 kDa characteristic of the HDL and apoA-I associated with the high molecular weight components as had been seen in serum and defined above as region 1 (size range of LDL) and region 2 ranging from 815 to 345 kDa (Figs. 1 and 5). Finally, it should be noted that each apoA-I band seen in lipoprotein subfractions can be observed directly in serum, including those in the 390–275 kDa range upon longer exposure.

In contrast to apoA-I, the distribution of apoA-II in the different density subfractions of serum is remarkably constant and limited to a narrow size range of lipoproteins (Fig. 5). LDL does not contain any significant level of apoA-II (not illustrated) while in HDL, apoA-II-containing lipoproteins range in size between 390 to 130 kDa. In VLDL and in the protein fraction, apoA-II is associated with smaller lipoproteins from 305 to 130 kDa. Only traces of free apoA-II can be observed in any of the density sub-classes or in serum.

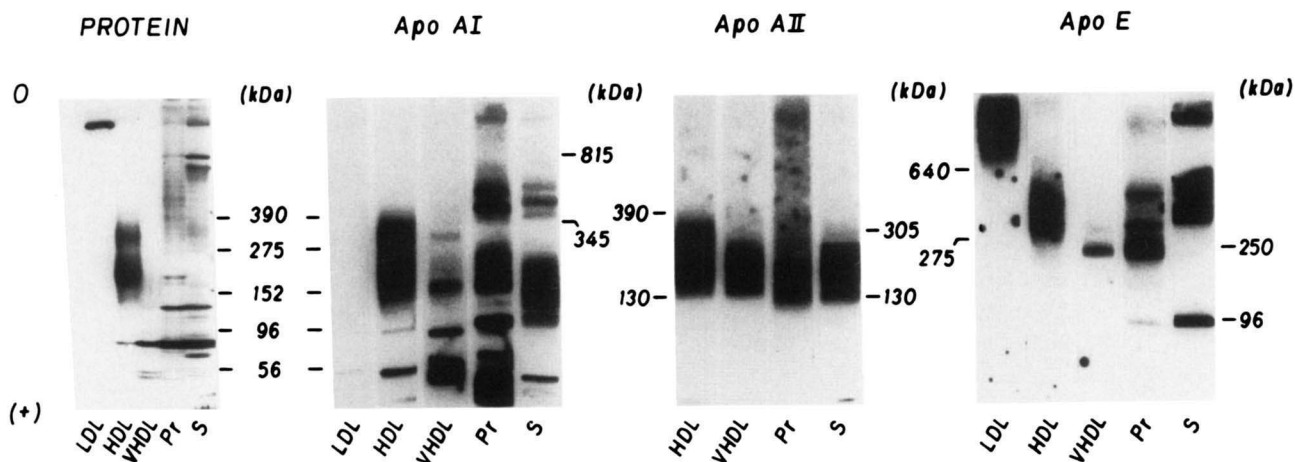


Fig. 5. Gel stained for protein with Coomassie Blue (left) or autoradiographs of replicas (right), probed for apoA-I, apoA-II, and apoE, of serum lipoprotein density classes electrophoresed on 4–30% polyacrylamide gradient gel electrophoresis. For the immunoblots with anti-apoA-I, the following amounts of each fraction were electrophoresed: LDL, HDL, VHDL (100 μ g of protein), protein fraction (200 μ l), and total serum (75 μ l). For the immunoblots with anti-apoA-II, we used: HDL, VHDL (100 μ g of protein), protein fraction (75 μ l), and total serum (75 μ l). For the immunoblots with anti-apoE, we used: LDL, HDL, VHDL (300 μ g of protein), protein fraction (200 μ l), and total serum (200 μ l).

The size distribution of apoE-containing particles in the serum density subclasses is distinct from that of apoA-I and apoA-II (Fig. 5). Unlike apoA-I, significant amounts of apoE are associated with lipoproteins of LDL size in the LDL fraction. In HDL, apoE is associated with large lipoproteins ranging from 640 to 275 kDa, while in VHDL and in the protein fraction, apoE is present mostly in lipoproteins of well-defined size centered at 250 kDa. In addition, three other well-defined bands containing apoE can be observed in the protein fraction as well as in serum at 515, 355, and 96 kDa (Figs. 4 and 5). However, the apoE band at 250 kDa seen in VHDL and the protein fraction is not observed in serum.

