Characterization of Electrophoretic Lipoprotein Fractions: Immunochemical and Electron Microscopic Studies

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To improve the characterization of electrophoretic lipoprotein subfractions, we developed two new techniques for analyzing lipoproteins after electrophoresis on thin agarose layers. Overlay with antisera exactly localizes specific apoproteins without any distortion caused by antigen diffusion; electron microscopy of eluted fractions determines the varying particle-size distribution. Applied together, these methods can detect individual differences between hyperlipemic samples that are not immediately apparent in the electrophoretic pattern, and should provide valuable new insight into the classification of hyperlipoproteinemias.

Additional Keyphrases antibody overlay technique
- agarose gel electrophoresis
- nephelometry
- hyperlipoproteinemia phenotyping
- protein localization on electrophorograms

To a considerable extent the phenotyping of hyperlipoproteinemias with electrophoresis is an operational classification (1). Despite the wide acceptance of this procedure as a clinical tool, interpretation of observed patterns is based largely on information obtained by correlating electrophoretic findings with independent chemical and ultracentrifugal analyses. Very little has been done to characterize the isolated separated lipo-

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phoresis, a few drops of monovalent α- or β-lipoprotein antiserum (Behring Diagnostics, Inc., Woodbury, N.Y. 11797) were placed on the undried gel and were rolled with a glass tube, to form a liquid film over its entire surface. Purity of antisera was confirmed by immunoelectrophoresis. The overlaid gel was then placed in a humid chamber for a few hours to allow the antiserum to diffuse into it and precipitate specific antigen. The unprecipitated serum proteins and antiserum were eluted for about 3 h in agitated isotonic saline, and the gel was dried for protein staining.

**Electron microscopy.** Electrophoretic fractions, identified from a stained pattern run in parallel, were cut with a razor blade from the undried gel, immersed in isotonic saline, and agitated gently for 24 h at room temperature. This mixture was centrifuged carefully to remove the gel from the lipoproteins, which were now in the supernatant fluid. A drop of supernatant fluid was fixed with an equal drop of phosphate-buffered osmium tetroxide (10 g/liter) for direct electron microscopic visualization by the method of Jones and Price (3). Electron micrographs were taken with a Philips EM-300 electron microscope and particle sizes were estimated from enlarged photographs.

**Lipid assays:** Total lipids were determined with the sulfo-phospho-vanillin method (4), cholesterol according to Babson et al. (5), and triglyceride glycerol according to Carlson (6).

**Nephelometry:** To estimate particulate fat, serum turbidity (10-fold dilutions with isotonic saline) before and after ultrafiltration was measured with the Amino-Bowman fluorometer (American Instrument Co., Silver Spring, Md. 20910), used with a red primary filter but no secondary filter. The serum turbidity removed by ultrafiltration through filters of 50-nm nominal pore diameter (Sartorius Division, Brinkman Instrument Co., Westbury, N.Y. 11590) represents mainly a chylomicron fraction, whereas the serum turbidity remaining after ultrafiltration corresponds to pre-β-lipoproteins. Results are reported in arbitrary light-scattering units (1/21). In normal fasting serum, less than 15 1/21 are removed by filtration, and less than 25 1/21 remain after filtration, as described previously (7).

**Results**

Figure 1 demonstrates the specific precipitation of α- and β-lipoproteins after they have been separated on gels of different agarose content. After protein stain (patterns A) the β-lipoproteins appear as a sharp line between the two β-bands. After lipid stain (patterns D) a narrow band of β-lipoproteins and a broader band of pre-β-lipoproteins are seen. To make migration comparable, it was necessary to use only a 1-μl sample. This is insufficient to render the α-lipoproteins as clearly visible as in routine lipid patterns, in which 6-μl samples are used. The agarose content of the gel does not influence the migration of the β-lipoproteins, which are smaller, but the mobility of the larger pre-β-lipoproteins increases in the looser gels. Overlay with β-lipoprotein antiserum (patterns B) precipitates both β- and pre-β-lipoproteins. Overlay with α-lipoprotein antiserum causes a precipitate in the α-region with a trail and a second, rather faint band corresponding to the pre-β-lipoproteins (patterns C). In all subsequent experiments, gels with 9 g of agarose per liter were used, because this separates β- and pre-β-lipoproteins adequately without permitting the latter to move too far into the α-zone.

