Electron Microscopy of Human Plasma Lipoprotein Separated by Ultracentrifugation

By D. E. BEECHER, PH.D.

The particle size distribution of the S_f 30 "class" of serum lipoprotein was determined by the electron microscope. Most of the lipoprotein molecules are not spherical but have the shape of ellipsoids of rotation with an axis ratio of 1.5. The molecules of a homogeneous flotation "class" show differences in the reactivity of their lipid moiety demonstrated by their staining properties with osmium tetroxide.

A CERTAIN degree of correlation between increased levels of plasma lipoproteins and the incidence of arteriosclerosis, stressed by Gofman and his collaborators, has aroused widespread interest in this little known class of conjugated proteins. Ultracentrifugal studies of the Berkeley group and the work on lipoproteins of the Harvard Laboratory of Physical Chemistry, indicate that hydrated β-lipoprotein has a molecular weight of several millions. The shape of these molecules was found to be almost globular. Particles of this size and shape (diameter about 25 millimicrons) are well in the range of the resolving power of the electron microscope. A first attempt to demonstrate the particle size of the S_f 10-20 fraction in electron microscopic pictures of plasma lipoprotein was made by Prendergast and Teague. The following communication extends these observations.

EXPERIMENTAL PROCEDURE

Lipoprotein fractions were separated by the ultracentrifuge in a procedure using the negative sedimentation rates introduced by Gofman and collaborators. The lipoprotein of the serum of healthy persons was first concentrated in a Spinco Model L "preparative" ultracentrifuge at 30,000 r.p.m. for 15 hours. The top fraction of 1 ml., which contains all lipoprotein lighter than 1.063, was further separated at 52,640 r.p.m. in a Spinco Model E ultracentrifuge. After 60 minutes at full speed a photographic picture of the distribution of the lipoprotein was taken. The centrifuge was then decelerated, the cell immediately removed from the rotor and turned 90 degrees. The layers of the lipoprotein are only slightly disturbed by the described manipulation. This will be avoided in future experiments by the use of a swinging bucket rotor. Samples of 0.05 ml. were withdrawn from different levels of the analytic cell by means of a small pipette. The photographic picture of the distribution served as a guide for the identification of the level from which the sample was withdrawn. The visible borderline between the optically empty salt solution and the yellowish tainted lipoprotein provided another means of orientation.

For the preparation of the electron microscopic specimen, the lipoprotein fractions were diluted 1:1000 with distilled water. A drop of this solution was placed on a "Formvar" membrane on a stainless steel screen. After evaporation of the water at room temperature, the lipoprotein was fixed and stained by a drop of 0.2 per cent buffered osmium tetroxide. The fixative stayed in contact with the specimen for an interval of five minutes. It was subsequently washed off by dipping the screen with the specimen into distilled water. This washing removes also the remaining sodium chloride from the specimen. After drying at room temperature, most of the specimens were shadowed with palladium in an RCA vacuum chamber.

The micrographs were made with the North American Philips EM-100 electron microscope at an original magnification of 20,000 on the viewing screen; they were later enlarged by light to the reproduced magnification. For counting and measuring the particles, the micrographs were ruled into convenient sections.

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FIG. 1. Molecules of the ultracentrifugally separated lipoprotein, assumed to be connected with the incidence of arteriosclerosis. Different staining depth of the molecules indicates heterogeneity of the lipid content within a certain flotation "class." (X 60,000)

**OBSERVATIONS**

Electron microscopic pictures of specimens with different flotation rates show that the particles in the fastest floating components may be only several microns in size. Most of these have a globular shape, as previously found by the light microscope. In this study, particular interest concentrated on the fine-structure of the lipoprotein emulsions with flotation rates below $S_f = 100$. Figure 1, as an example, shows an electron microscopic picture of a sample with a rate of about 30 Svedberg flotation units. The homogeneous appearance and the absence of large particles in this picture are criteria of the effectiveness of the sampling technic.

A diagram of the particle size distribution illustrated in figure 1 is plotted as curve A in figure 2. It has two maxima: one peak at 23 millimicrons and another one below 10 millimicrons. The first maximum indicates the mean particle diameter of the $S_f = 30$ fraction. The second maximum is approximately 10 times higher than the first and discloses the presence of a great number of particles with diameters below 10 millimicrons. The size of these particles approaches the limit of the resolving power of the electron microscope, and they could not be measured accurately. The minor peak at 40 millimicrons, described by Prendergast and Teague, was not observed.

Figure 1 shows clearly that particles of the same size were stained by osmium tetroxide in different shades. The majority were only lightly stained, about 15 per cent were much darker in appearance and deviated visibly from a circular form. Their average length was 1.5 times their width. The same elongated structure was observed to a lesser extent in the lighter stained particles. Similar observations were made in a number of subjects.

