Characterization of Apolipoprotein B from Human Serum Low Density Lipoprotein in *n*-Dodecyl Octaethyleneglycol Monoether: An Electron Microscope Study

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ABSTRACT We have studied the structure of the totally delipidated polypeptide (apolipoprotein B [apo B]) present in low-density serum lipoprotein in detergent (*n*-dodecyl octaethyleneglycol monoether) solution by electron microscopy. The protein-detergent complex appears as a rod-shaped particle, 75–80 nm long and 4.5–5.5 nm wide. The volume of this particle is consistent with the previously published composition reported by Watt and Reynolds (1980, *Biochemistry* 19:1593–1598) of two copies of apo B and five to six equivalent micelles of detergent. The asymmetric particle possesses a high degree of flexibility and a strong tendency to self-associate in an orderly fashion. The extent of this association is pH dependent.

The main vehicle for plasma cholesterol transport is low-density lipoprotein (LDL), a quasi spherical particle ~20–23 nm in diameter. The major subspecies of LDL in human plasma is LDL_2, defined as a relatively homogeneous population of particles having a hydrated density of 1.02–1.063 g/cm^3. Numerous studies of holo-LDL_2 have suggested that it is composed of a hydrophobic core of triglycerides and cholesteryl esters that are rendered water soluble by an as yet undefined "packaging" property of the protein (apolipoprotein B [apo B]) and associated phospholipids. Nuclear magnetic resonance studies have demonstrated that all phospholipid head groups are accessible to the aqueous milieu (23).

Data from numerous laboratories have shown an invariant mass (510,000 daltons) of apo B in all LDL and very low-density lipoprotein (VLDL) particles (for a review, see reference 19). Empirical estimates of the polypeptide chain molecular weight by SDS gel electrophoresis (7, 11, 14) and chemical cleavage (2), have been reported and yield conflicting results. However, the molecular weight in concentrated solutions of guanidinium chloride has been determined by sedimentation equilibrium (a rigorous thermodynamic technique) in two laboratories and found to be 255,000 daltons (13, 17). Therefore, LDL_2 must contain two copies of apo B per particle (dimeric).

Both ionic and nonionic detergents have been used to study the protein component of holo-LDL_2 (6, 8, 14, 16, 21). Investigation of the properties of apo B (5, 12) in the presence of bound detergent (such as SDS, Triton X-100, and *n*-dodecyl octaethyleneglycol monoether [$C_{12}E_8$]) indicates that the dimeric form (8, 16, 21) of this protein is retained when all native lipids are removed. Additionally, the nonionic detergent, $C_{12}E_8$, maintains the secondary structure of apo B as assessed by circular dichroism (21).

Unlike holo-LDL_2, apo B in this detergent appears to be highly asymmetric with a frictional ration ($f/f_{min[unhydrated]}$) of 2.0–2.3. Hydrodynamic studies with spherical or nearly spherical globular proteins characteristically give frictional ratios between 1.0 and 1.3. Because $f/f_{min(unhydrated)}$ is a measure of both the deviation of a particle from a spherical shape and the degree of hydration, the previous hydrodynamic studies of the apo B-$C_{12}E_8$ complex cannot be unambiguously interpreted. Consequently, we have used electron microscopy to examine the shape of the complex directly. These studies demonstrate a highly flexible, rod-shaped particle with dimensions compatible with the protein molecular weight, detergent binding, and hydrodynamic properties observed in solution. We previously noted a tendency toward self-association between the dimeric species of apo B in detergent, particularly at neutral pH, and this phenomenon is also observed in our morphological studies. It is of interest because the association appears highly ordered in a lateral direction.

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MATERIALS AND METHODS

Materials

$C_{12}E_8$ was obtained as a homogeneous product from Nikko Chemicals, Co. (Tokyo, Japan). Sepharose 4B, Sepharose CL-4B, and chromatographic columns were products of Pharmacia Fine Chemicals, Div. Pharmacia Inc. (Piscataway, N. J.). Regenerated cellulose membranes were from Schleicher & Schuell, Inc. (Keene, N. H.). All other materials were as previously described (21).

Methods

Preparation of Holo-LDL_2 and Delipidation

Preparation of holo-LDL_2 was carried out as described previously (16, 21) using blood from fasting, normal human volunteers. After isolation, the two free sulfhydryls per 250,000 g protein were alkylated with iodoacetamide and the reaction terminated by dialysis against 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer, pH 7.4, ionic strength 0.3. Holo-LDL_2 was then passed through sterile 0.22-μm filters (Millipore Corp., Bedford, Mass.) into sterile test tubes and stored at 4°C. The purity of the preparations was routinely assessed by SDS gel electrophoresis on either 3.3 or 5% polyacrylamide gels (22).

