Molecular Weights of Apoprotein B Obtained from Human Low-Density Lipoprotein (Apoprotein B-PI) and from Rat Very Low Density Lipoprotein (Apoprotein B-PIII)†

John Elovson

Research Service, Veterans Administration, Wadsworth Medical Center, Los Angeles, California 90073, and Department of Medicine, School of Medicine, University of California, Los Angeles, California 90024

James C. Jacobs, Verne N. Schumaker,* and Donald L. Puppione

Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024

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ABSTRACT: Human low-density lipoproteins (LDL) were isolated from single donors by differential centrifugation between densities of 1.020 and 1.050 g/mL. The LDL were reduced and alkylated in 7 M guanidine hydrochloride, and the lipid was removed by multiple extractions in the cold with a mixture of diethyl ether and ethanol. Sedimentation studies on the resultant human apoprotein B (apoprotein B-PI) at low concentrations in 6.00 M guanidine hydrochloride showed a single sharp boundary with a sedimentation coefficient of 2.15 ± 0.04 S at 25 °C, uncorrected for viscosity or density. Diffusion experiments performed in the same solvent at low speeds in the analytical ultracentrifuge gave a D_{25} = 0.694 ± 0.043 Fick. Combining these values with an apparent specific volume of 0.703 mL/g yielded a molecular weight of 387 000, indistinguishable from that obtained by sedimentation equilibrium analysis in 7 M guanidine hydrochloride. Similar values were also obtained by calibrated sedimentation analysis, by Sepharose 2B chromatography in guanidine hydrochloride, and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Rat very low density lipoproteins (VLDL), isolated from sera of Triton WR1339 treated animals, were used as the source of rat apoprotein B-PIII. The delipidated VLDL were solubilized in sodium dodecyl sulfate, and apoprotein B-PIII was isolated by Sepharose 4B chromatography. With appropriate corrections for density and viscosity, the behavior of rat apoprotein B-PIII was identical, upon analytical ultracentrifugation, in 6 and 7.7 M guanidine hydrochloride, corresponding to sedimentation and diffusion coefficients of 1.47 S and 0.92 Fick, respectively, in 6 M guanidine hydrochloride. These data may be combined to yield a molecular weight of 210 000. Similar values were obtained by calibrated sedimentation analysis, by Sepharose 2B chromatography in guanidine hydrochloride, and by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Kane et al. (1980) reported the existence of three novel human apoprotein B peptides (termed B-74, B-26, and B-48 by these authors) which migrated more rapidly on sodium dodecyl sulfate (SDS)[1]-containing polyacrylamide gels than did the major apoprotein B peptide (B-100) classically found in human LDL. B-74 and B-26 appeared to be minor fragments produced by a unique scission of the large peptide (B-100); however, the third novel peptide (B-48), which was about half the size of B-100, appeared to be a separate protein by its amino acid composition. Most significantly, this novel peptide accounted for essentially all the apoprotein B found in lymph and plasma chylomicrons but was absent from plasma VLDL and LDL. Thus, in the human, the liver appeared to secrete VLDL containing exclusively the large apoprotein B peptide, while the intestine appeared to produce chylomicrons containing exclusively the novel smaller peptide.

This concept was fully supported by the very interesting report (Malloy et al., 1981) of a patient who completely lacked VLDL and LDL, i.e., the hepatic lipoproteins which contain the large peptide (B-100), while possessing normal B-48-containing chylomicrons and good tolerance for dietary fats.

In the rat, on the other hand, the situation is clearly different in that circulating VLDL in this species contains predominantly the smaller apoprotein B peptide. Although Krishnaiah et al. (1980) emphasized the fact that rat intestine elaborated almost exclusively the smaller apoprotein B peptide, Elovson et al. (1981) directly demonstrated the presence of this peptide in nascent VLDL isolated from rat liver Golgi apparatus and provided clear kinetic evidence that the large amounts of this peptide found in circulating rat VLDL must be of hepatic rather than intestinal origin. The incorporation of labeled amino acids into rat plasma lipoproteins in vivo (Windmuller, 1981) by perfused livers (Sparks et al., 1981) and by isolated hepatocytes (Davis & Boogaerts, 1982) has directly confirmed the fact that unlike the situation in the human, rat liver secretes both the large and small apoprotein B peptides in comparable amounts.

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* Address correspondence to this author at the Molecular Biology Institute.

[1] Abbreviations: EDTA, disodium (ethylenedinitrilo)tetraacetic acid; LDL, low-density lipoprotein(s); VLDL, very low density lipoprotein(s); SDS, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; GSH, reduced glutathione; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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Elovson et al. (1981) use the nomenclature PI (peptide I) for the largest rat apoprotein species, which comigrates exactly with the large human species (B-100) on SDS–polyacrylamide gels, and PIII for the smaller rat peptide, which also comigrates with the smaller human species (B-48), with an additional rat peptide, PII, migrating slightly faster than PI. Since the "centile" nomenclature of Kane et al. (1980) was based on their estimates of apparent molecular weights on polyacrylamide gels, which as shown here are problematic, we have adopted the nomenclature of Elovson et al. (1981) and will refer below to the large human apoprotein B peptide as human apoprotein B-PI and to the smaller rat apoprotein B peptide as rat apoprotein B-PIII.

The above studies supported the pioneering work of Smith et al. (1972), who clearly demonstrated that human apoprotein B isolated from LDL had a very high molecular weight, using for this purpose three different techniques: sedimentation equilibrium analysis, gel permeation chromatography, and polyacrylamide gel electrophoresis. Although other studies, referenced by Morrisett et al. (1975), by Olofsson et al. (1980), and by Socorro et al. (1982), have continued to suggest smaller molecular weights, these probably reflect the fact that apoprotein B is easily degraded by factors yet poorly understood, as discussed by Lee et al. (1981), Steele & Reynolds (1979a), Schuh et al. (1978), Chapman & Kane (1975), and Krishnaiah & Wiegandt (1974).

Even though the molecular weights of the B apoproteins now appear to be very large, the actual published values have varied considerably and have been complicated by the suggestion of Steele & Reynolds (1979b) that, unlike almost all other proteins, human apoprotein B in SDS solutions exists as a noncovalently associated dimer, leading to difficulties in interpreting the apparent molecular weights obtained by polyacrylamide gel electrophoresis in the presence of this detergent. Thus, the apparent molecular weight of 550 000 for human apoprotein B-PI estimated by Kane et al. (1980), using this method, would be in good agreement with that of a dimer of peptides having the monomer molecular weight of 255 000 determined by Smith et al. (1972). However, other authors report considerably lower and very similar apparent molecular weights for human apoprotein B-PI on SDS–polyacrylamide gels: 335 000 (Kirshnaiah et al., 1980), 340 000 (Chapman et al., 1977), 350 000 (Williams, 1979; Sparks et al., 1981), 360 000 (Walsh & Atkinson, 1983), and 400 000 (Schuh et al., 1978). The estimated molecular weights for apoprotein B-PIII by the same method are 264 000 (Kane et al., 1980), 240 000 Krishnaiah et al., 1980), and 220 000 (Elovson et al., 1981), again raising the question whether these values represent the apparent molecular weight of a single peptide or that of a dimer containing two peptides of approximately half that size.

