Plasma very low density lipoproteins contain a single molecule of apolipoprotein B

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Abstract Rat and human very low density lipoproteins (VLDL) were fractionated by zonal ultracentrifugation, yielding sharply defined fractions with narrow sedimentation limits. Sedimentation coefficients for the individual fractions were determined at two densities with the analytical ultracentrifuge, and the results were analyzed to yield buoyant densities and molecular weights for the particles in each fraction. For the rat lipoproteins, the weight concentrations of triglycerides, cholesterol, phospholipid, and protein were determined for each fraction, and their molar concentrations of apolipoprotein B were measured with a radioimmunoassay. For the human lipoproteins the corresponding values were taken from Patsch et al. (Patsch, W., J. R. Patsch, G. M. Kostner, S. Sailer, and H. Braunsteiner. 1978. Isolation of subfractions of human very low density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **253**:4911–4915). From these data, a ratio of the number of apoB peptides to the number of lipoprotein particles was calculated for each fraction. This ratio was close to 1 for all VLDL fractions, ranging in particle diameter from about 40 to 80 mm and 30 to 50 mm, respectively, for rat and human VLDL. The majority rat VLDL contain B-48 rather than B-100 as their (single) apoB peptide. Based on these data, we proposed that only a single copy of B-48 is required for VLDL assembly in rat liver, unless nascent hepatic VLDL contain additional apoB peptides which are uniformly lost from the plasma VLDL particles when they are analyzed.—**Elovson, J., J. E. Chatterton, G. T. Bell, V. N. Schumaker, M. A. Reuben, D. L. Puppione, J. R. Reeve, Jr., and N. L. Young.** Plasma very low density lipoproteins contain a single molecule of apolipoprotein B. *J. Lipid Res.* 1988. **29:** 1461–1473.

Supplementary key words zonal ultracentrifugation • buoyant densities • radioimmunoassay • apoB-48 • assembly of VLDL particles

All mammalian triglyceride-rich lipoproteins contain apoB, which appears to be obligatory for their assembly and secretion (1). Thus, VLDL[2] and chylomicrons are exclusively made in liver and intestine, the only adult tissues capable of expressing apoB (2). Although the actual mechanisms for assembly and secretion are unknown, it has long been known that human plasma LDL contain about 500 kDa apoB per particle, originally assumed to represent two copies of a 250 kDa peptide (3). Recently, however, the peptide molecular mass was estimated to be close to 400 kDa (4), prompting the proposal that the LDL particle contains one copy, rather than two, of apoB. The presence of a single apoB peptide on LDL has now been established by cloning of B-100, which showed its true molecular mass to be 512 kDa (5–9). Considering the tight association of apoB with its lipoprotein particle it seems highly likely, therefore, that the precursor VLDL also contain a single B-100 per particle. In fact, several years ago Eisenberg et al. (10) found the same amount of "apoLDL" protein (determined to be about 370 kDa per mole at the time) in three subfractions of human VLDL as in human LDL.

Several years ago a number of laboratories independently discovered the two major size isoforms of apoB (11–14). In human and most other mammals the larger isoform (called apoB-100 or PI or $apoB_h$) and smaller (B-48 or PIII or $apoB_l$) are exclusively produced by adult liver and intestine, respectively (15); however, adult rat and mouse livers synthesize both peptides (12,14,16,17). Previously available evidence suggested that B-48 corresponded to the N-terminal moiety of B-100 (8,18). This has now been conclusively established by Powell et al. (20), who have shown that

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; RIA, radioimmunoassay.

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[2]Unless otherwise stated, VLDL refers to plasma VLDL.

a C→U transition in B-48 mRNA converts glutamine$_{2153}$ into an in-frame stop codon. Thus, B-48 contains the first 2152, or roughly one half, of the 4536 amino acids in B-100. This raises the additional question of whether assembly of a TG-rich lipoprotein requires apoB on a mass or molar basis: that is, do B-48-containing particles carry one or two B-48 peptides? We have examined these questions in regard to rat and human VLDL.