Immunoprecipitation of serum apoA-I and apoA-II

To characterize the association of apoA-I and apoA-II in lipoprotein species of different sizes, we subjected serum to immunoprecipitation with antibodies against apoA-I and apoA-II, either separately or in combination. It should be noted that, due to presence of high concentrations of added BSA as a carrier, the relative position of the bands in these experiments cannot be compared to those in the preceding figures. When serum was immunoprecipitated with anti-apoA-I, all signals for apoA-I were removed except those associated with bands of high molecular weights (Fig. 6A, lane 2) which correspond to the bands of 815 to 345 kDa defined above. These high molecular weight bands which are positive for apoA-I probably do not contain lipids since they are found in the protein fraction of serum upon centrifugation (Figs. 4 and 5). They may represent apoA-I bound to high molecular weight proteins and this association may prevent accessibility to the epitope for the immunoprecipitation but not

for the immunodetection. Similar results were obtained with different monoclonal antibodies against apoA-I which were shown to be efficient in the precipitation of 125 I-labeled HDL (14). Although immunoprecipitation with anti-apoA-I removed all immunodetectable apoA-I in the lipoproteins of 400 to 150 kDa, which correspond to the particles found in HDL, some immunodetectable apoA-II remained in this region of the gel (Fig. 6B, lane 7). This may indicate that some lipoproteins with apoA-II may exist which do not contain apoA-I. As we have shown earlier, the antibody against apoA-II can precipitate up to 80% of labeled HDL apoA-II (14). Likewise, here, we observed that after immunoprecipitation with anti-apoA-II, some immunoreactive apoA-II was still detected in the region of 400 to 150 kDa (Fig. 6, lane 8) as well as immunoreactive apoA-I (Fig. 6, lane 3). However, combination of antibodies against apoA-I and apoA-II succeeded in removing all immunodetectable apoA-I and apoA-II in this size range (Fig. 6, lanes 4 and 9).

These experiments show that lipoproteins with both apoA-I and apoA-II are distributed across the size range of 400 to 150 kDa and that, in addition to the lipoproteins with apoA-I and without apoA-II found by others (15), some lipoproteins may also exist which contain apoA-II but no apoA-I. However, it cannot be ruled out that these apoA-II-containing lipoproteins contain apoA-I that is not immunoprecipitable or immunoreactive with the antibodies used.

Size heterogeneity in lipoproteins of HepG2 culture medium

When total HepG2 culture medium was electrophoresed on 2–16% GGE, a single well-defined band of apoB was