To resolve these difficulties, we report here the sedimentation and diffusion properties of both apoprotein B-PI and apoprotein B-PIII in guanidine hydrochloride. We have been particularly careful to study preparations essentially free of aggregated or degraded material as seen in the ultracentrifuge, i.e., those which showed a single, symmetrical boundary which yielded the expected diffusion coefficient at high and low centrifugal velocities, indicating that a single molecular weight component was present. We find values corresponding to molecular weights of 387 000 ± 11 000 and 210 000 ± 10 000 for human apoprotein B-PI and rat apoprotein B-PIII, respectively. Approximately the same values are obtained by calibrated sedimentation analysis, after analysis by gel filtration, by polyacrylamide gel electrophoresis, and, for apoprotein B-PI, by sedimentation equilibrium analysis.

Materials and Methods

Materials. Myosin heavy chain was obtained by gel exclusion chromatography of reduced and alkylated myosin in 6 M Gdn·HCl/50 mM NH_4HCO_3, pH 8, on a 48 × 1 cm Sepharose 2B-CL column. *Escherichia coli* β-galactosidase was a generous gift of Dr. I. Zabin. Triton WR 1339 was obtained from Ruger Chemical Co., Irvington, NJ. Sepharose 2B-CL and 4B-CL, blue dextran, and the Perspex peristaltic pump were from Pharmacia. Gentamicin was obtained from Sigma, St. Louis, MO. Guanidine hydrochloride, ultrapure grade, was obtained from Schwarz/Mann, Orangeburg, NY.

Isolation of Human LDL. Fasting blood was obtained from two normal volunteers at the UCLA Blood Donor Center. Each unit of fresh, citrated plasma was brought to a final concentration of 0.05% reduced glutathione (GSH), 0.05% azide, 0.10% EDTA, 0.005% gentamicin, and 15 μL of diisopropyl fluorophosphate. These concentrations were maintained during the isolation procedure except that no more diisopropyl fluorophosphate was added. Successive centrifugations were carried out at densities of 1.006, 1.063, 1.020, and 1.055 g/mL by using either a saturated NaCl solution or else a 1.006 g/mL solution to adjust the density. All centrifugations were performed with a Ti 70 rotor (Beckman, Palo Alto, CA) at 12 °C and 45 000 rpm for 20 h.

Isolation of Human Apoprotein B-PI from LDL. The LDL were dialyzed against 2 mM EDTA, 0.05% glutathione, 0.02% azide, and 0.01% gentamicin. In a typical extraction, 1 mL of 1 M Tris-HCl, pH 8.9, was added to 12 mL of LDL containing 5 mg/mL apoprotein B. Solid guanidine hydrochloride (Gdn·HCl) was added to 7.0 M, and apoprotein B was reduced by the addition of 50 μL of 1 M dithiothreitol (DTT). After 30 min at room temperature, the protein was alkylated by the addition of an excess (0.3 mL) of 0.5 M iodoacetic acid, adjusted to pH 8.9 with Tris base. After 15 min, when no detectable thiol remained, excess iodoacetate was destroyed by addition of another 50 μL of 1 M DTT and 0.25 mL of 5% GSH. The solution was divided between four screw-capped 25-mL Corex tubes, chilled on ice, and extracted 3 times with 8 mL/tube of ether/ethanol (3:1) using a rotator (1 rps) at 4 °C. Phases were separated between extractions by centrifugation for 5 min at 5000 rpm. Under these conditions, all of the original yellow color was removed from the aqueous phase during the first extraction, with little or no interfacial material remaining after centrifugation. Next, three extractions were performed with 10 mL of pure ether per tube; again, the aqueous solution remained clear, and no interfacial material was observed. The combined aqueous phases were concentrated to 10 mL (Amicon centricones, M_r 50 000 cutoff), bromophenol blue was added as a V_t marker, and apoprotein B was fractionated on a 100 × 2.5 in. Sepharose 2B-CL column in 6 M Gdn·HCl, 20 mM Tris, 2 mM EDTA, and 0.02% GSH, pH 8.3 at 4 °C. Six-milliliter fractions were collected at a flow rate at 12 mL/h. Apoprotein B emerged as a symmetrical peak, with varying amounts of material in a small leading shoulder, all well within the included volume. The six peak fractions were pooled, concentrated as above, and run through a small column (20 × 1.5 cm) of Sepharose 4B-Cl to exchange the solvent for the final buffer containing 6 or 7 M Gdn·HCl/10 mM Tris-HCl, pH 8.3, but not EDTA or GSH, to minimize background absorption during analytical ultracentrifugation. Upon sedimentation velocity analysis, this material contained well over 90% of a single component, with small amounts of slower and faster moving materials at the leading and trailing edges of the sedimentary boundary.

Isolation of Total VLDL Apo B from Rat. Ad libitum fed male Sprague-Dawley rats (400–500-g body weight) were exsanguinated through the aortic bifurcation 5–6 h after intrajugular injection of 0.3 mL/100 g body weight of 20% (w/v) Triton WR 1339 in saline. Serum obtained after 1 h at 4 °C was made 1.5 mM in Na_2EDTA, adjusted to d = 1.019 g/mL with solid KBr, and centrifuged in the Beckman SW 28 rotor at 28 000 rpm for 20 h. The solid plugs of lipoprotein were removed from the top of the tubes with a spatula and washed twice by resuspension and recentrifugation as above. The washed Triton–VLDL, resuspended to one-tenth of the original serum volume, was dialyzed, reduced, and alkylated in 7 M Gdn·HCl and delipidated essentially as described above for human LDL. The final aqueous phase was concentrated on Amicon centricones to about 3 mg of protein/mL, dialyzed against two changes (200 mL, 4 h) of 6 M urea, 2 mM EDTA, and 50 mM NH_4HCO_3 to remove guanidine hydrochloride, made 1% (w/v) in SDS, and finally dialyzed against 1 L of 0.1% SDS in 2 mM EDTA and 50 mM NH_4HCO_3 (buffer A).

Preparation of Apo B-PIII by Gel Exclusion Chromatography in SDS and 6 M Gdn·HCl. The above VLDL protein solution (14 mg of apoprotein B and 63% apoprotein B-PIII) was applied to a 1.5 × 90 cm column of Sepharose 4B-CL equilibrated and eluted with buffer A at a flow rate of 5.5 mL/h by using a peristaltic pump. Thirty-minute fractions were collected and analyzed by quantitative SDS–polyacrylamide gel electrophoresis. Fractions 30–38 of this first run (Figure 2A), which contained predominantly apoprotein B-PIII, were reapplied to the column in sequence, and elution was continued with buffer A. After fractions 1–30 of the second run were collected, the column outlet was connected to the inlet to give recirculation for the equivalent of fractions 31–47, followed by renewed collection and analysis of fractions 48–80. Fractions 60–68 were pooled to give 4.7 mg of apoprotein B-PIII, which contained less than 4% of the larger molecular weight apoprotein B components.

The SDS in the pooled material was removed by three cycles of 10-fold concentration and redilution with water followed by one cycle in 6 M urea, using the Amicon XM 100 membrane; the final concentrate was then dialyzed overnight against 200 mL of 6 M Gdn·HCl in 50 mM NH_4HCO_3/2 mM EDTA, pH 8.3 (buffer B). A 1.5-mL portion of this material containing 2.5 mg of apo B-PIII was applied to a 110 × 1.0 cm Sepharose 2B-CL column equilibrated and eluted with buffer B at 2.6 mL/h with collection of 30-min fractions. One-tenth-milliliter samples of selected fractions were dialyzed against 6 M urea and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Fractions 55–65 (195 mg of apoprotein B-PIII) were pooled, concentrated in Amicon centricones, and dialyzed against 6 M Gdn·HCl/2 mM EDTA/10 mM NH_4HCO_3 prior to analytical ultracentrifugation. A second preparation was performed similarly, except that the final material was dialyzed against 7.7 M Gdn·HCl.