MATERIALS AND METHODS

Preparation of rat plasma VLDL

Ad libitum chow-fed male retired breeder rats were exsanguinated through the aortic bifurcation under halothane anesthesia. Blood was collected into syringes containing 200-fold concentrated stocks to give final concentrations of no less than 2 mM EDTA, pH 7.4, 0.1% ϵ-amino caproic acid and 0.001% each leupeptin and antipain. After low-speed centrifugation to remove blood cells, the plasma was further adjusted to 2 mM phenyl methyl sulfonyl fluoride and 0.001% each pepstatin, chymostatin, and BHT from 200-fold stocks in DMSO. The 300 ml of plasma recovered was layered in 25-ml aliquots under 12 ml of inhibitor-containing saline in 12 polyallomer tubes for the Beckman Ti-50.2 rotor and centrifuged 16 hr at 45,000 rpm in the Beckman L8-55M instrument at 20°C. The combined VLDL supernatant fractions, about 30 ml, were made 5% with respect to sucrose, overlayed with 2 mM EDTA-saline in two tubes, and refloated as before. The plugs of floating VLDL were collected with the top 5 ml of each tube, using a Beckman tube-slicer, and gently redispersed with a loose-fitting, hand-held Teflon-glass homogenizer.

Preparation of human plasma VLDL

Blood was drawn from a normolipemic donor following a 12-hr fast and plasma was isolated by the UCLA Blood Bank. EDTA, sodium azide, and gentamicin sulfate were added to 135 ml of plasma to final concentrations of 0.04%, 0.05%, and 0.005%, respectively. VLDL were obtained by centrifugation at 45,000 rpm at 16°C for 24 hr in a Beckman Ti-70 rotor and Beckman L5-65 ultracentrifuge. VLDL were recovered with a pipette in the top 3 ml of each tube.

Zonal ultracentrifugation

Rat and human VLDL were subfractionated by zonal ultracentrifugation in a Beckman Ti-14 zonal rotor and Spinco Model L ultracentrifuge (21), modified to accommodate loading, running, and unloading this rotor. The sodium bromide gradient spanned a density range of 1.00–1.15 g/ml; it was linear with respect to the rotor volume (650 ml) and contained a constant background EDTA concentration of 1.4 mM, pH 7.6. Ten ml of the VLDL adjusted to a solvent density of 1.19 g/ml was loaded at the bottom of the gradient (22), and centrifuged at 18,200 rpm at 23°C for 1.25 hr (rat) or 30,000 rpm at 26°C for 1.25 hr (human). Twenty-two 30-ml fractions were collected by introduction of a concentrated NaBr solution at the bottom of the gradient (22). Rotor effluent absorption was monitored at 280 nm during fractionation using an ISCO UV Analyzer.

Fractionated rat VLDL were concentrated by ultracentrifugation at 24,000 rpm at 16°C for 18.5 hr in a Beckman SW-27 rotor and Beckman L5-65 ultracentrifuge. The concentrated fractions were dialyzed into 1.68 M NaCl containing 0.04% EDTA, 0.05% NaN_3, and 0.005% gentamicin sulfate (d 1.063 g/ml at 26°C), and aliquots of the odd-numbered fractions were taken for analytical ultracentrifugation. Remaining materials were dialyzed against three changes of 1 mM EDTA, pH 8, to remove salts, and stored in liquid nitrogen until analyzed for chemical composition.

Fractionated human VLDL were first dialyzed into 1.684 M NaCl containing 0.7 mM EDTA, pH 7.6. They were then concentrated by centrifugation at 40,000 at 16°C for 18 hr in a Beckman Ti-50 rotor and Beckman L2-50 ultracentrifuge.

Analytical ultracentrifugation

Analytical ultracentrifugation of rat and human VLDL was performed at room temperature (monitored during the experiment) in a Beckman Model E ultracentrifuge using the schlieren optical system. Three fractions could be analyzed simultaneously in the AN-F rotor using double sector cells, two of which were equipped with 1° positive or negative wedge windows. Two consecutive centrifugations, at speeds ranging from 12,590 to 35,000 rpm depending upon particle size, were first made on aliquots of the dialyzed lipoprotein fractions in an aqueous NaCl solvent of density 1.063 g/ml at 26°C, with redispersion by gentle shaking of the removed ultracentrifuge cells between the runs. The remaining samples were then redialyzed into an aqueous NaCl solvent of density 1.125 g/ml (rat VLDL) or 1.190 g/ml (human VLDL), and the runs were repeated as before. Peak positions were measured using a Nikon Model 6C microcomparator, and flotation rates were determined from ln ρ vs. $\omega^2 t$ plots. The buoyant density, ρ_b, of each fraction was determined from the x-intercept of an ηs vs. ρ plot, and flotation rates were converted to standard S_f^0 values, defined as the negative sedimentation coefficient in an aqueous