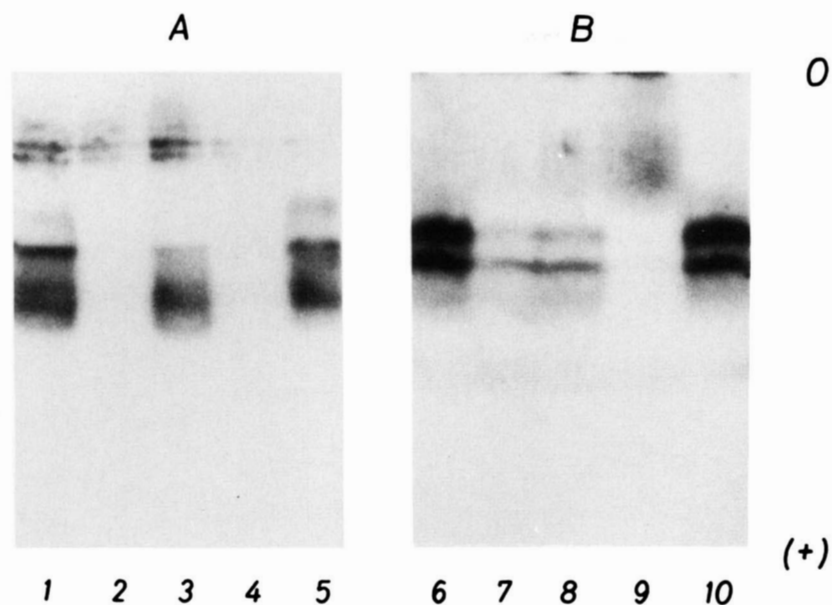


Fig. 6. Autoradiograph of immunoblots of serum electrophoresed on 2.5-27% polyacrylamide gradient gels after immunoprecipitation. An aliquot (150 μ l) of supernatant was applied to the gel after precipitation with: non-immune antiserum, lanes 1 and 6; anti-apoA-I, lanes 2 and 7; anti-apoA-II, lanes 3 and 8; anti-apoA-I + anti-apoA-II, lanes 4 and 9. Lanes 5 and 10 show the starting serum. A: The blot was incubated with anti-apoA-I; B: the blot was incubated with anti-apoA-II.

seen which corresponded to the general position of LDL (Fig. 7A). There were four distinct bands for apoA-I, some of which were aligned with bands for apoA-II and apoE (Fig. 7A). However, a better resolution of the lipoproteins present in HepG2 culture was obtained by separation on 4-30% GGE (Fig. 7B). Lipoproteins containing apoA-I were separated into three major classes centered at 265, 120, and 67 kDa (Fig. 7) which are significantly smaller than the apoA-I-containing lipoproteins of serum (Fig. 1). Minimal exposure of the autoradiograph of apoA-I immunoblot demonstrated that the two major lipoproteins with apoA-I had mean molecular weights of 120,000 and 67,000 (Fig. 7B, last lane). In contrast, the apoA-II-containing lipoproteins of the HepG2 culture medium are separated into three classes with mean molecular weights of 305,000, 230,000, and 170,000 (Fig. 7B), values which are very similar to those observed for apoA-II-containing lipoproteins of serum (Fig. 1). Similarly, apoE-containing lipoproteins of HepG2 medium are separated into three classes centered at 800,000, 580,000, and 400,000 (Fig. 7B) which correspond generally to the molecular weight range observed in serum for the lipoproteins with apoE.

When the lipoproteins of the HepG2 culture medium were separated by sequential ultracentrifugation and electrophoresed on 4-30% gradient gels, we could verify that the majority of apoA-I was in fact associated with lipids and floated in the HDL range (Fig. 7C). Only the apoA-I

which is seen in the band at 60 to 67 kDa and which may be an apoA-I dimer was present in the protein fraction (Fig. 7C).

DISCUSSION

As reported initially by other groups (1-4), gradient gel electrophoresis provides an excellent resolution of lipoproteins by size for VLDL, IDL, LDL, and HDL. The localization of the separated lipoproteins by lipid stain is much more efficient for the large and lipid-rich particles than for particles in the HDL size range. The latter are best identified by protein stains but this requires the initial separation of lipoproteins either by centrifugation (4) or by immunoaffinity (15). The detection of lipoproteins by immunoblotting with specific antibodies is clearly advantageous in that it can be applied to the characterization of lipoproteins of any size as they occur in plasma or serum.

The original applications of GGE were directed toward the determination of lipoprotein size which is obtained by application of high voltage, i.e., 3000 volt \cdot hr, that ensures the migration of lipoproteins to the exclusion limit of the polyacrylamide gel pores (3). However, when lipoproteins are blocked in their vertical migration by the pore size, they are also blocked for the horizontal migration required in the electrophoretic transfer to nitrocellulose. These