Analytical Gel Exclusion Chromatography in 6 M Guanidine Hydrochloride. This was performed in buffer B using a 110 × 1 cm column of Sepharose 2B-CL. The elution positions of reduced and alkylated samples of human apoprotein B-PI, rat apoprotein B-PIII, myosin heavy chain, *Escherichia coli* β-galactosidase, phosphorylase *a*, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* were determined by quantitative SDS–PAGE of fractions dialyzed against 6 M urea.

Analytical Ultracentrifugation: Translational Diffusion. Low-speed diffusion coefficient measurements were made in a Beckman Model E analytical ultracentrifuge equipped with an ultraviolet scanner, using synthetic boundary, double-sector cells with aluminum-filled Epon centerpieces, at 10 000 rpm in an AN-J rotor. The average temperature was close to 25 °C, but temperature controls were off to avoid cycles of heating and cooling, which could lead to convective mixing and a disturbed boundary. The temperature drifts during the 6–8-h diffusion experiments were 2–3 °C.

Electrical signals from the ultraviolet scanner were sent to an A/D converter to give a digital output, and the numbers were stored on flexible disks. The data were eventually transferred to a DEC 11/45 computer where they were processed to give values of absorbance as a function of radial position and sedimentation and diffusion coefficients. The equation used for diffusion analysis employs the ratio of absorbances $A(r)/A(r_p)$, where $A(r)$ is the absorbance at any position r within the boundary and $A(r_p)$ is the average absorbance within the plateau region beyond the boundary. If the freely diffusing boundary obeys Fick's second law and is Gaussian, then this ratio of absorbances obeys the well-known Gaussian integral:

$$\frac{A(r)}{A(r_p)} = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{y} e^{-z^2}\, dz \qquad (1)$$

where z is a dummy variable and y is given by

$$y = \frac{r - r_0}{\sqrt{2}\sigma} \qquad (2)$$

In eq 2, r denotes radial positions within the boundary, as before, and r_0 is located at the boundary center. The quantity $\sigma^2 = 2Dt$ is the variance of the Gaussian curve; D is the diffusion coefficient, and t is time.

The computer program for diffusion analysis first calculates the ratios of absorbances across the boundary. Then it determines the values of y from eq 1 by interpolation from a table of the Gaussian integral which is stored in the computer program. These values of y are plotted as a function of r, as shown in Figure 5C, and a straight line is placed through the central points by least-squares analysis. The slope of this line is $(\sqrt{2}\sigma)^{-1}$, from which the program determines σ. Finally, the program calculates values of $\sigma^2 = 2Dt$ by analyzing each scan in this manner, plots these values as a function of time, and determines $2D$ and, hence, the diffusion coefficient from the slope of the least-squares line, as shown in Figure 5D.

The measured diffusion coefficients were corrected to 25 °C. Because of the low protein concentrations used, 0.2–0.4 mg/mL, concentration corrections were assumed to be negligible.

High-speed diffusion analysis at 60 000 rpm was performed on boundaries which moved one-third the length of the cell. By comparing the low-speed and high-speed diffusion coefficients, one may estimate the degree of sedimentation heterogeneity.

Sedimentation Velocity. Sedimentation coefficient measurements using the ultraviolet scanner were made in a double-sector cell with aluminum-filled Epon centerpieces at 60 000 rpm in a Ti-ANF rotor. The computer generated a derivative plot from the scanner output, and the sedimentation coefficients were calculated from the log r vs. t plots of the maximum ordinate. The sedimentation coefficients were corrected to 25 °C, assuming negligible concentration corrections.

Molecular Weights from s and D Values Obtained by Analytical Ultracentrifugation. The measured s_{25} and D_{25}

values in 6 M guanidine hydrochloride were combined to give a molecular weight by using the Svedberg equation:

$$\frac{s}{D} = \frac{M(1 - \phi\rho)}{RT} \quad (3)$$

The value of 0.703 mL/g reported by Smith et al. (1972) for human apoprotein B was also used for the apparent specific volume (ϕ) for rat apoprotein B-PIII.

Sedimentation Equilibrium. Sedimentation equilibrium studies of apoprotein B-PI in 7.0 M guanidine hydrochloride and 0.01 M Tris, pH 8.9, were performed by using the UV scanning optical system of the analytical ultracentrifuge and the AN-J rotor at 11 000 and 13 000 rpm. The heater was not used, but refrigeration was employed at a high setting, and the temperature remained close to 24 °C in all experiments. Equilibrium required 72 h, and meniscus depletion was achieved for apoprotein B, as was apparent from the flat base line at the meniscus. Control experiments with myosin were run simultaneously.

The analog signal from the scanner was digitized and stored on disk and later analyzed by a computer program, according to the method of Roark & Yphantis (1969) which computes weight, number, and z-average molecular weights at each point within the cell.

Quantitative SDS–PAGE was performed as described by Elovson (1980). Gdn·HCl-containing fractions were dialyzed against 200 volumes of 6 M urea before sample preparation.

Other Methods. SDS was determined by the methylene blue extraction method (Ray et al., 1966). Protein was measured by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard. Standard proteins were reduced and alkylated by sequential treatment for 15 min at room temperature with 50 and 150 mM iodoacetamide in 6 M Gdn·HCl/0.2 M NH_4HCO_3, pH 8.

Results

Isolation and Hydrodynamic Analysis of Apoprotein B from Human LDL. The presence of glutathione, as suggested by Lee et al. (1981), and EDTA seemed critical to inhibit degradation of apoprotein B during the prolonged centrifugation stages involved in LDL isolation and purification. Gentamicin and diisopropyl fluorophosphate were routinely added to the plasma; these may be superfluous, but we have not tried to do without them. During LDL isolation and purification, the pH was checked and adjusted to pH 7.4 after each salt addition or dilution. The reduction and alkylation of apoprotein B in the presence of 7.0 M guanidine hydrochloride seemed essential to prevent the development of aggregated material during the subsequent steps of lipid extractions. Passage of ether through activated alumina to remove peroxides just prior to lipid extraction seemed to yield a better product with the elimination of the last traces of aggregated material. We typically isolated 10 mg of apoprotein B from human LDL, with excellent recoveries and no insoluble material remaining at the interface.

In the analytical ultracentrifuge, apoprotein B isolated from human LDL sedimented as a single, very sharp boundary. Values for the solvent viscosities and densities were measured to four significant figures, and temperature was measured to 0.1 °C, so errors due to these sources should be less than 1%. Accurate values for the sedimentation coefficient were determined at 25 °C and 0.25 mg/mL, and five values were obtained separately on the same sample, in 6.0 M Gdn·HCl. The average and standard deviation of these values is 2.15 ± 0.04 S, which is very close to the value which we have also obtained for the major component of several other samples in which some aggregated or degraded material was present. Diffusion experiments were also performed at 10 000 rpm in the analytical ultracentrifuge in the same solvent. The results of five separate diffusion experiments yielded an average value and standard deviation of 0.694 ± 0.043 Fick at 25 °C. When the sedimentation and diffusion values are combined with an apparent specific volume of 0.703 ± 0.004 mL/g, as measured by Smith et al. (1972), and a value of 1.142 g/mL for the density of 6.00 M guanidine hydrochloride, we obtain an average molecular weight for human apoprotein B-PI, including all sources of error, of 387 000 with a standard error of the mean (SEM) of ±13 000.