NaCl solvent of density 1.063 g/ml at 26° at infinite dilution. VLDL molecular weights were calculated by solving two simultaneous equations for molecular weight, M, and particle radius, R:

$$S = M(1 - \bar{v}\rho)/(N\ 6\ \pi\eta R\ f/f_o) \qquad Eq.\ 1)$$

$$(4/3)\pi R^3 = M\bar{v}/N \qquad Eq.\ 2)$$

where $\bar{v}$, the partial specific volume, is taken as the reciprocal of the buoyant density, ρ_b. The solvent density, ρ, and viscosity, η, equal 1.063 g/ml and 0.01022 Poise, respectively, at 26°C. N is Avogadro's number. Values for the fractional ratio, f/f_o, were obtained by assuming that the VLDL are spherical particles with a constant 11Å shell of hydration for all particle sizes. This involved a reiterative calculation, and parameter values became invariant after three cycles. The 11 Å value corresponds to the $f/f_o = 1.11$ value for LDL forced by the molecular weight of this particle, which now can be directly calculated from its composition and the 512,000 protein molecular weight of its single B-100 peptide as determined from its cDNA sequence. It also corresponds to the measured value of $f/f_o = 1.11$ determined by sedimentation and diffusion analysis for LDL (23).

Radioimmunoassay for rat apoB peptides

Rabbit antisera against reduced and alkylated rat B-100 and B-48 were raised as described (24,25) with continued boosting over one year's time. B-100-specific antiserum was obtained by absorption with B-48 as described (24).

Standard rat B-100 and B-48 were prepared without reduction and alkylation from delipidated Triton-VLDL by preparative SDS-PAGE. About 5 mg total apoB was applied per standard-sized 1.5-mm 5% acrylamide slab gel. After staining for 15 min in 0.025% Coomassie Blue in 5% acetic acid-40% isopropanol, gels were destained in several changes of 10% isopropanol followed by distilled water to remove all traces of acetic acid. The appropriate bands were cut out, weighed, and homogenized briefly in twice their weight of 50 mM Tris·HCl, pH 8.5, using a Polytron homogenizer at low speed. SDS was added to a final concentration of 0.1%, and the suspensions were stirred slowly in the cold overnight. After centrifugation in the Sorval SS34 rotor at 10,000 rpm for 5 min and recovery of the supernatants, the gels were reextracted as before, and the combined supernatants were precipitated with four volumes of ethanol at −20°C. The precipitated apoB peptides were collected by centrifugation as above, resuspended to about 1 mg/ml in 0.3% SDS, heated at 90°C until dissolved, with brief sonication if needed, and dialyzed overnight against 0.1% SDS in the cold. After brief heating to dissolve any precipitated SDS, the preparations were stored at −74°C, and reheated before use.

Micro-Lowry protein assays (26) employing a final volume of 0.25 ml were performed in microfuge tubes, extracting with an equal volume of chloroform as needed to remove lipid turbidity. The concentrations of the BSA standards were determined from their absorbance at 280 nm, using an extinction coefficient of 0.67 ml/mg·cm (27).

Absolute concentrations of rat apoB standards were obtained by quantitative amino acid analysis, using norleucine internal standards. Precisely measured standard samples of BSA, rat B-100, and rat B-48, each containing about 1 μg of protein per nmol norleucine, were hydrolyzed for 24 and 72 hr, each in duplicate. The molar ratio of each of their component amino acids to the norleucine standard (except cysteine, methionine, proline, and tryptophan, which are not measured by this procedure) was determined on a Beckman 6300 analyzer, using the 3000 Chromatograph Data System (Nelson Analytical Inc., Cupertino, CA). Values for threonine, serine, and tyrosine were obtained by linear extrapolation to 0 time. The mole percent of each measured amino acid was then calculated using the known values for cysteine, methionine, proline, and tryptophan for BSA, and assuming the same combined contribution of these amino acids to the rat apoB peptides as in human B-100 and B-48. The total amino acyl mass (including cysteine, methionine, proline, and tryptophan) per nmole norleucine for each protein was then compared to the corresponding value obtained by Lowry assay.