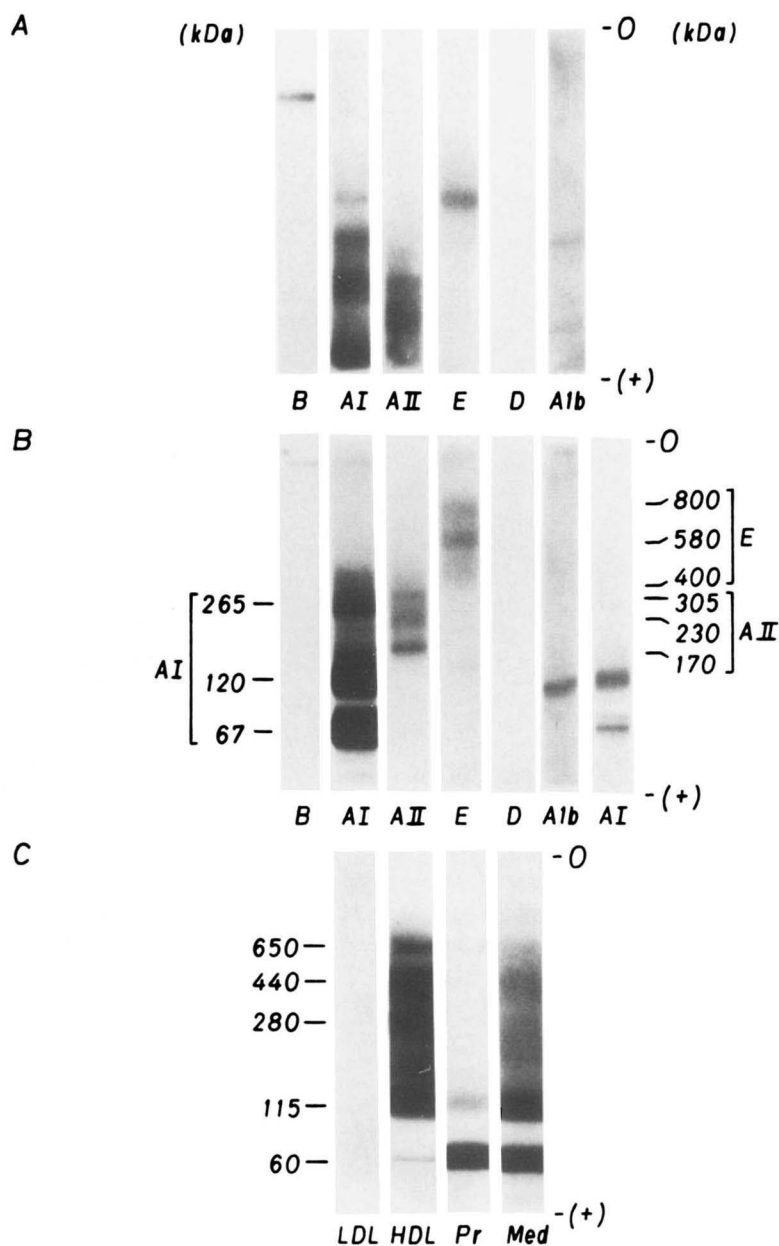


Fig. 7. Autoradiograph of immunoblots of HepG2 medium and HepG2 secreted lipoproteins electrophoresed on polyacrylamide gradient gels. Replicas were incubated with antibody specific for the indicated apolipoprotein. A: HepG2 medium (1 ml) on 2-16% gels. The equivalent of 3.5 ml was applied to probe for apoA-II. B: HepG2 medium (1 ml) on 4-30% gels. C: HepG2 secreted lipoproteins (25-50 µg of protein) separated on 4-30% gradient gels and probed for apoA-I.

considerations led us to decrease the voltage applied during the migration to 1600 volt·hr, which was found to be sufficient for a nearly optimum size separation without blocking the lipoproteins and thus ensuring efficient transfer for immunoblotting which was nearly complete for all proteins and lipoproteins after 72 hr. However, both the losses occurring through the pores and the limited capacity of nitrocellulose paper do not permit a complete blotting of compounds that differ widely in their concentration. For these reasons, immunoblotting of lipo-