Holo-LDL_2 was delipidated using $C_{12}E_8$ as described previously (21). $C_{12}E_8$ (at 30 mg/mg protein) was incubated with 3–4 mg LDL protein for 2 h at room temperature. The solution was applied to a 1.5 × 90 cm column of Sepharose 4B (or CL-4B), equilibrated, and eluted with a buffer solution containing 1 mM $C_{12}E_8$. The protein-detergent complex which eluted with a $K_d = 0.35$ was used for the studies presented here.

Protein concentrations were determined using a modified Lowry procedure (10) that incorporated SDS in all samples and standards and used bovine serum albumin as the standard. Lipid phosphorous was assayed according to the micromethod of Bartlett (1). $C_{12}E_8$ concentrations were measured using the method of Garewal (4) except that absorbance readings were made at 319 nm.

Morphological Studies

SHADOW CASTING: Suspensions of the protein-detergent complex in buffers at ionic strength 0.3 were applied to freshly prepared carbon-coated grids

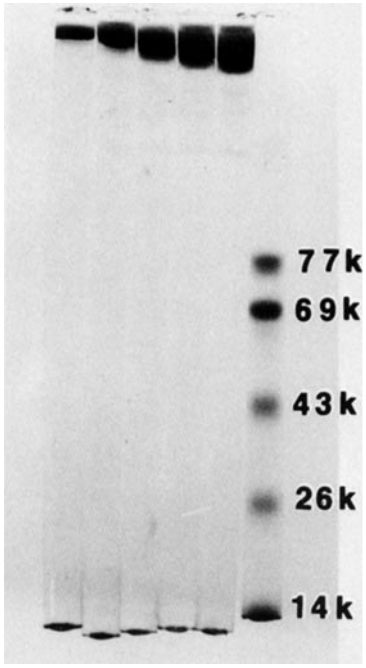

FIGURE 1 SDS-polyacrylamide gel electrophoresis of holo-LDL_2. Freshly prepared alkylated holo-LDL_2 was solubilized by the addition of solid SDS to a final concentration of 5% and boiled for 90 s in the presence of β-mercaptoethanol (22). From left to right 10, 20, 30, 40, and 50 μg of protein were applied to the top of the 5% acrylamide gels. The gel at the far right was loaded with the following standards: horse transferrin (76,000); bovine serum albumin (69,000); hen egg ovalbumin (43,000); bovine pancreas α-chymotrypsinogen A (25,000); and egg lysozyme (14,300).

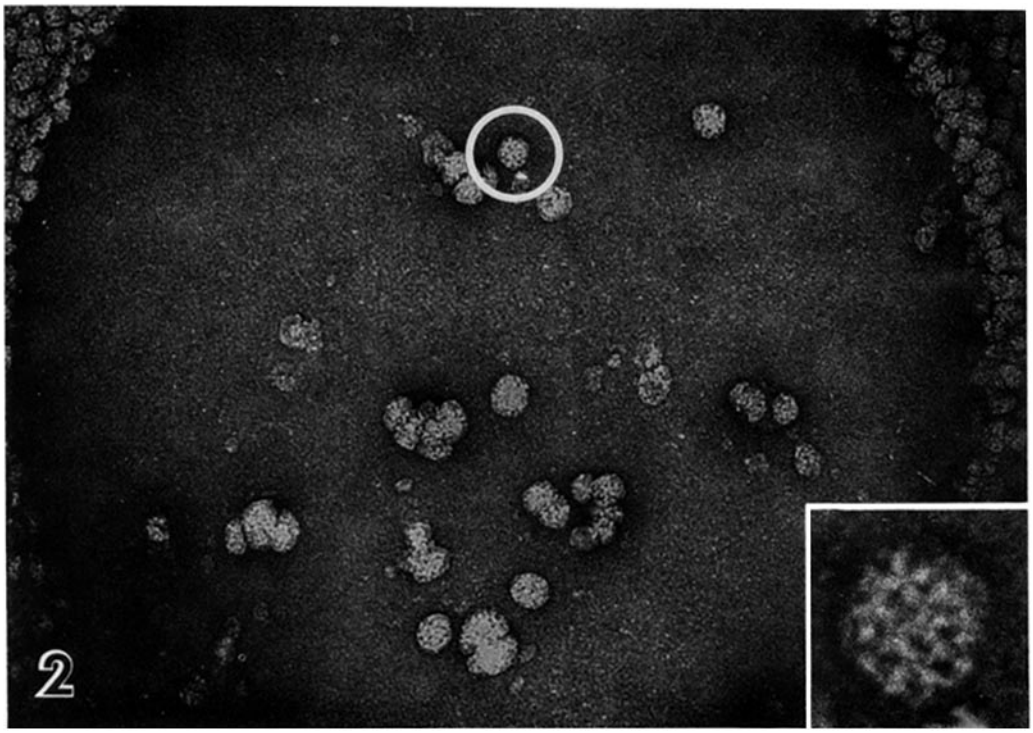

FIGURE 2 Uranyl acetate negative-stained preparation of human holo-LDL_2. Holo-particles are embedded in a film of stain covering a hole in the carbon support film. The edges of the hole can be seen at the sides of the figure. × 160,000. The particle encircled is shown at higher magnification in the inset. × 870,000.