Sedimentation Equilibrium. As shown in Figure 1A,B, both myosin and human apoproteins B-PI display log OD vs. r^2 plots with negative curvature in concentrated Gdn·HCl, implying that the preparations were sufficiently free of both lower and higher molecular weight contaminants to allow the nonideality term, the second virial coefficient, to predominate for these large, randomly coiled molecules. The myosin data were similar to those obtained by Gazith et al. and served as a control. The Roark and Yphantis plots (Figure 1C,D) show the reciprocals of the number-, weight-, and z-average molecular weights as a function of position in the cell. For cases showing negative curvature of the log OD vs. r^2 plots, that is, when the second virial coefficient predominates, the apparent number-average molecular weight is expected to be greater than the apparent weight-average molecular weight, which in turn should be greater than the apparent z-average molecular weight. This is indeed seen to be the case for myosin, and to a lesser extent for apoprotein B, probably reflecting a small degree of molecular weight heterogeneity in the latter samples.

Molecular weights for both myosin and apoprotein B were taken from five separate determinations. Molecular weights for the myosin data averaged 205 000 ± 7300 while those for the apoprotein B-PI data averaged 370 400 ± 10 000 (SEM).

Isolation and Characterization of Rat Apoprotein B-PIII. The particles in the fraction with density less than 1.019 g/mL which accumulated upon Triton treatment had an apoprotein B-PI:apoprotein B-PIII ratio of about 1:2, very similar to that for untreated animals (Elovson et al., 1981). A striking feature, previously noted by Ishikawa & Fidge (1979), is the absence of apoproteins E and C in VLDL from animals treated with Triton in vivo (Figure 2A). As shown in Figure 2B, comparison of its mobility on SDS–PAGE relative to bovine serum albumin, phosphorylase b, β-galactosidase, and rabbit muscle myosin heavy chain gave an apparent molecular weight for apoprotein B-PIII of 220 000. Also included in Figure 2B is the migration of human apoprotein B-PI run with the same standards under the same conditions, yielding an apparent molecular weight value of 335 000.

Due to the inability of sodium dodecyl sulfate to solubilize the large amounts of nonpolar lipids present in VLDL, the latter were first delipidated by two-phase extraction with ethanol/ether in the presence of 7 M Gdn·HCl, followed by removal of the Gdn·HCl by dialysis against urea, before addition of sodium dodecyl sulfate. Since resolution of the peptides by sodium dodecyl sulfate–Sepharose chromatography (Figure 2A) also was limited, the sample was cycled through a 90-cm column 3 times to obtain the desired separation. Although for reasons of clarity apoprotein B-PI and apoprotein B-PII are not plotted individually in Figure 3A, the former did elute slightly earlier, suggesting that the small difference in their mobilities on sodium dodecyl sulfate gel electrophoresis reflects an actual difference in size. Figure 3B shows the

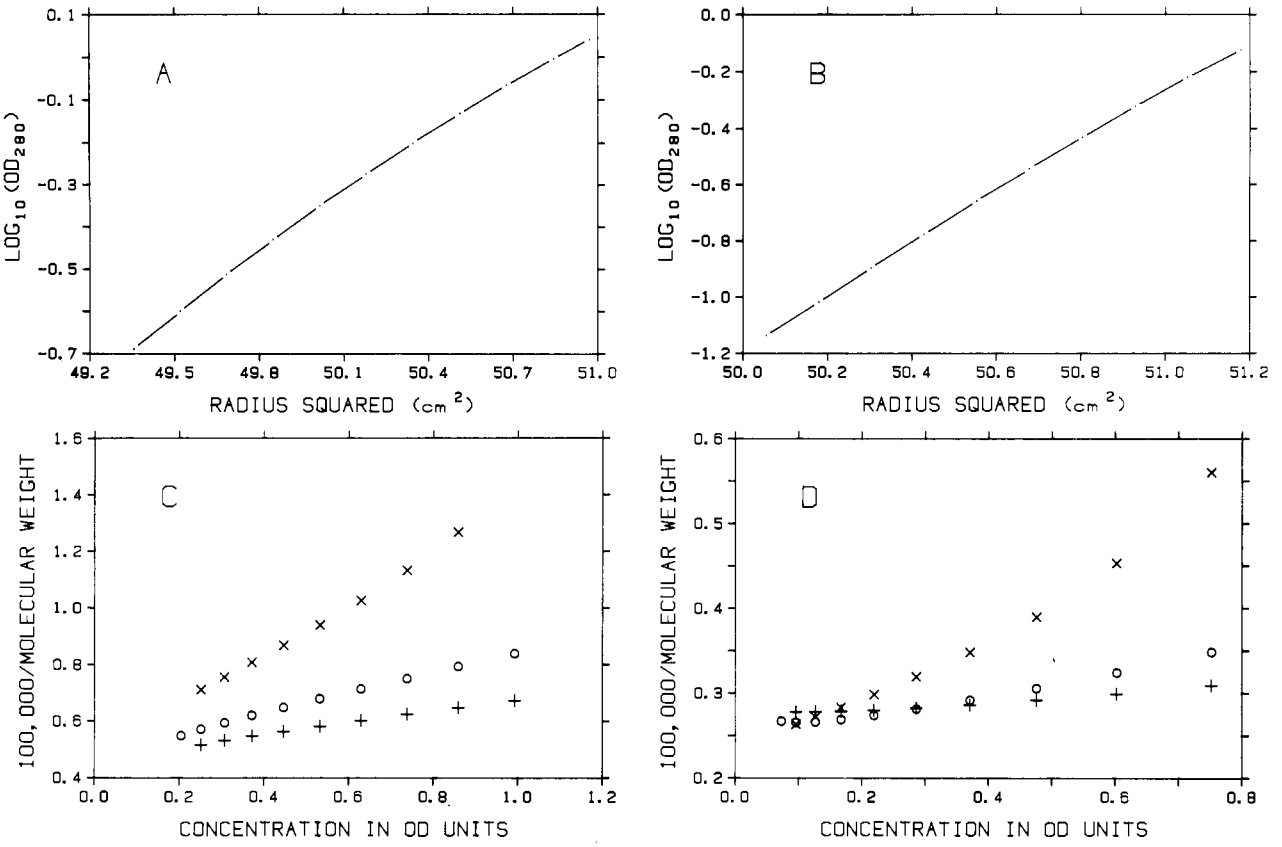

FIGURE 1: Sedimentation equilibrium of myosin and apoprotein B-PI. Both of these experiments are at 13 000 rpm and 24 °C. The solvent for myosin is 6 M Gdn·HCl, and that for apoprotein B is 7.1 M Gdn·HCl. (A) Log OD vs. r^2 plot for myosin showing the negative curvature characteristic of nonideality but molecular weight homogeneity. (B) Log OD vs. r^2 plot for apoprotein B also showing negative curvature. (C) Roark and Yphantis plots of reciprocal average molecular weights as a function of concentration (in OD units) for myosin: (+) reciprocal number-average molecular weight; (O) reciprocal weight-average molecular weight; (×) reciprocal z-average molecular weight. (D) Roark and Yphantis plot of reciprocal average molecular weights for apoprotein B.

elution profile obtained with the final cycle through the Sepharose 4B column.

Apoprotein B-PIII from the Sepharose 4B column was freed of most of the unbound SDS by repeated ultrafiltration through an XM 100 membrane, which was partly permeable to the detergent micelles but effectively retained the apoprotein B-PIII–detergent complex. The last cycle of dilution and concentration was performed in 6 M urea and reduced the SDS to apoprotein B-PIII weight ratio to about 1:10.