The radioimmunoassays for total apoB and B-100 (24) were modified as follows. For radioiodination, 1 μg rat B-48 or B-100 in 5 μl 1% SDS/1 M sodium phosphate, pH 7.8, was mixed with 3 μl Na ^{125}I (100 mCi/ml, 13–17 mCi/μg I (NEN)), followed by 3 μl 0.03% chloramine-T. After 15 min at room temperature, 5 μl each of 1% NaI and 0.5% BSA were added, and the reaction mixture was transferred with a 50-μl water wash onto a spincolumn prepared by centrifuging 2 ml of a 50% slurry of Sephadex G-50 fine (Pharmacia) in buffer A (0.5% SDS, 0.017% bovine serum albumin, 5 mM EDTA, 0.1% sodium azide, and 1 mM phenylmethylsulfonyl fluoride in 50 mM sodium phosphate, pH 7.8) in a disposable column (Bio-Rad) at 2000 rpm (Sorval GLC-2) for 2 min. The eluate and a 100-μl buffer A wash were collected by recentrifugation as above, diluted to 500 μl with buffer A, stored at −70°C, and used within 4 weeks. Labeled antigens averaged 10^5 cpm/ng, and were greater than 95 and 50% precipitable with trichloroacetic acid and excess anti-apoB, respectively. For the assay, 1 ng of labeled apoB peptide and 5 to 700 fmol of unlabeled standard

or unknown apoB, each in 100 μl buffer A, were mixed in a conical microcentrifuge tube, heated at 90°C for 10 min, and cooled to room temperature. The appropriate antiserum was added in 200 μl buffer B (6% Triton X-100, 3% sodium sarcosylate, 0.3% bovine serum albumin, 5 mM EDTA, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 0.3% soybean trypsin inhibitor, and 0.001% each leupeptin, antipain, pepstatin, and chymostatin in 50 mM sodium phosphate, pH 7.8), and the tubes were incubated overnight at 4°C. After addition of 20 μl of a 1% suspension of washed *S. aureus* cells (insoluble protein A, Sigma), and a 15-min incubation at room temperature, 100 μl dibutyl phthalate was added to each tube, and bound antigen was recovered by pelleting the *S. aureus* cells. The supernatant and most of the dibutyl phthalate layer was removed by aspiration, the inverted tubes were allowed to drain for 10 min, and the pellet was recovered for gamma counting without further washing by cutting off the tip of the tube. Blanks by this procedure contain less than 0.5% of input counts.

The assays for total apoB used ^{125}I-labeled B-48 and 100 nl anti-B-48; those for B-100 used ^{125}I-labeled B-100 and 100 nl B-48-absorbed anti-B-100 antisera, respectively. Each assay contained a series of two-fold dilutions of the appropriate apoB standard as well as controls without unlabeled antigen and blanks without antibody, all in triplicates. Each unknown was assayed at three twofold dilutions, each in duplicate. The intraassay coefficient of variation (SEM/mean) within each such six-aliquot set averaged 4%, with dilution curves for standards and unknowns showing the same slopes. Intrassay coefficient of variation averaged 5%.

Lipid analyses

Triglyceride glycerol (28), total cholesterol (29), and phospholipid phosphorus (30) were determined as described. Values for VLDL triglycerides and phospholipids were calculated using averages of the fatty acid compositions reported by Dunn, Wilcox, and Heimberg (31) and DePury and Collins (32). Free and esterified cholesterol were determined separately after thin-layer chromatography on precoated Silica Gel G plates (Merck) in 10% ether in hexane.

RESULTS

Fractionation of rat VLDL

The separation of VLDL obtained from about 300 ml plasma from *ad libitum*-fed rats on the zonal gradient is shown in **Fig.1** As performed here zonal centrifugation separates VLDL primarily according to size, and as seen in Fig 1, the fractions collected covered a broad distribution throughout the gradient. The factions produced sharp peaks in the analytical ultracentrifuge (**Fig. 2**), indicating that the VLDL in each fraction were relatively uniform in size. The actual size distribution of the various zonal fractions was estimated from the widths of the individual schlieren peaks. After correction for diffusion, the peak widths reflect the spread of flotation coefficients within each fraction. Thus, since the square root of the flotation coefficient is directly proportional to particle diameter