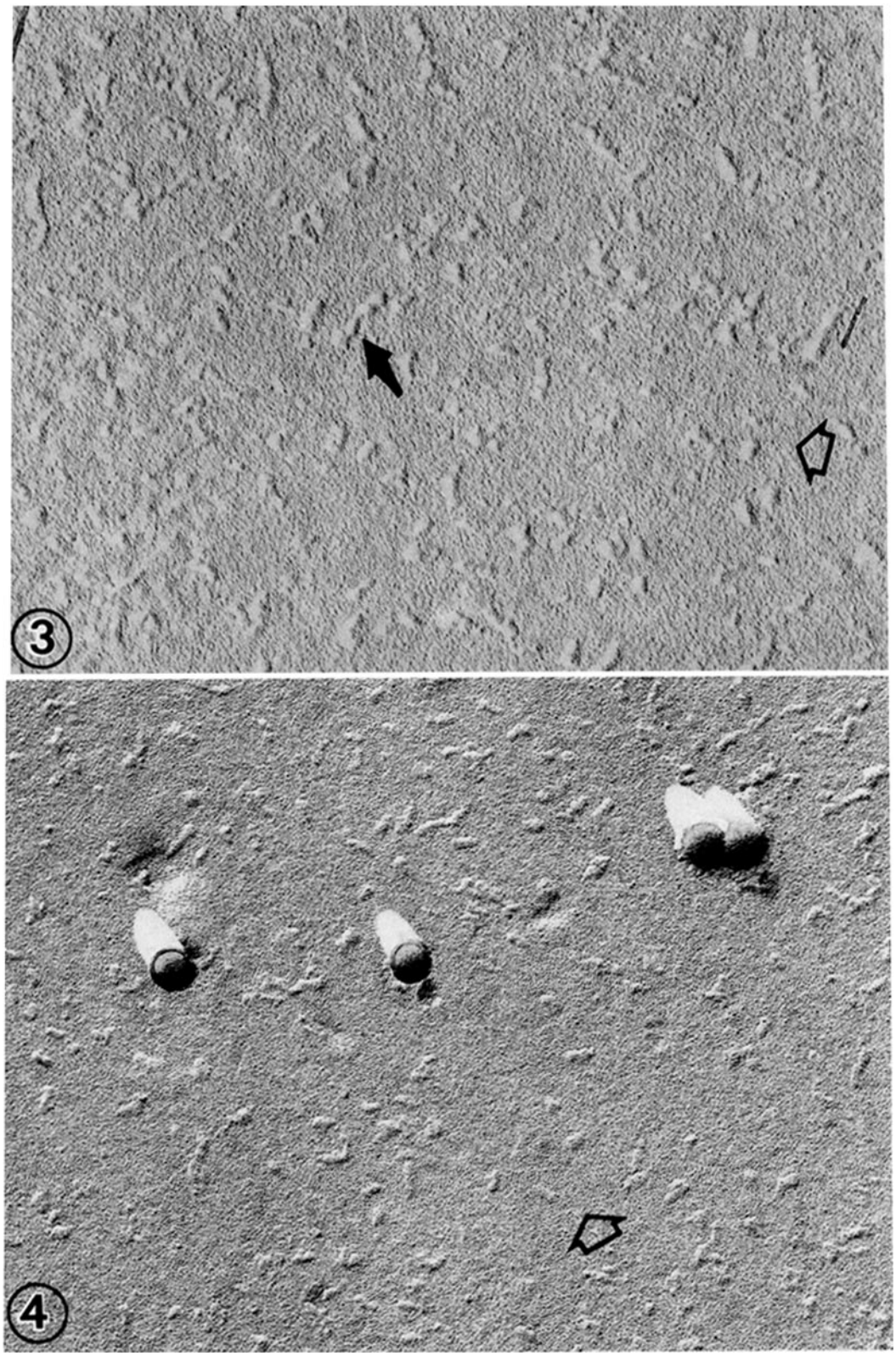

FIGURE 3 Totally delipidated apo B in $C_{12}E_8$ prepared at pH 10.0 and shadowed with platinum and carbon at ~12°. The open arrow indicates the direction of the shadowing. The arrow points to rod-shaped particles suggesting the presence of substructure. × 98,000.