Apoprotein B-PIII was finally dialyzed into 6 M Gdn·HCl and chromatographed on Sepharose 2B-CL in that solvent. As seen in Figure 4A, the material absorbing at 280 nm eluted as a symmetrical peak with a leading shoulder, only part of which could be accounted for by the remaining small amounts of apoprotein B peptides larger than apoprotein B-PIII, suggesting the presence of some aggregated material in the early part of the apoprotein B-PIII peak. The fractions from the later part of the peak (Figure 3A) contained pure apoprotein B-PIII (Figure 2A, lane c) which appeared homogeneous in the centrifuge (see below).

Sedimentation and Diffusion Studies on Rat Apoprotein B-PIII. The results of sedimentation and diffusion studies on rat apoprotein B-PIII in 6.00 M Gdn·HCl are shown in Figure 5. In two runs, single, sharp, symmetrical sedimenting boundaries were obtained with measured sedimentation coefficients at 25 °C in the above solvent of 1.45 and 1.49 S. The diffusion coefficient was 0.92 Fick at 25 °C in 6.0 M Gdn·HCl, and this, together with the average sedimentation coefficient, yielded a molecular weight of 201 000. More extensive studies were performed on a second preparation in 7.7 M Gdn·HCl, with seven determinations of the sedimentation coefficient averaging 0.915 ± 0.03 (SD) S, a value which when corrected for viscosity and density corresponded to a sedimentation coefficient of 1.43 S in 6.00 M Gdn·HCl. For the diffusion coefficient in 7.70 Gdn·HCl, the averaged value of five measurements was 0.626 ± 0.04 (SD) Fick, yielding an average molecular weight for apoprotein B-PIII in 7.70 M Gdn·HCl of 210 000 ± 10 000 (SEM). As a control, two diffusion experiments with myosin heavy chains in 6 M Gdn·HCl, 25 °C, gave values of 0.97 and 0.98 Fick, which, when combined with s_{25} = 1.44 S and a $\bar{v}$ of 0.73 cm^3/g, yielded a molecular weight of 195 000.

Molecular Weights Estimated from Calibrated Sedimentation Velocity. The sedimentation coefficients were measured for eight different proteins which had been dialyzed against three changes of 6.00 M guanidine hydrochloride and 10 mM dithiothreitol (reduced form). The protein concentrations employed were low (absorbances at 280 nm of 0.2 or less), giving less than 3% concentration corrections to the sedimentation coefficients, as calculated from the intrinsic viscosities (Tanford et al., 1967). The sedimentation coefficient for myosin was determined 11 times to establish a reliable "swing" point close to the value for apoprotein B-PIII. The sedimentation data for the eight calibration proteins are presented in Table I and plotted in Figure 6. As shown, rat apoprotein B-PIII cosedimented with myosin, while the behavior of human apoprotein B-PI corresponded to an apparent molecular weight of 440 000.

Molecular Weight from Sepharose 2B-CL Chromatography. The elution of the apoprotein B peptides from Sepharose 2B in 6 M guanidine hydrochloride was also compared with that of reduced and alkylated standard proteins. As shown

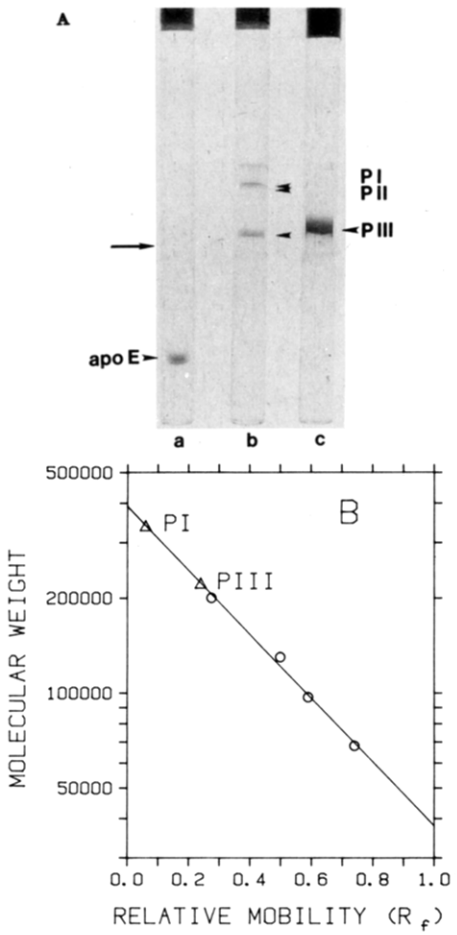

FIGURE 2: SDS–PAGE of rat apoproteins. (A) Composite gels. The arrow shows the junction between 6% (upper) and 11% (lower) acrylamide sections. Lane a, rat apoprotein E standard. Lane b, VLDL apolipoproteins isolated from animals treated with Triton WR 1339. Note the complete absence of apoprotein E (and apoprotein C). Lane c, apoprotein B-PIII purified from the above by SDS–Sepharose 4B chromatography. (B) Plot of log M_r vs. R_f for bovine serum albumin, phosphorylase, *E. coli* β-galactosidase, myosin, rat apoprotein B-PIII, and human apoprotein B-PI on 5% polyacrylamide gels. Apparent molecular weights of 220000 and 335000 are estimated for apoprotein B-PIII and apoprotein B-PI, respectively.

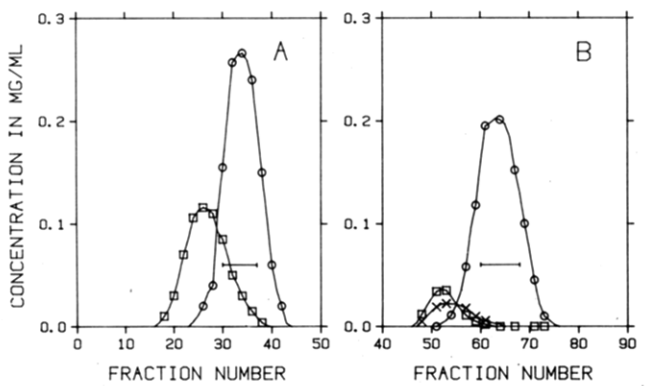

FIGURE 3: Elution profiles of rat apoprotein B-PI and apoprotein B-PIII from sodium dodecyl sulfate–Sepharose 4B-CL chromatography. (A) Elution profiles of apoprotein B-PI and apoprotein B-PII (□) and of apoprotein B-PIII (○) after one pass through a 90-cm column. (B) Purified apoprotein B-PIII (○) after the third pass as described in the text. Small amounts of apoprotein B-PI (□) and an intermediate-sized peptide (×) are also shown. The plotted values were estimated by quantitative sodium dodecyl sulfate gel electrophoresis.

in Figure 4B, apoprotein B-PIII migrated very close to myosin heavy chain, at the position expected for peptides of about

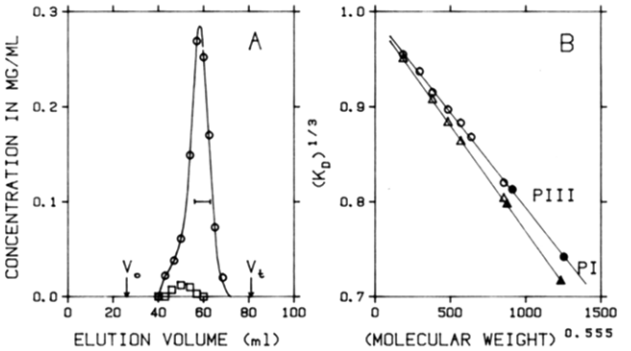

FIGURE 4: Six molar guanidine hydrochloride chromatography of rat apoprotein B-PIII and human apoprotein B-PI. (A) Elution profile of sample from Figure 3B. The bar shows fractions of rat apoprotein B-PIII (○) pooled for ultracentrifugal studies. (B) Results of two separate experiments with marker proteins, apoprotein B-PI, and apoprotein B-PIII plotted as $K_D^{1/3}$ vs. $M^{0.555}$, where K_D = (elution volume − column volume)/(void volume − column volume). Marker proteins are cytochrome *c*, chymotrypsin, ovalbumin, bovine serum albumin, phosphorylase, β-galactosidase, and myosin.