proteins separated by GGE can only give approximate values for the distribution of a given apolipoprotein in lipoproteins of different sizes, and, accordingly, one should not attempt to quantify apolipoprotein distribution with this methodology. Despite these reservations, the immunodetection of lipoproteins separated by GGE is clearly superior in sensitivity and specificity to that afforded by the staining techniques. This approach has also allowed us to characterize the apparent molecular weights of particles containing specific apolipoproteins in HDL

and VHDL and in the plasma protein fraction. Because the voltage that we used in the electrophoresis was different from that in the literature (4), the apparent molecular weights of nine major lipoprotein bands seen in LDL, HDL, and VHDL have been measured after migration for 1600 volt · hr and compared to those obtained at 3000 volt · hr. They were found to be lower after 1600 volt · hr by less than 8% (range 4–10%) without any bias toward the largest or smallest particles. Therefore, each molecular weight reported here should be multiplied by a factor of 1.08.

We chose to isolate the lipoproteins by single-spin density gradient centrifugation in order to minimize losses of apolipoproteins from each density class of lipoproteins. However, it is important to note that the densities at which these lipoproteins were isolated does not coincide exactly with those commonly used in sequential ultracentrifugation. In addition, since we used the sera of normal fasting subjects which contained very low levels of VLDL, we did not attempt to optimize the electrotransfer of VLDL or IDL which could be achieved with gradient gels of 2–16% acrylamide. Thus apoB-containing lipoproteins larger than LDL have not been studied in the present report. We initially suspected that the presence of apoA-I, A-IV, D, and E in the size range of LDL and above (Fig. 1) represented apolipoproteins associated with chylomicron remnants or other large lipoproteins; however, they were also found in the protein fraction (Figs. 4 and 5). At first it also appeared that albumin might be specifically associated with LDL (Fig. 1) and might represent an apolipoprotein, but the strength of the signal was much decreased when serum was electrophoresed on 2–16% acrylamide gels (Fig. 2). Consequently albumin should be considered as a contaminant.

By comparison with other apolipoproteins, serum apoA-II is unusual in that it is associated with lipoproteins of limited size heterogeneity ranging from 305 to 130 kDa, whereas apoA-I, A-IV, D, and E can be seen associated with a wide range of particles extending from the size of LDL to the migration front (Figs. 1 and 2). Even when the strength of the signal is increased by electrophoresis of an isolated lipoprotein fraction such as HDL and VHDL, apoA-II is only present in lipoproteins of limited heterogeneity ranging from 390 to 130 kDa in HDL, and from 320 to 130 kDa in VHDL (Fig. 4). In HDL, we observed a bimodal distribution of apoA-I at 390–275 kDa (11.9–10.2 nm) and 275–152 kDa (10.2–8.4 nm) which overlaps with the distribution of apoA-II. These lipoproteins are somewhat larger than the HDL₂ (10.57 and 9.16 nm) and the HDL₃ (8.44, 7.97, and 7.62 nm) measured by Blanche et al. (1) or than the Lp (A-I without A-II) (10.8 and 8.5 nm) and the Lp (A-I with A-II) (9.6, 8.9, and 8.0 nm) measured by Cheung and Albers (15). The larger apparent size measured in our experiments cannot be the

result of the lower voltage which we applied during the migration in order to improve the electrotransfer of the separated lipoproteins since this lower voltage tends, on the contrary, to decrease the apparent sizes of particles. In addition, it is intriguing that we observed the existence in serum of Lp (A-I without A-II) identified as fraction 4 in Fig. 1 which migrates between 130 and 90 kDa (8.1 and 7.5 nm) but no Lp (A-I with A-II) which should also have been expected in that region (15). It is unlikely that these apparent discrepancies could be related to the specificities of the antibodies used since we have obtained the same results with polyclonal antisera against apoA-I. We would rather hypothesize that the identification of lipoprotein bands by immunoblotting need not agree exactly with the bands seen upon staining with Coomassie blue as done by others (15). In serum, we have also observed that lipoproteins containing apoA-II can be seen as three populations of discrete sizes which are identified as fractions 3.1, 3.2, and 3.3 on Fig. 1 and which may correspond to the three populations of Lp (A-I with A-II) cited above (15).