FIGURE 4 Apo B-$C_{12}E_8$ preparation at pH 10.0 shadowed on the grid at 30°. The arrow indicates the direction of shadow. × 98,000.

and allowed to stand at room temperature for 30 s. The grids were washed with buffer solution containing 1 mM $C_{12}E_8$, 1% glycerol, and an aliquot of latex particles (mean diameter 0.057 μm). We studied two conditions: (*a*) apo B-$C_{12}E_8$ complexes in 10 mM sodium carbonate buffer, pH 10.0, containing 1 mM $C_{12}E_8$, and (*b*) apo B-$C_{12}E_8$ complexes in 10 mM TES buffer, pH 7.4, containing 1 mM $C_{12}E_8$. The grids were shadowed in a Balzers BAE-120 apparatus (Balzers Corp., Hudson, N. H.) and the thickness of the platinum-carbon film controlled with a quartz crystal thin film monitor QSG 201. The shadowing angle was varied from 10° to 30°. The local angle of shadow was measured directly from the length of shadow cast by polystyrene latex spheres deposited on the support film before shadowing.

NEGATIVE STAINING: Dilute suspensions of holo-LDL_2 particles (0.05–0.7 mg/ml) were applied to carbon-coated holey grids, washed with stain solution (2% uranyl acetate in water), and blotted with filter paper. Holes (~0.5–1.0 μm

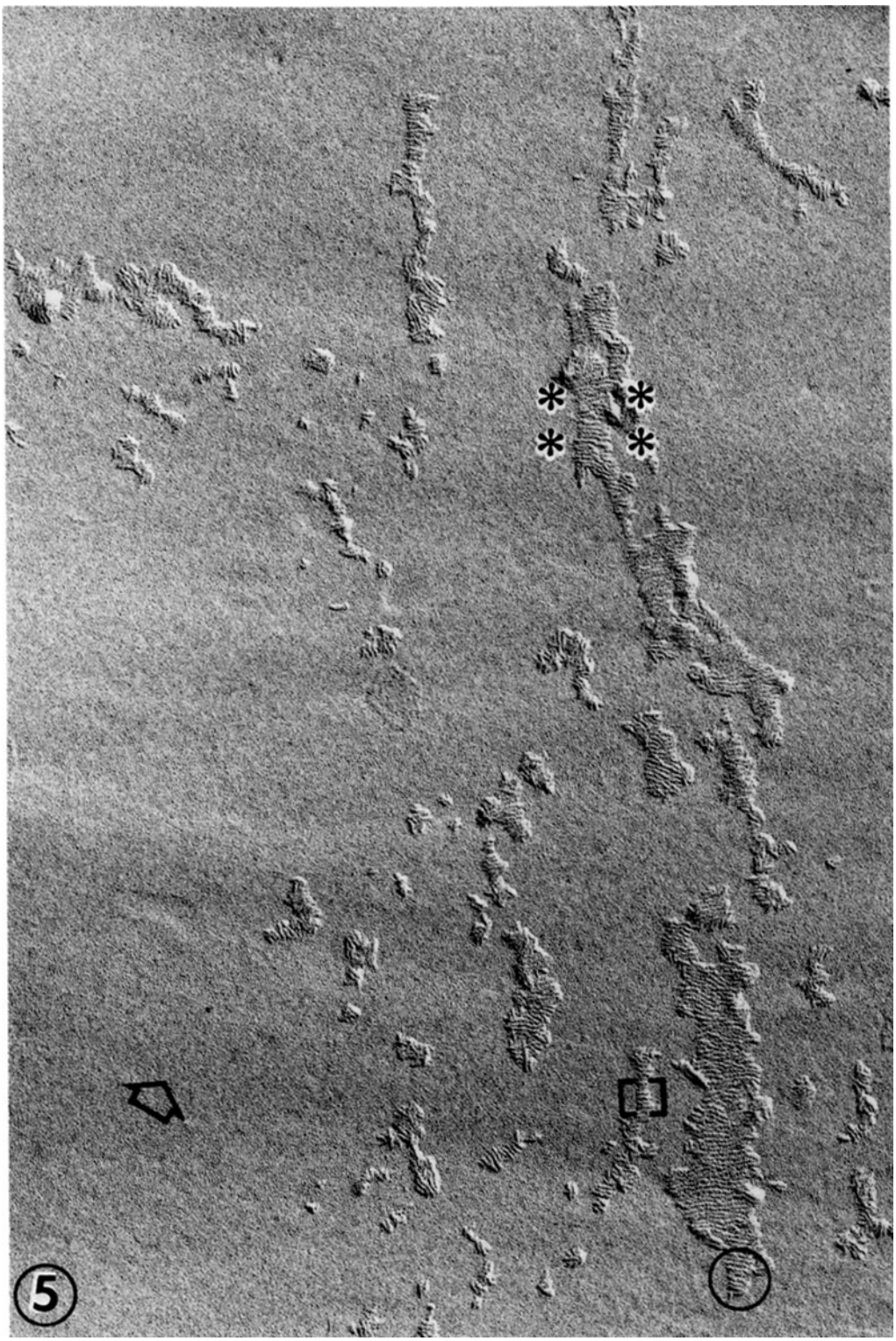

FIGURE 5 Apo B-$C_{12}E_8$ prepared at pH 10.0 showing a region of the grid where the sample was aggregated. The arrow indicates the direction of the shadow. The areas specified by the circle, brackets, and stars show rod-shaped particles of different lengths but constant width. × 66,000.

in diameter) in the carbon-support films which were completely covered with a layer of stain containing the particles were selected at low magnification. They were then imaged, while we took care to avoid excessive radiation damage.