Table I: Calibrated Sedimentation Analysis of Reduced Proteins in 6 M Guanidine Hydrochloride

protein	s_{25}(6 M Gdn·HCl) (S)	ϕ	n^a
apoprotein B-PI	2.15	0.703	3997[b]
apoprotein B-PIII	1.47	0.703	1654[b]
myosin	1.42 (±0.01)[c]	0.71	1791
galactosidase	1.11, 1.12	0.72	1057
phosphorylase *b*	0.99	0.736	841
bovine serum albumin	0.96	0.714	582
aldolase	0.60, 0.68	0.733[d]	361
chymotrypsinogen	0.56, 0.61, 0.64	0.712	245
myoglobin	0.39, 0.47	0.74	153
ribonuclease	0.45	0.694	124

[a] Values of *n* were obtained from the Atlas of Protein Structure, except for myosin and phosphorylase where *n* was estimated as $M/110$. [b] Values of *n* for apo B-PI and apo B-PIII were determined from Figure 6. [c] Average of 11 separate ultracentrifuge measurements. [d] Reisler & Eisenberg (1969). Values of ϕ for the other calibration proteins were from Tanford et al. (1967).

210000 daltons. Thus, the size of the apoprotein B-PIII peptide as a random coil in 6 M guanidine hydrochloride corresponds closely to that deduced from the mobility on SDS–PAGE. In the same set of experiments, the elution volume of human apoprotein B-PI corresponded to apparent molecular weights of 374000 and 405000.

Diffusion Coefficients Determined from High-Speed Sedimentation Experiments. Because of the *s* vs. *c* effect, the trailing portion of a sedimenting boundary sediments faster than the leading portion, opposing the spreading caused by diffusion. Thus, for a homogeneous material, the observed diffusion coefficient will be less at high than at low rotor speed. The broadening of the sedimenting boundaries for apoprotein B-PI and apoprotein B-PIII during the course of sedimentation velocity experiments at 60000 rpm may be analyzed to yield such diffusion coefficients. Values of 0.47 and 0.71 Fick were found for apoprotein B-PI and apoprotein B-PIII, respectively, at 60000 rpm; these values were about 30% less than the corresponding values of 0.69 and 0.92 Fick found at 10000 rpm. These decreases in diffusion coefficient were those expected for the concentration dependence of the sedimentation coefficient across the boundary, in sedimentation studies of very high molecular weight random coil molecules, and were consistent with homogeneity. On the other hand, even a small degree of sedimentation heterogeneity would have resulted in a marked increase in the apparent diffusion coefficients.

Using the 60000 and 10000 rpm values to extrapolate to

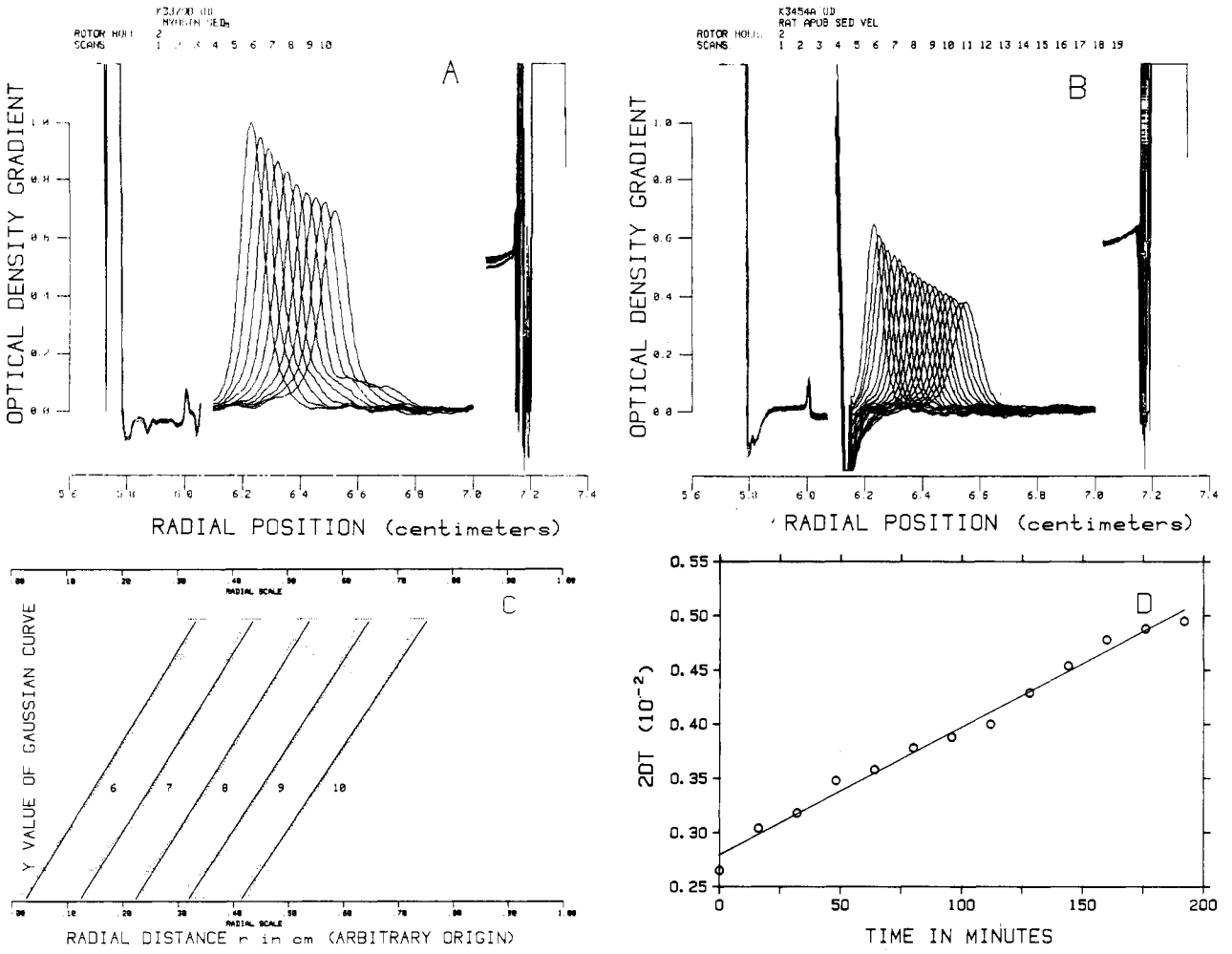

FIGURE 5: Sedimentation profiles for rabbit muscle myosin and rat apoprotein B-III and diffusion analysis for rat apoprotein B-PIII. Panels A and B show superimposed scanner traces converted by the computer to the derivative form for myosin and apoprotein B-III taken at 16- and 8-min intervals, respectively, at 60 000 rpm and 25 °C in 6.0 M guanidine hydrochloride (data sets K3379B and K3454A). Note the presence of some aggregated material in this myosin preparation which appears as a leading component. Panels C and D show plots of y vs. r and of $2Dt$ vs. t, respectively, for rat apoprotein B-PIII, 48-min intervals, at 10 000 rpm and 25 °C in 6.0 M guanidine hydrochloride (data set K3475A).