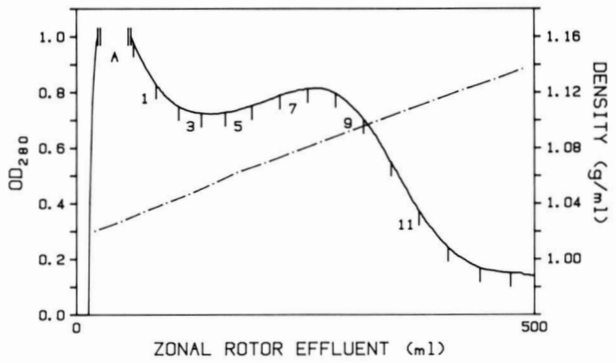

Fig. 1. Absorbance (at 280 nm) profile of rotor effluent and terminal density gradient following zonal ultracentrifugation of rat VLDL. Absorbance at 280 nm (solid line) was monitored continuously during fractionation. Double vertical bars indicate off-scale absorbance readings; single vertical bars separate fractions. Region A contained essentially no protein and was not used in these experiments. The high absorbance of the early fractions is primarily due to light scattering; thus, fraction 1 actually contained about one-third as much material as fraction 11 (see Table 4). The terminal density gradient (broken line) was determined by measuring the fraction density for a "blank" zonal run.

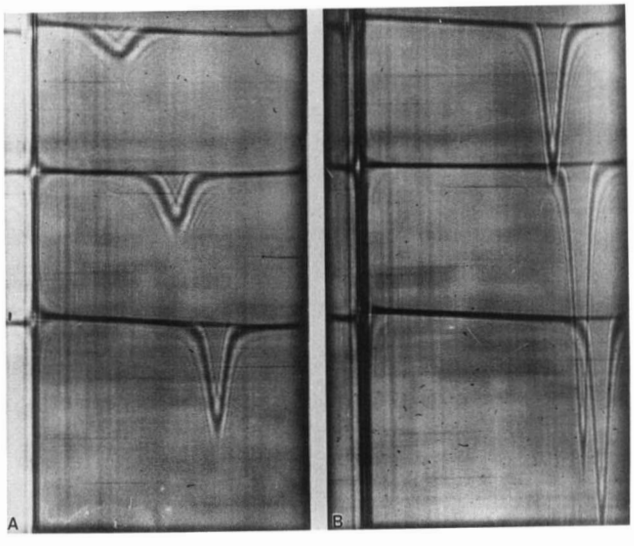

Fig. 2. Schlieren patterns for rat VLDL fractions. Photographs displayed were taken after centrifugation for 28 min at 12,590 rpm in an aqueous NaCl solvent of density 1.063 g/ml at 26°C. Flotation is from right to left. A, (top to bottom) fractions 1, 3, and 5. B, (top to bottom) fractions 7, 9, and 11.

TABLE 1. Hydrodynamic properties of rat VLDL zonal subfractions

VLDL Fraction	S_f^0	$\bar{v}$	f/f_o	VLDL Mol. Mass	Particle Diameter	Fraction Width
	S	*ml/g*		*MDa*	*nm*	*nm*
1	425.3	1.070	1.028	146.0	79.1	3.9
3	299.7	1.067	1.033	90.0	67.3	3.6
5	214.5	1.062	1.038	58.2	58.1	3.2
7	153.0	1.055	1.044	38.5	50.5	2.8
9	108.9	1.050	1.050	24.9	43.6	2.9
11	77.1	1.041	1.057	17.0	38.3	3.3

S_f^0 is the concentration-corrected average flotation coefficient for the fraction determined in an aqueous NaCl solvent with density 1.063 g/ml and viscosity 0.01022 Poise at 26°C. $\bar{v}$ is taken as the reciprocal of buoyant density determined from an ηs vs. ρ plot. The frictional coefficient, f/f_o, is estimated by assuming that the VLDL are perfect spheres with an 11 Å shell of hydration. Fraction width is one standard deviation in particle diameter estimated from the diffusion-corrected peak width in the schlieren patterns. This assumes that diameter is directly proportional to the square root of the flotation coefficient when a small correction for particle density variation across the peak is ignored.

(ignoring a small correction for particle density variation across the peak), the corrected widths provide estimates of the standard deviation of particle sizes in each fraction. These estimates are listed in the last column of **Table 1.** It should be noted that the sharpness of the VLDL fractions is characteristic of the zonal rotor procedure (21,22), where loading at 3000 rmp avoids the interfacial instability problems (33) associated with layering macromolecular solutions on top of supporting density gradients at unit gravity.