Figure 2 shows typical particles sampled from the origin and from the β-, the pre-β-, and the α-region. After an overnight fast, few chylomicrons are visualized in normal human serum. Particles from the β-region are uniform and about 30 nm in diameter, whereas particles from the pre-β-region are heterogeneous, ranging from 30 to 60 nm diameter. However, the heavy metal coat deposited on lipoproteins during shadowing for electron microscopy increases the diameter of al

![Fig. 1](image-url)
particles by 10 to 15 nm regardless of their native size (Sata, T., Jones, A. L., and Havel, R. J.: personal communications). Very few, if any, of the small particles from the α-region can be visualized after they are eluted from agarose.

Though obtained from fasting subjects, all samples in Figure 3 were turbid. In one sample (subject R.T.), chylomicrons hardly are distinguishable in the electrophoretic pattern, whereas in the other two samples (subjects A.B. and J.B.) chylomicrons visibly trail from the origin to the β-region. This makes the β-band less distinct, but their strong blue staining distinguishes β-lipoproteins from chylomicrons. Antiserum overlay confirms the distinction: β-lipoprotein antiserum precipitates a strong β-band that is wider but less sharply demarcated than in the controls, whereas the broader pre-β-band is similar to the controls. Antiserum to α-lipoprotein precipitates the α-band and precipitates a band in the pre-β-region, but none in the β-region. Electron micrographs of these three specimens (Figure 4) reveal particles of quite similar size at the origin and also in the pre-β-region. However, particles in the β-region are dissimilar. Here subject A.B. has β-together with some pre-β-lipoproteins; subject R.T., in addition, has occasional chylomicrons; whereas subject J.B., whose serum was icteric, has very few β-lipoproteins but many small chylomicrons with 100 nm diameter or greater. The different lipoprotein size distribution is confirmed by ultrafiltration. In subject A.B. a large fraction of the particles causing turbidity passes the filter of 50-nm pore diameter, whereas the same filter retains most of these particles in subject J.B.

Figure 5 shows a specimen with increased serum triglyceride owing, in large part, to increased pre-β-lipoproteins, as evidenced by the marked turbidity of the sample even after ultrafiltration. The electron micrographs (Figure 6) show chylomicrons moving even beyond the β-region. It is further noted that β-lipoproteins trail into the pre-β-region (region 5 of the electrophoretic pattern in Figure 5).

Discussion

Electrophoresis is now commonly used to phenotype hyperlipoproteinemias. While some hereditary forms are quite clear-cut entities [i.e., type II of Fredrickson (1) or hyperbetalipoproteinemia], other forms are less well defined. Direct

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**Fig. 2.** Electron micrographs of electrophoretic lipoprotein fractions from a normal, fasting subject

*Upper left:* chylomicrons sampled at the origin. *Upper right:* sample from the β-region. *Lower left:* sample from the pre-β-region. *Lower right:* sample from the α-region. Magnification 13,000×

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**Fig. 3.** Electrophoretic patterns of subjects R.T., A.B., and J.B.

Protein stain (A), lipoprotein stain (B and E), α-lipoprotein antiserum overlay (C), and β-lipoprotein antiserum overlay (D). 1-μl samples were used for patterns A through D. 6-μl samples were used for patterns E to achieve stronger lipid stain. With this larger sample, migration rates differ from those of patterns A through D. Fractions sampled for electron microscopy are indicated by hatches and numbers. Antigen excess may cause soluble immunocomplexes so that the center of the α-precipitate appears clear.
information about the lipoprotein classes separated by electrophoresis is desirable because electrophoresis on most supports only separates the serum proteins into overlapping fractions. As immunoelectrophoresis demonstrates, any position on the electropherogram is occupied by different proteins that respond similarly to the separating force. Because the specific apoprotein together with particle size are major factors determining the electrophoretic migration of lipoproteins, the described immunochemical and electron microscopic techniques were developed to analyze these characteristics.