**Particle Size Distribution.** The particle size distribution found in this study is compared in figure 2 with findings of Prendergast and Teague (curve B). One curve has a peak at 22,
The dimensions of certain ultracentrifugally isolated lipoproteins were recently calculated by Oncley and Gurd using values from published studies of Gofman and associates. These data are reproduced in table 1. In addition, the table shows data for the diameters of the dehydrated lipoprotein molecules computed for this study under the assumption that the lipoproteins contain 0.6 Gm. of water for each gram of dried weight (Oncley, Scatchard and Brown) and that the density of the dehydrated lipoprotein is 1.1.

The data for the diameters of the dehydrated molecules, compiled in table 1, seem to indicate that the sample in figure 1 with a mean particle diameter of 22 millimicrons was taken from the

<table>
<thead>
<tr>
<th>Lipoprotein Sf</th>
<th>Hydrated density</th>
<th>Hydrated molecular weight M × 10^6</th>
<th>Hydrated diameter A</th>
<th>Dehydrated diameter* A</th>
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<tbody>
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<td>2</td>
<td>1.050</td>
<td>1.1</td>
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<td>130</td>
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<td>1.040</td>
<td>1.7</td>
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<td>150</td>
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<td>175</td>
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<td>2.7</td>
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<td>180</td>
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<td>13</td>
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<td>190</td>
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<tr>
<td>40</td>
<td>0.96</td>
<td>5.9</td>
<td>270</td>
<td>235</td>
</tr>
</tbody>
</table>

* Computed from hydrated diameter assuming 0.6 Gm. of water for each gram of dried weight and a density of 1.1 for the dried substance.

Factors Influencing the Electron Microscopically Observed Particle Size. A number of factors may influence the size of a single lipoprotein particle in the electron microscopic picture. The necessity of drying the specimens causes changes of the particle size which are difficult to evaluate. The values for the diameter of dried lipoproteins (table 1) were computed to allow a preliminary comparison of the electron microscopically determined diameters with the hydrated diameters which determine the flotation rate.

During the drying process the particle size of a certain fraction may increase by deposition of smaller size molecules on the larger particles. This effect which was investigated on latex particles by Watson and Grube may, to a certain extent, also influence the particle size of lipoproteins, as the presence of a large number of small molecules was detected in the electron microscopic pictures.
Furthermore, the size of lipoprotein particles, apparent in the electron microscopic pictures, may depend on the degree of flattening of the lipoprotein droplets on the supporting membrane. This flattening of particles was observed in electron microscopic studies of synthetic latices by Maron, Moore and Powell.7

It amounted, for this material, generally to less than 10 per cent, but this factor may be more pronounced in the case of lipoproteins.

The fixing and staining of the lipoprotein specimen by buffered osmium tetroxide may possibly change the particle size. Very little is known about the action of this fixing agent on lipoproteins. A study by Porter and Kallman6 of the properties of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy revealed its reaction with certain plasma proteins. The fixative forms gels which are remarkably free from evidence of coarse coagulation. The use of buffered osmium tetroxide solutions, following a recommendation by Palade, may in addition contribute to retain the original particle size during the fixation process.

Finally, the electron beam itself may, by heating and charging, change the particle size of the lipoproteins. A decrease of the particle diameter under the influence of prolonged electron bombardment was noticed in latex specimens by Watson and Grube.4 This evaporation is very likely negligible in osmium-fixed preparations, since the vapor pressure of this metal is low. The fixation may also protect the particles from electrostatic charging by the electron beam. Specimen charging, investigated by Ellis9 for polystyren latex, changes the apparent particle size only in the absence of electrical conduction in the specimen and in the supporting material.

The enumeration of the different factors which may influence the particle size in electron microscope pictures of lipoproteins should caution against an overemphasis of the results; on the other hand, these considerations will pave the way to a more detailed knowledge of this most interesting class of natural emulsions.

Particle Shape. As already mentioned, the electron microscopic pictures disclose a high percentage of the deep stained and 20 per cent of the lighter stained particles as deviating from a spherical shape. Considered as ellipsoids of rotation, the length of these particles is about 1.5 times larger than their width. The possibility of preparation or fixation artefacts accounting for this deviation was ruled out. It seems possible, however, that the small observed deviation from the spherical shape escaped notice in previous investigations which considered β-lipoproteins as molecules of globular shape.

Heterogeneity of the Lipid Content within a Certain Flotation Class. The striking difference in the staining properties of the lipoprotein particles by osmium tetroxide seems to indicate that a certain fraction of the particles (about 20 per cent) is richer in lipid material than the rest. A recent study of the immunochemical behavior of human plasma β-lipoproteins by Gitlin10 reaches similar conclusions and it is suggested “that immunochemical heterogeneity exists within the various flotation classes.” His data also indicates “that normal human plasma contains a lipid-poor protein that is immunochemically related to the protein moiety of β-lipoprotein.”

The possibility of a chemical analysis of single, large-size molecules opens a new field for electron microscopic investigations. In the case of serum lipoproteins, it leads to the interesting conclusion that large molecules which show the same sedimentation rate in the centrifugal field of force, implying that they have similar size, shape, and density, may have different chemical composition and reactivity.

REFERENCES

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