ELECTRON MICROSCOPY: Most observations were performed with a Philips EM 301 electron microscope equipped with an anticontamination device cooled at liquid nitrogen temperature. An objective aperture of semiangle of 5 $\times 10^{-3}$ radians was used. The state of focus was determined by studying the contrast transfer function (3) of the micrographs. A magnification, on the plate, of 25,000 was used throughout.

RESULTS

The purity and homogeneity of holo-LDL_2 preparations used in this study were routinely monitored by SDS polyacrylamide gel electrophoresis and negative staining. Fig. 1 shows SDS gels of holo-LDL_2 loaded at five different protein concentrations (10–50 μg) and stained with Coomassie Blue. A single band near the top of the gel was observed, regardless of the loading concentration. Note that all the material entered the gel and that no smaller species were present. This Coomassie Blue staining pattern is in good agreement with that shown by Triplett and Fisher (20) and Steele and Reynolds (16), but is markedly different from that reported by Huang and Lee (7), and Socorro and Camejo (14). These discrepancies could be due to the susceptibility of apo B to enzymatic cleavage (9, 15).

Homogeneity with regard to size was assessed by negative staining. The preparation consisted solely of spherical particles, 20–25 nm in diameter. No indication of contamination by other lipoprotein particles (i.e., chylomicra, VLDL, high-density lipoprotein [HDL], etc.) was observed. Careful examination at higher magnification provided additional structural information on the surface of the holo-LDL_2 particle.

Fig. 2 shows LDL_2 particles suspended in a film of stain covering a hole in the supporting carbon coat. The holo-LDL_2 particles are spherical in shape with diameters ranging from 20 to 25 nm. The holo-particle inside the white circle is shown at higher magnification in the inset of the figure. The surface of the holo-LDL_2 particle consists of electron-lucent rings, each containing a central electron-dense pool of stain spaced ~6 nm (center-to-center) apart. These observations strongly suggest the presence of an orderly arrangement of domains on the surface of the particle (see also reference 12). The apparent order seen in Fig. 2 is clearly different from the tetrahedral arrangement of domains described by Gulik-Krzywicki et al. (5) using freeze-etching electron microscopy. The differences could be because the two techniques are showing different parts of the particle. (For example, we do not know where the fracture plane is located in the holo-LDL_2 particle.) Thus, it is difficult at this time to determine unambiguously the symmetry of the particle, and pursuing the structural analysis any further with the use of these techniques would not be fruitful.

Shadow Casting of the Apo B-$C_{12}E_8$ Complex

Apo B-$C_{12}E_8$ complexes were obtained as described in Methods. Negative staining is of limited value to study apo B-$C_{12}E_8$ complexes, largely because of technical problems. For example, uranyl acetate produces an artifactual aggregation of the sample, probably because of the low pH (4.5) of the stain solution. Similarly, sodium phosphotungstate at neutral pH is known to react with polyoxyethylene detergents producing an insoluble precipitate. Therefore, to circumvent these problems the samples were studied by shadow casting techniques at different shadowing angles.

EXPERIMENTS AT pH 10.0: Fig. 3 shows apo B-$C_{12}E_8$ complexes at pH 10.0 shadowed on the grid at ~12°. Both fields revealed the presence of elongated structures of variable length (40–120 nm) and 4.5–5.5 nm in thickness. Usually, these asymmetric structures possessed smooth surfaces, but in a few instances (Fig. 3, arrow) the rod-shaped particles seemed to be composed of distinct domains. In those cases, the elongated particles cast a serrated shadow consistent with the presence of substructure.

Fig. 4 shows the same apo B-$C_{12}E_8$ complexes at pH 10.0 shadowed at ~30°. Once more, the particles were seen as elongated particles of variable length and ~5 nm thickness. The surface of these structures did not exhibit a clear indication of substructural domains, as described in Fig. 3 obtained at a lower shadowing angle.