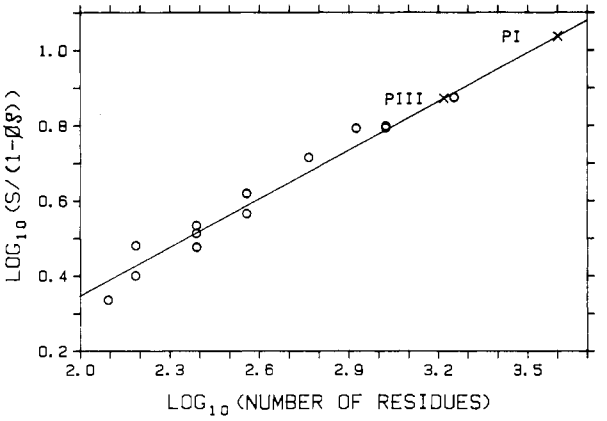

FIGURE 6: Calibrated sedimentation velocity analysis in 6.0 M guanidine hydrochloride and 10 mM dithiothreitol of marker proteins, rat apoprotein B-PIII, and human apoprotein B-PI. The data are plotted as $\log_{10} n$, where n is the number of amino acid residues, as a function of $\log_{10} [s/(1 - \phi\rho)]$. Values of n, s, and ϕ are listed in Table I. Although 11 separate determinations were made of the myosin sedimentation coefficient, it was given a weighting factor of 6 instead of 11 because of uncertainty in the myosin v in calculating the least-squares line through the experimental points.

zero force yields less than a 1% correction to the measured diffusion coefficient at 10 000 rpm, and this small correction was ignored in calculating the molecular weights for apoprotein B.

Discussion

The pioneering work of Tanford, Reynolds, and collaborators suggested a unifying picture of human LDL apoprotein B as being a very large peptide, present as two copies on the native LDL particle, which remained associated as a dimer upon delipidation and solubilization in detergents, including SDS; however, transfer of the delipidated peptides into high concentrations of Gdn·HCl resulted in the formation of random coil monomers with hydrodynamic properties corresponding to a molecular weight of 255 000, two of which therefore would account for the approximately 500 000 daltons of protein commonly reported to be present on human LDL particles.

Recently, several laboratories independently discovered that apoprotein B exists in different forms with different apparent molecular weights on SDS–PAGE, and the work reported here was undertaken to analyze the molecular properties of the novel, smaller apoprotein B-PIII peptide from rat VLDL in light of the above human apoprotein B paradigm. The results show that rat apoprotein B-PIII behaves in concentrated Gdn·HCl as expected for a random coil, with a peptide molecular weight of approximately 210 000; however, they also show that human apoprotein B-PI in such solvents behaves as a species with a molecular weight of 387 000 rather than the 250 000 reported earlier.

This reopens the question of the stoichiometry of apoprotein B-PI on human LDL: if correct, a 387 000 molecular weight

for apoprotein B requires reevaluation of the absolute LDL protein mass compatible with an integer number of such peptides per particle. The results of such an analysis will be presented elsewhere. Here, we must first evaluate the reliability of four molecular weight values for the two apoprotein B species.

Hydrodynamic measurements are perturbed by heterogeneity, concentration dependence, preferential solvation, and convective disturbances. We have reduced initial sample heterogeneity by gel exclusion chromatography to obtain samples which showed little or no aggregated or degraded material, as evidenced by their sedimenting as single symmetrical boundaries which showed the expected decreased diffusional broadening at high rotor velocities. Concentration dependence has been avoided by working at concentrations of about 0.25 mg/mL for both the apoprotein B species, where corrections to the ratio of s to D should be negligible. We have not attempted to measure preferential solvation but instead have relied upon the value of 0.703 mg/g for ϕ determined by Smith et al. for apoprotein B-PI in 7.6 M Gdn·HCl, who measured "the densities of several protein solutions (all containing 1–1.5 mg/mL) and the densities of the guanidine hydrochloride solutions against which they had been dialyzed for several days". An error of ±0.01 g/mL in $\bar{V}$ would cause an error of approximately ±25 000 in the molecular weight, or 6.5%. Convective disturbances during diffusion experiments were minimized by adding a very small amount of water to the upper Gdn·HCl solution used to form the initially sharp boundary in the center of the synthetic boundary cell. This created a small, superimposed but rapidly diffusing Gdn·HCl boundary which could be seen with the schlieren optical system, but which did not affect the absorbance difference across the boundary, which was entirely due to protein. This small, superimposed Gdn·HCl gradient should have completely prevented convection during the diffusion measurements.

With these precautions, the molecular weights calculated from the s and D values are 387 000 ± 13 000 (SEM) and 210 000 ± 10 000 (SEM) for human apoprotein B-PI in 6 M Gdn·HCl and for rat apoprotein B-PIII in 7.7 M Gdn·HCl, respectively. For the rat pepptide, an additional value in 6 M Gdn·HCl of 201 000 was determined from s and D values obtained concurrently with measurements for rabbit muscle myosin in the same runs, which gave a molecular weight of 201 000 ± 10 000 (SD), essentially identical with published values. The quality of these measurements as evidenced by their standard error of the mean and by the appearances of the sedimenting boundaries in Figures 1 and 6 offers no suggestion of any systematic error or of abnormal behavior for the apoprotein B samples.

Molecular weights calculated from the Svedberg equation are independent of the shape of the molecule. However, for both apoprotein B peptides, there is in fact close agreement between the calculated molecular weights and the apparent molecular weights obtained by reference to the sedimentation (Figure 6) or gel exclusion (Figure 4) behavior of standard proteins which form random coils in 6 M Gdn·HCl. This is consistent with the apoprotein B peptides themselves forming random coils in that solvent, i.e., showing the same dependence of Stoke's radius on peptide chain length as do the standard proteins. In other words, if the s value for apoprotein B-PI in Figure 6 actually were to reflect the sedimentation behavior of a species which was either much larger and more asymmetric or much smaller and more compact than a random coil of about 387 000 daltons, it would have displayed a correspondingly much larger or smaller apparent molecular weight on Sepharose 2B-CL in Figure 6. This is not the case. Furthermore, looking at the rat apoprotein B-PIII peptide, its sedimentation, diffusion, and gel exclusion chromatographic behavior in 6 M Gdn·HCl is essentially indistinguishable from that of the rabbit muscle myosin standard.

It should be noted that the slope of the log $[s/(1 - \phi\rho)]$ vs. log η plot of Figure 6 is different from that given previously by Tanford et al. (1961), largely due to their use of a literature value for myosin which seemed low. Thus, we measured the sedimentation coefficient for myosin in 6 M guanidine hydrochloride 11 times. The measured s and D values of our myosin sample give the correct molecular weight for the myosin polypeptide, so we believe that our sedimentation values for myosin are correct as well as precise. A useful relationship is given by the straight line in Figure 6 which has the formula

$$\log_{10} [s/(1 - \phi\rho)] = -0.51523 + 0.43096 \log_{10} (\text{number of residues})$$

Smith et al. (1972) also reported an apparent molecular weight for human apoprotein B-PI of 250 000 by gel filtration in guanidine hydrochloride, as compared to the average value of 390 000 found here. This difference may be more apparent than real, however, since the apoprotein B-PI peptide, which is well included on the Sepharose 2B-CL used in the present study, elutes too close to the void volume of the Sepharose 4B used by Smith et al. to allow precise estimates of its apparent molecular weight.