The determination of the buoyant densities for the odd-numbered fractions is shown in **Fig. 3.** When the average S_f^0 value for each fraction was plotted as a function of buoyant density, a smooth relationship was obtained, permitting the estimation of buoyant densities for the even-numbered fractions from the flotation coefficients alone. Using the average floatation coefficients and buoyant densities for each fraction, the average molecular weights and particle radii can be calculated from equations 1 and 2, as described in Methods. The experimentally determined values of S_f^0 and buoyant density, as well as the calculated values of f/f_o, molecular weight and radius for the odd-numbered zonal fractions, are listed in Table 1.

Inspection of the data presented in Table 1 shows that the rat VLDL fractions contain particles with average diameters ranging from about 40 to 80 nm, which differ substantially in buoyant densities from about 0.96 to 0.93 g/ml, respectively. The fractions are relatively narrow, with particle size deviations of about ± 6% from the mean.

Radioimmunoassay of rat ApoB

Compared to our previously reported version (25), the sensitivity and accuracy of the RIA for total apoB was improved by using higher-affinity antibodies raised by extended immunization with rat B-48. As previously shown for the lower-affinity antibodies used at that time (25), rat B-100 and B-48 are equally effective on a molar basis in displacing labeled B-48 from the higher-affinity antibodies used here (**Fig. 4**). Thus, as before, this assay directly measures molar amounts of total rat apoB. Conversely, since rat B-48 fails to displace labeled rat B-100 from B-48-absorbed anti-rat B-100 antiserum (25), such displacement constitutes a specific assay for B-100.

It should be emphasized that both assays were designed to measure differences in actual apoB peptide mass, using denaturation of all antigens in hot SDS to eliminate differences in antigen presentation between samples. This is essentially the same proce-

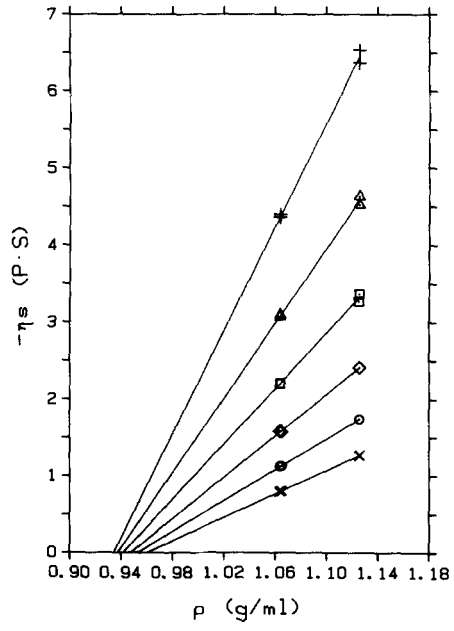

Fig. 3. Buoyant density determination for odd numbered rat VLDL fractions 1–11. The product of solvent viscosity, η (in units of Poise, P), and flotation coefficient, -s (in units of Svedbergs, S), was plotted as a function of solvent density, ρ. The x-intercept, $-\eta s = 0$, yields the buoyant density of the lipoproteins. Fraction 1, +; fraction 3, △; fraction 5, ⊡; fraction 7, ◇; fraction 9, ⊙; and fraction 11, x. All measurements are duplicates with single symbols representing closely overlapping values.

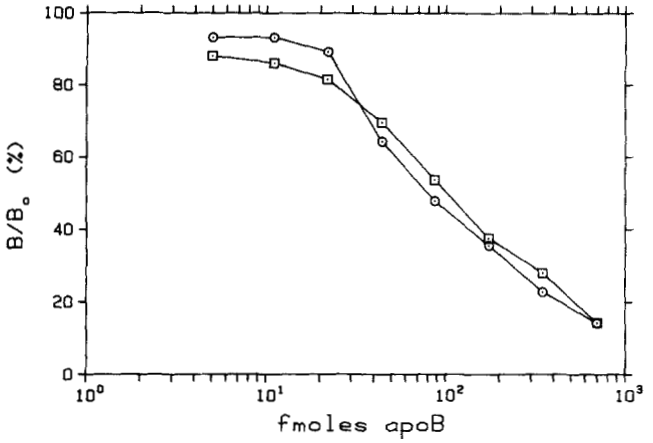

Fig. 4. Molar equivalence of rat B-48 and B-100 in radioimmunoassay for total rat apoB. The assay was performed as described in Materials and Methods, using unlabeled B-100 (circles) and B-48 (squares) to compete against labeled B-48 for binding to polyclonal antibodies against rat B-48. Abscissa: femtomoles unlabeled apoB per assay. Ordinate: labeled B-48 bound in the presence of competing antigen, as percent of that bound in the absence of the latter.