Previous ultracentrifugation experiments (21) demonstrated

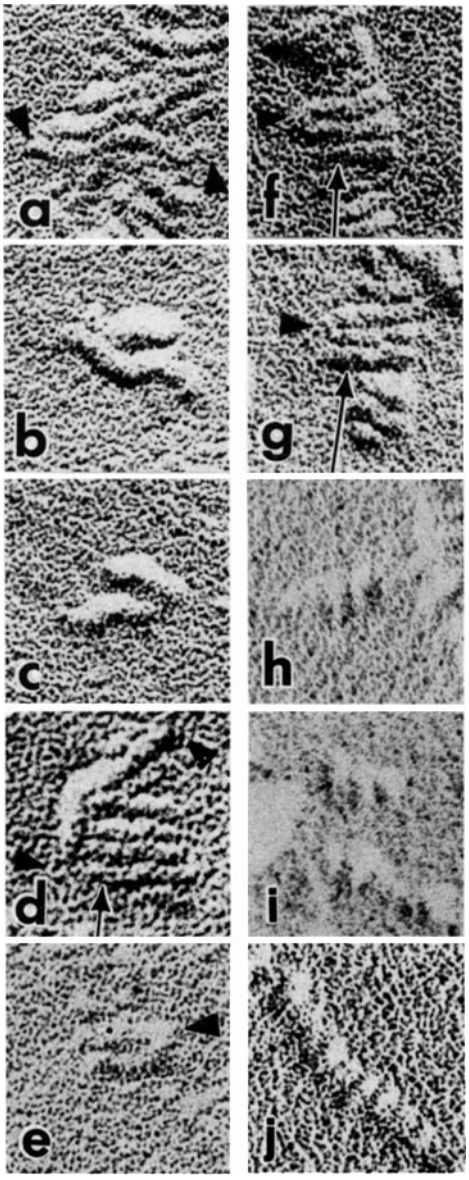

FIGURE 6 Selected views of apo B-$C_{12}E_8$ complex prepared at pH 10.0 and imaged from those regions showing aggregation. The figure illustrates the different shapes that the rod-shaped particle can undertake. The direction of the shadow in each picture is roughly from bottom to top. The solid arrowhead in d, e, and f points to the connection of a bent rod-shaped particle. × 300,000.

that from 10–20% of apo B-$C_{12}E_8$ complexes at pH 10.0 are aggregated. This phenomenon is also observed in some regions of the grid. In those areas the rod-shaped particles associate laterally, providing a better estimate of the shape and dimensions of the individual particles. Fig. 5 shows a low magnification view of these aggregated protein-detergent complexes. The width of a single particle, now measured directly (i.e., instead of from the shadow length) is 4–5 nm, whereas the length is variable. For example, the areas enclosed by stars in Fig. 5 contains particles of ~80 nm in length, while encircled complexes possess a length of ~40 nm, and those species bracketed are ~20 nm long.

Fig. 6 is a gallery of selected views of the rod-shaped particle, demonstrating the different shapes observed under our experimental conditions. Among these shapes we clearly distinguished a fully elongated, ~80-nm-long rod-shaped particle (Fig. 6 *a* and *d*, the black arrowheads indicate the ends of the rod), a shorter rod-shaped particle ~40–50 nm long (arrowhead, Fig. 6 *e* and arrows, Fig. 6 *f* and *g*), and a particle having two distinct curved regions whose connection is difficult to resolve at this particular shadowing angle (Fig. 6 *b* and *c*). Also, Fig. 6 *e–g* show a horseshoe-shaped structure connected by a region clearly observable (small black arrowheads). Finally, we have detected structures composed of ellipsoidal segments spaced ~12–13 nm apart (Fig. 6 *h–j*), which could be ~30–35 nm long (Fig. 6 *h* and *i*) or longer (Fig. 6 *j*). Because ultracentrifugation experiments (21) demonstrate homogeneity in particle weight, these structures must be reconciled with a species of constant mass. A single flexible, rod-shaped particle of dimensions ~80 nm by 4–5 nm is consistent with both morphological and solution data as elaborated in Discussion.

EXPERIMENTS AT PH 7.4: Previous hydrodynamic studies from this laboratory have indicated that apo B-$C_{12}E_8$ complexes at pH 7.4 undergo time-dependent association in solution (21). We studied, as a control, this aggregated species by shadow casting. Fig. 7 shows a representative view of the sample. In contrast to the appearance of the particles observed at pH 10.0 (Figs. 3–6), the protein-detergent complex produced large, thin sheets that seemed to be composed of numerous, individual elongated particles preferentially associated laterally.

DISCUSSION

All our experimental observations of the apo B-$C_{12}E_8$ complex are consistent with a highly asymmetric particle, rodlike in shape, with dimensions of ~80 nm in length and 4.5–5.5 nm in thickness. These dimensions yield an axial ratio, a/b, of 15. Previous studies (21) have demonstrated that each particle contains two copies of apo B [500,000/(6×10^{23})g protein] and 580,000 ± 140,000/6×10^{23} g detergent. Using the known partial specific volume for each species ($\bar{v}_{protein} = 0.725$ and $\bar{v}_{detergent} = 0.973$), an anhydrous volume per particle is calculated as $1.6 \pm 0.2 \times 10^{-18}$ cm^3. The volume of the rod-shaped particle observed in the microscope studies is $1.0 \pm 0.3 \times 10^{-18}$ cm^3 (prolate ellipsoid) or $1.5 \pm 0.3 \times 10^{-18}$ cm^3 (cylinder), well within experimental error of that obtained in solution.