In HDL, the signals for apoA-I, A-II, A-IV, and D are overlapping (Fig. 4) and are therefore compatible with the association of two or more of these apolipoproteins on the same particles in agreement with the apolipoprotein heterogeneity that was observed previously by isoelectric-focusing on HDL₂ and HDL₃ (16). However both apoD and apoE tend to be associated with the larger lipoproteins that are present in the HDL fraction. This size difference is most pronounced with apoE, which is present only in lipoproteins of 640 to 275 kDa (Fig. 4). These results corroborate the observations of others (17–19) who have shown by gel filtration of plasma that a significant proportion of apoE-rich lipoproteins is found in particles of sizes intermediary between LDL and HDL. We have observed in HDL that the smaller lipoproteins of 275 to 150 kDa may contain apoA-I, apoA-II, and some apoD and apoA-IV but no apoE, whereas the largest of the apoE-containing lipoproteins do not contain significant amounts of apoA-I, apoA-II, or apoD. HDL with apoE have been isolated by heparin–Sepharose chromatography and have been shown to contain both apoA-I and A-II (20, 21). In addition, apoE-containing HDL₂ were also shown to be larger than HDL₂ without apoE (21) with an average size of 12.2 nm, a value in agreement with our present results. In HDL, we have also identified a clearly delimited lipoprotein band at 96 kDa which may contain both apoA-I and D but no apoA-II, A-IV, or E that has not been reported previously. A similar band at 96 kDa is also seen in VHDL and in the plasma protein fraction, which indicates that this lipoprotein is probably poor in lipids. A band at 56 kDa is also seen in both HDL and VHDL which contains only apoA-I, and which could be either a monomer or a dimer of apoA-I associated with some lipids.

Whereas in GGE of HDL most apolipoproteins are seen as associated with lipoproteins with overlapping polydisperse size distributions, consistent with the presence of many different lipoproteins containing for the most part two or more apolipoproteins, GGE of VHDL (containing here some HDL₃) shows such polydisperse size distributions only for apoA-I (unimodal at 175–136 kDa) and for apoA-II (bimodal at 250–175 kDa and at 175–136 kDa). Therefore, lipoproteins containing both apoA-I and A-II may also be present in VHDL but only in the smaller size range. The major apoA-I signal which is seen in both VHDL and the protein fraction at 56 kDa may represent apoA-I that was stripped off from the larger lipoproteins containing both apoA-I and A-II during centrifugation. However this apoA-I band at 56 kDa is also seen in GGE of HDL and serum; therefore, it may demonstrate the existence of such particles in native serum or it may result from the stripping of apoA-I dimer during electrophoresis. The signals for the other apolipoproteins appear as discrete bands without clear overlap (Fig. 4). One possible exception is a band at 250 kDa which could represent a lipoprotein containing apoA-II, D, and E with traces of apoA-I and A-IV. While this could be construed as evidence for the existence of single lipoprotein families in that density, GGE of whole serum does not show the presence of an apoE-containing lipoprotein at 250 kDa (Fig. 5). Consequently, the apoE band that is seen at 250 kDa in both VHDL and the plasma protein fraction is probably an artefact generated by ultracentrifugation, and we ought to be cautious in the interpretation of the other apolipoprotein bands that are seen in these density fractions if they are not also observed in serum.

GGE of the protein fraction of serum also unexpectedly demonstrated the presence of apoA-I, A-IV, and E in particles of high molecular weights (350 kDa). Since these particles are found in the fraction of $d > 1.21$ g/ml, it is highly unlikely that their large size is related to high lipid-protein ratios. Also, since these large particles exist in serum as demonstrated by the corresponding signals for apoA-I, A-IV, and E (Figs. 1 and 5), these apolipoprotein-containing particles are not artefacts of the centrifugation. We hypothesize that they represent apolipoproteins bound to specific proteins, possibly by hydrophobic interactions. The protein-bound forms of apoA-I were also shown to be inaccessible to specific antibodies against apoA-I, which otherwise can immunoprecipitate all lipoprotein associated apoA-I (Fig. 6). We assume, therefore, that these proteins are bound to apoA-I and mask the epitopes which are normally exposed when apoA-I is associated with lipids.