In the usual immunoelectrophoresis technique, both antigen and antibody diffuse into a “spacer” gel to precipitate in arcs (“double diffusion”). The “spacer” assists the resolution of multiple precipitation arcs obtained with a polyvalent antiserum, but diffusion into an arc limits precise localization of antigen, even with a monovalent antiserum (the length of the arc exceeds the width of the original antigen band). By contrast, only the antibody diffuses in the described antiserum overlay technique, and diffusion through the entire depth of the gel is rapid on a thin agarose layer (“single diffusion”). Therefore, the antigen is quickly immobilized by precipitation and can be localized without distortion by diffusion.

No electron microscopic studies of lipoproteins after electrophoresis have been reported. Removing lipoproteins from most electrophoretic supports is difficult because β-lipoproteins bind to agar (8) and chylomicrons stick to paper as well (9). However, lipoproteins can be eluted from
thin agarose layers, because agarose contains no charged chemical groups (10) and the diffusion path out of the gel is short. Chylomicrons can be demonstrated in electron micrographs even when they are not detected by visual inspection of serum or by electrophoresis (a relatively insensitive method) and only nephelometry reveals their presence (7). In turbid sera, large particles migrated as a trail from the origin to the β-region because the gel we used for electrophoresis is sufficiently loose to allow chylomicrons to penetrate. More concentrated gels sterically restrict movement of large particles (10); more polar supports such as agar (8) or paper (9) retard or prevent their migration by adsorption. On the latter support, the broadening of the β-band in chylomicronemia also does not occur. If this effect seen on agarose were due to some association of β-lipoproteins with chylomicrons, it would be of physiological interest. Although both kinds of particles were found together in the β-region, the electron micrographs showed no such relationship after fixation. Neither α- nor β-apoprotein was demonstrable between the origin and the β-region where chylomicrons migrate, even in very turbid or milky sera, but the small protein content of the chylomicrons may not provide sufficient antigen to produce a visible precipitate. However, after gel filtration the largest lipid particles do not produce an immunoreaction with these antisera either (11).

The majority of particles in the β-region were homogeneous in size, of about 30 nm diameter, and precipitated only with β-lipoprotein antiserum. Remarkable heterogeneity of particles, usually with 30 to 60 nm diameter, was encountered in the pre-β-region. In this zone a variety of sizes occurred among different samples. Here β-lipoprotein antiserum always formed a precipitate, but α-lipoprotein antiserum often did as well. This confirms findings with pre-β-lipoproteins separated according to size by gel filtration (11). α-Apoprotein was almost always demonstrable immunologically up to quite some distance on the anodic side of the albumin region. Lipid-staining material in front of the albumin band has been found on various electrophoretic supports and is usually interpreted to be fatty acids bound to albumin. However, no positive proof has ever been given for this interpretation. Although our patterns clearly showed the presence of α-apoprotein in this zone, the small α-lipoproteins were not seen with certainty in eluates.

Both described techniques should be of considerable value in further clinical research of human lipoprotein subfractions in health and disease as improved electrophoretic methods increase the number of resolved bands. Additional serum lipoprotein apoproteins have been shown to occur in cholestasis (12) and hyperlipoproteinemia type III according to Fredrickson’s classification (13), and a precise means of localizing such proteins is useful to define their exact electrophoretic position. A method of collecting lipoproteins subfractions from agarose is applicable to microchemical much as to morphologic analysis. Antiserum overlay on thin gels can be used to localize other proteins as well as lipoproteins.

References