$C_{12}E_8$ micelles are hydrated to the extent of 0.8 g H_2O/g detergent (18). If we assume that this is the major contribution to the hydration of the particle in solution, we calculate a minimum hydrodynamic radius of 8.2 ± 0.3 nm, an $f/f_{0(hydrated)}$ of 1.8 ± 0.1 and an axial ratio of 15 ± 1 for the apo B-$C_{12}E_8$ complex studied in solution. This is in excellent agreement with the morphological data indicating no severe perturbation of the protein-detergent complex as the result of experimental

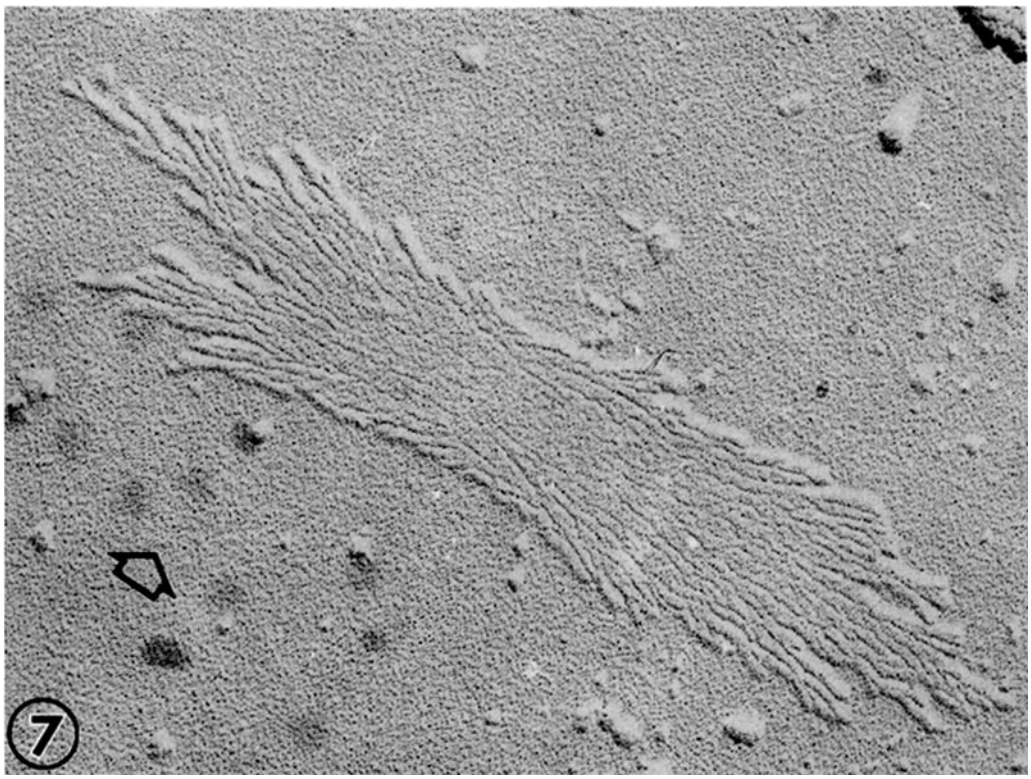

FIGURE 7 Apo B-$C_{12}E_8$ prepared at pH 7.4 and shadow casted on the grid. The rod-shaped particle aggregates, forming thin sheets as the one presented in this figure. The arrowhead indicates the direction of the shadowing. × 98,000.

manipulations involved in sample preparation for microscopy studies.

The most striking feature consistently seen throughout our experiments is the large variability in the length of the particle while its width remains constant. Selected views of the complex (Fig. 6) indicate that the variability in length can be accounted for by its considerable flexibility. For example, the horseshoe appearance (Fig. 6 *d–f*) can be explained if the 80-nm-long rod is bent in the middle. Similarly, the appearance of the particle observed in Fig. 6 *g–i* can arise by coiling of the rod around a central axis.

In agreement with previous solution studies (21) we note a strong tendency of the complex to self-associate, particularly at pH 7.4 where the protein-detergent complexes previously observed as individual particles now form large, thin sheets. These aggregates confirm the size and shape of the individual prolate ellipsoids of the nonaggregated material observed at pH 10.0. It has been suggested previously that the pH-dependent aggregation arises from interactions between the hydrophilic domains of the protein rather than their hydrophobic domains that are protected by interaction with the detergent. Increasing the solution pH results in an increase in the total charge on the molecule and thus weakens ionic interactions.