We believe the difference between the 255 000 molecular weight estimate for human apoprotein B-P I in concentrated Gdn·HCl contained by Smith et al. (1972) and the value of 387 000 found here is due to the combined effects of heterogeneity and nonideality, which were not considered by them. This is considered in detail below; however, since the 255 000-dalton estimate was obtained by sedimentation equilibrium centrifugation in 7.6 M Gdn·HCl and since the 387 000-dalton estimate obtained by sedimentation-diffusion in 6 M Gdn·HCl is halfway between 255 000 and 510 000 daltons, the remote possibility had to be considered that the 387 000 value could represent the average for a rapid equilibrium between a 255 000-dalton monomer and a 510 000-dalton dimer species. To examine this question and obtain ultracentrifugal data more directly comparable to those presented by Smith et al. (1972) and Steele & Reynolds (1979), we performed sedimentation equilibrium analysis of human apoprotein B-PI in 7.1 and 7.7 M Gdn·HCl as presented in Figure 1. The value which we obtained for the molecular weight of apoprotein B-PI by sedimentation equilibrium, 370 400, was probably a little low due to some heterogeneity in molecular weight which we suspect was still present in these samples. Therefore, we prefer the slightly higher value of 387 000 obtained by sedimentation and diffusion measurements.

The determination of molecular weights by sedimentation equilibrium is very sensitive to the combined effects of nonideality and molecular weight heterogeneity, since these effects cause opposite curvatures of the log c vs. r^2 plots, and can partially or completely cancel, leaving reasonably straight lines with incorrect slopes, as has been pointed out by Munk & Cox (1972). A number of workers, including Lapanje & Tanford (1967), Castellino & Barker (1968), and Munk & Cox (1972), have evaluated the second virial coefficients for various proteins which had been reduced and alkylated in concentrated Gdn·HCl. Substantial values are found for the second virial coefficients, and these are expected to be functions of the polypeptide molecular weight; thus, apoprotein B should show

an even more pronounced concentration dependence than myosin. The values of the second virial coefficient which may be calculated from the slopes of the weight-average data presented in Figure 1A,B are actually less for apoprotein B than for myosin, probably due to some molecular weight heterogeneity in the apoprotein B preparation. Nonetheless, we find extrapolated values for the molecular weights ranging between 354 000 and 381 000, entirely consistent with the molecular weights determined by the other methods. Furthermore, any negative curvature of the log c vs. r^2 plot is incompatible with a rapid monomer–dimer equilibrium, which would produce a pronounced positive curvature in Figure 1B and an extrapolated value of 0.4 at zero concentration in Figure 1D, with $M_z > M_w > M_n$.

Careful examination of the published sedimentation equilibrium data of Smith et al. (1972) shows plots of ln c vs. r^2 which are straight between concentrations of 37 and 619 μg/mL. If we assume a value for the second virial coefficient for apo B of 72.5×10^{-5} mol mL^{-1} g^{-2}, equal to the value observed by us for myosin and which may also be calculated from the data of Gazith et al. (1970) (Figure 5), then the variation in molecular weight as a function of concentration may be determined from the well-known relationship $1/M = 1/M_0 + 2Bc$, where M, M_0, B, and c are the apparent molecular weight, the true molecular weight, the second virial coefficient, and the concentration, respectively. At 619×10^{-6} g/mL, the apparent molecular weight will drop by 19%, even assuming a true molecular weight for apo B of 255 000, and will drop much more than this if the molecular weight is actually 400 000. Clearly, a straight ln c vs. r^2 plot over this concentration range is not compatible with a protein homogeneous in molecular weight when nonideality is taken into account. Apo B preparations are well-known to contain variable amounts of B-74 and B-26, fragments produced by proteolytic cleavage of apo B-100 by thrombin and kallikrein; it also spontaneously decomposes upon storage. Even our best preparations are never completely free of apo B fragments unless we size purify our material as described under Materials and Methods. Certainly, some fragments must have been present in the studies conducted by Smith et al. because size purification was not a part of their isolation procedure. The presence of such fragments would have produced positive curvature in the ln c vs. r^2 plots, which could have then masked the negative curvature due to nonideality. Moreover, the sedimentation equilibrium molecular weight values would be heavily weighted toward the smaller fragment sizes. In support of this thesis, one of the curves shown in their study, at 9341 rpm, actually exhibits positive curvature over its entire length. For this particular curve, the concentrations were so low that nonideality should not have produced significant curvature to offset the positive curvature due to size heterogeneity.

Reynolds and collaborators also concluded from sedimentation equilibrium experiments that human apoprotein B in SDS solutions behaved as a dimer of their proposed 255 000-dalton peptide. Actually, the three curves shown in Figure 3 of Steele & Reynolds (1979b) were extrapolated by them to molecular weights of about 415 000, 475 000 and 490 000. Recently published work by Patterson et al. (1984) reports sedimentation equilibrium data which show that the protein portion of nonionic detergent complexes with apo B has a molecular weight of 400 000 ± 39 000. Thus, the ranges of molecular weights found by three separate groups overlap: our values of 370 000–440 000, those of Steele and Reynolds of 415 000–490 000, and those of Patterson et al. of 360 000–440 000. In light of these values, we feel that the case for dimer formation is rather tenuous. Whatever the actual case may be, the conclusion of Reynolds raised the obvious question of whether apoprotein B peptides also migrate as dimers on SDS–PAGE, and this investigation was in part prompted by the resultant uncertainty regarding the interpretation of the apparent molecular weight of the newly discovered apoprotein B-PIII on SDS–PAGE. As it turns out, the apparent molecular weight of 220 000 is in fact in good agreement with the 210 000 molecular weight for the monomeric apoprotein B-PIII peptide as determined here by its hydrodynamic properties in 6 and 7.7 M Gdn·HCl; thus, the migration of apoprotein B-PIII on SDS–PAGE does *not* correspond to that of a dimer containing two peptides of approximately half this size. Furthermore, the apparent molecular weight for human apoprotein B-PI on SDS gels of 335 000 is in reasonable agreement with its true molecular weight of 387 000; thus, both apoprotein B peptides appear to behave as monomeric species on SDS–PAGE. In contrast, Kane et al. reported an apparent molecular weight of 550 000 for human apoprotein B-PI in a preparative 2.3% acrylamide PAG system and used this value, which is close to that for the dimer of 255 000-dalton peptides proposed by Steele and Reynolds, as the basis for their "centile" nomenclature for the apoprotein B peptides. However, this estimate is considerably larger than the apparent molecular weights reported by most other groups, who have used >3% acrylamide gels to arrive at values in the 330 000–400 000 range, i.e., consistent with the 387 000 molecular weight for apoprotein B-PI found in the present paper. The most notable exception, of course, is the original report by Smith et al., who found an apparent molecular weight on SDS–PAGE of 255 000, i.e., identical with their proposed *monomer* molecular weight.

We can only assume that these differences are due to a lack of readily available molecular weight standards in this range to enable the calibration of this empirical procedure between different laboratories. It is certainly difficult to imagine that the single apoprotein B-PI bands obtained by Smith et al. (1972) and Kane et al. (1980), using essentially the same SDS–PAGE system, should represent the exclusive occurrence of the monomeric and dimeric species, respectively.

The apoprotein B-PI samples we have studied were obtained from two male donors in their 20's, and their apoprotein B-PI appeared to have identical mobilities on SDS gels and identical sedimentation coefficients, as well as very similar molecular weights.

In conclusion, we find that the 95% confidence limit for the molecular weight of rat apoprotein B-PIII is 210 000 ± 20 000 (2 × SEM), and the value for human apoprotein B-PI is 387 000 ± 26 000 (2 × SEM). This latter value raises the interesting question of whether there are one or two apoprotein B-PI polypeptides on human LDL.

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