Owing to the ease with which apoA-IV can be dissociated from lipoproteins during centrifugation, there is much uncertainty as to the proportion of apoA-IV that is associated with lipids in serum (22). Our immunoblotting of apoA-IV on serum lipoproteins separated by GGE indicates that most of apoA-IV is associated with a small

particle that comigrates with albumin (Fig. 1). However, some apoA-IV present in particles of 130–150 kDa can be seen by direct immunoblotting of serum separated by GGE, where it comigrates with lipoproteins containing apoA-I and A-II (Fig. 1, fraction 3.3). Bisgaier and colleagues (23) have also noted the presence of apoA-IV in analogous small particles (7.8–8.0 nm) isolated by gel filtration of plasma which also contained apoA-I. These authors have noted that some apoA-IV appears to be displaced during electrophoresis from these small lipoproteins which were initially isolated by gel filtration. It appears, therefore, that apoA-IV can be easily lost from lipoproteins during electrophoresis as well as centrifugation but it is clear in our experiments that some apoA-IV remains associated with centrifugally prepared lipoproteins. When HDL are electrophoresed on gradient gels (4), some apoA-IV is also associated with lipoproteins of a size similar to that of Lp (A-I without A-II) and Lp (A-I with A-II). ApoA-IV, like apoA-I, is also present in particles of high molecular weight that are found in the protein fraction of serum and that probably represent protein-bound forms of apoA-IV.

The lipoproteins of HepG2 culture supernatants have a distribution different from that of serum (Fig. 7). In agreement with Thrift et al. (24), we have observed that apoE is present in large lipoproteins that range in size from 800 to 400 kDa. These apoE-containing lipoproteins do not contain any apoA-I or A-II and may represent Lp(E). The major apoA-I-containing lipoproteins are centered at 120 and 67 kDa and do not contain any apoA-II. Three distinct bands of lipoproteins with apoA-II are seen at 305, 230, and 170 kDa which overlap but do not coincide with the peak of lipoproteins with apoA-I at 265 kDa.

Therefore, most of apoA-I, A-II, and E found in HepG2 culture supernatant are associated with distinct and separate lipoproteins. ApoA-II and apoE are present in lipoproteins of the same size range whether in HepG2 medium or in serum, but apoA-I is not. Newly secreted hepatic apoA-I is found mostly in small lipoproteins, and metabolic events that take place in the circulation cause its association with larger serum lipoproteins that also contain apoA-II and/or apoE. It is a novel observation that HepG2 secretes mostly Lp (A-I without A-II), and little if any Lp (A-I with A-II) although Lp (A-II without A-I) may be present.

Immunoblotting of apolipoproteins after GGE of serum or HepG2 lipoproteins provides a powerful tool for the analysis and characterization of lipoproteins. We anticipate that this technique, used in conjunction with immunoprecipitation of specific lipoprotein families, will allow us to define the specific associations of different apolipoproteins in lipoprotein species, most especially in the HDL and VHDL size range which are very well resolved in this system. This approach will also enable us to study how cholesterol esterification and lipid transfer processes

transform the small lipoproteins with apoA-I secreted by the liver into the larger and more complex apoA-I-containing lipoproteins that are found in the circulation.

In summary, while the size distribution of lipoproteins with apoA-I and with or without apoA-II which we observed is generally consistent with the results of Cheung and colleagues (15, 25), we have also noted some differences such as the presence of an Lp (A-I without A-II) at 130–90 kDa (8.1–7.5 nm) and of an Lp (A-I without A-II) but possibly with apoD and apoE at 96 kDa. We also report a distinct band of apoA-I (probably a dimer) which is seen at 56 kDa in HDL, VHDL, and protein fraction as well as in serum. In addition, we have presented new information on the size distribution of apoD, E, and A-IV in lipoproteins notably in LDL, HDL and VHDL, and in native serum. ■

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