The secondary structure (as measured by circular dichroism) as well as the quarternary state (dimeric) of apo B are maintained in $C_{12}E_8$. However, the asymmetric appearance of the detergent-apo B particle is strikingly different from the spherical shape of holo-LDL_2 (Figs. 3–7 compared to Fig. 2). The flexibility of the apo B-$C_{12}E_8$ particle seen in this study suggests that the protein can adopt a number of conformations in solution, one of which is stabilized in the holo-particle through interactions with bound lipid. It will be the task of reconstitution experiments to elucidate how apo B when combined with lipid directs the formation of a spherical, water-soluble lipoprotein.

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REFERENCES

1. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* 234: 466–468.
2. Bradley, W. A., M. F. Rohde, A. M. Gotto, Jr., and R. L. Jackson. 1978. The cyanogen bromide peptides of the apoprotein of low density lipoprotein (Apo B): Its molecular weight from a chemical view. *Biochem. Biophys. Res. Commun.* 81:928–935.
3. Erickson, H. P., and A. Klug. 1971. Measurement and compensation of defocusing and aberrations by Fourier processing of electron-micrographs. *Phil. Trans. Roy. Soc. Lond. Ser. B.* 261:105–118.
4. Garewal, H. S. 1973. A procedure for the estimation of microgram quantities of Triton X-100. *Anal. Biochem.* 54:319–324.
5. Gulik-Krzywicki, T., M. Yates, and L. P. Aggerbeck. 1979. Structure of serum low density lipoprotein. II. A freeze-etch electron microscopy study. *J. Mol. Biol.* 131:475–484.
6. Helenius, A., and K. Simons. 1972. The binding of detergents to lipophilic and hydrophilic proteins. *J. Biol. Chem.* 247:3656–3661.
7. Huang, S. S., and D. M. Lee. 1979. A novel method for converting apolipoprotein B, the major protein moiety of human plasma low density lipoprotein into a water-soluble protein. *Biochim. Biophys. Acta.* 577:424–441.
8. Ikai, A., and M. Hasegawa. 1978. Dimeric nature of apo-low density lipoprotein extracted with Triton X-100. *J. Biochem.* 83:755–759.
9. Krishnaiah, K. V., and H. Wiegandt. 1974. Demonstration of a protease-like activity in human serum low density lipoprotein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 40:265–268.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
11. Olofsson, O., K. Bostrom, U. Svanberg, and G. Bondjers. 1980. Isolation and partial characterization of a polypeptide belonging to apoprotein B from low-density lipoprotein of human plasma. *Biochemistry.* 19:1059–1064.
12. Pollard, H., A. M. Scanu, and E. W. Taylor. 1969. On the geometrical arrangement of the protein subunits of human serum low-density lipoprotein: evidence for a dodecahedral model. *Proc. Natl. Acad. Sci. U. S. A.* 69:304–310.
13. Smith, R., J. R. Dawson, and C. Tanford. 1972. The size and number of polypeptide chains in human serum low density lipoprotein. *J. Biol. Chem.* 247:3376–3381.
14. Socorro, L., and G. Camejo. 1979. Preparation and properties of soluble, immunoreactive apo LDL. *J. Lipid Res.* 20:631–638.
15. Steele, J. C. H., Jr. 1979. The effect of plasmin on human plasma low density lipoprotein. *Thromb. Res.* 15:573–579.
16. Steele, J. C. H., Jr., and J. A. Reynolds. 1979. Characterization of the apolipoprotein B polypeptide of human plasma low density lipoprotein in detergent and denaturant solutions. *J. Biol. Chem.* 254:1633–1638.
17. Steele, J. C. H., Jr., and J. A. Reynolds. 1979. Molecular weight and hydrodynamic properties of apolipoprotein B in guanidine hydrochloride and sodium dodecyl sulfate solutions. *J. Biol. Chem.* 254:1639–1643.
18. Tanford, C., Y. Nozaki, and M. F. Rohde. 1971. Size and shape of globular micelles formed in aqueous solution by n-Alkyl polyoxyethylene ethers. *J. Phys. Chem.* 81:1555–1560.
19. Tanford, C., and J. A. Reynolds. 1979. Structure and assembly of human serum lipoproteins. *In* The Chemistry and Physiology of Human Plasma Proteins. D. H. Bing, editor Pergamon Press, New York. 111–126.
20. Triplett, R. B., and W. R. Fisher. 1978. Proteolytic digestion in the elucidation of the structure of low density lipoprotein. *J. Lipid Res.* 19:478–488.
21. Watt, R. M., and J. A. Reynolds. 1980. Solubilization and characterization of apolipoprotein B from human serum low density lipoprotein in *n*-dodecyl octaethyleneglycol monoether. *Biochemistry.* 19:1593–1598.
22. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
23. Yeagle, P. L., R. B. Martin, and R. G. Landgon. 1978. Location and interactions of phospholipid and cholesterol in human low density lipoprotein from ^{31}P nuclear magnetic resonance. *Biochemistry.* 17:2707–2710.