dure we use to prepare samples for SDS gels, which show little or no degradation of the apoB peptides (4,25). Furthermore, as expected from the use of polyclonal antibodies raised against the SDS-denatured antigens, even extensive degradation of apoB standards or unknowns, whether artifactual or intentional, has little effect on their activity in our radioimmunoassays. Thus, all available data support the conclusion that the results reflect actual apoB concentrations, unbiased by the origin and history of the samples analyzed.

Accurate apoB measurements require accurate estimates of the protein concentrations of the apoB standards. As shown in **Table 2,** amino acid analysis performed as described in the Methods section gave quantitative recovery of BSA standard samples. However, the calculated amino acid recoveries for samples of rat B-48 and B-100 were only about 80% of those expected from their BSA-standardized Lowry protein values. Thus, as has been previously noted for human apoB (34), conventional Lowry assays overestimate rat apoB concentrations by about 25%. The alternative explanation, i.e., spuriously low amino acid recoveries in the apoB samples, receives no support by the data in Table 2. The extrapolated serine, threonine, and tyrosine values for BSA are in good agreement with those obtained from its amino acid sequence (35), and the corresponding experimental values for the rat apoB peptides should be equally reliable. The contents of cysteine, methionine, proline, and tryptophan are not measured at all in the procedure used; however, the combined amino acyl mass in the remaining amino acids accounts for 89% of the original Lowry protein in the BSA sample, identical to their fraction by weight in the amino acid sequence. Although the full cDNA sequence for the rat apoB peptides is not available, the proportions of the remaining amino acids are very similar to those of their human counterparts. Thus, it appears highly unlikely that the sum of cysteine, methionine, proline, and tryptophan residues in rat apoB differ substantially from the 8% by weight found in the cloned human peptides. Thus, the validity of the quantitative amino acid analysis to measure apoB protein mass, with the corollary 25% hyperchromicity of apoB in the Lowry assay compared to the BSA standards, seems well established for both rat and human apoB.

The concentrations of total apoB and B-100 in the eleven rat VLDL zonal fractions are shown in **Table 3.** Eighty percent of total apoB is recovered in the second half of the gradient (fractions 7–12), corresponding to particles of less than 40 MDa. This is even more pronounced for B-100, reflecting a progressive increase in its abundance from about 20 to 50 mole% of total apoB across the gradient, i.e., from one-third to two-thirds of total apoB by weight.

Composition of rat VLDL subfractions

Table 4 shows the concentrations of triglycerides, phospholipids, total cholesterol, and total protein in the fractions. The ratio of free to esterified cholesterol was determined to be 2.3:1 in fraction 9. Applying this ratio to all fractions, the absolute and relative amounts of lipoprotein triglyceride, phospholipids, free and esterified cholesterol (as cholesteryl oleate), and protein were also calculated (Table 4). The Lowry total protein values are given as uncorrected BSA equivalents, since the proportion of apoB is small and the actual chromogenicity of the other apoproteins is undetermined. As expected, triglycerides are the major component by weight in all subfractions, ranging from 77 to 64% of the mass in order of decreasing VLDL molecular weights, making them 50- to 20-fold more abundant than the estimated cholesteryl esters, the other core component. The proportion of surface components shows the expected increase with decreasing VLDL particle size, maintaining a roughly constant phospholipid to protein ratio of about 2.5:1 across the gradient. The fraction of total protein accounted for by apoB increases with decreasing VLDL particle size, as expected from the loss of non-apoB apoproteins with other surface components during lipolysis.

The molar concentrations of VLDL particles in each zonal fraction were calculated from their mass concentrations (Table 4) and molecular weights (Table 1),

TABLE 2. Quantitative amino acid composition and recoveries from rat apoB peptides

Amino Acid	BSA		B-100		B-48	
	Protein Sequence	Found	Human cDNA	Rat, Found	Human cDNA	Rat, Found
			mole%			
Asp	8.8	9.7 ± 0.4[a]	10.6	10.3 ± 0.1	10.6	9.8 ± 0.0
Thr	5.9	5.6[b]	6.6	6.2[b]	6.7	6.5[b]
Ser	4.8	5.4[b]	8.6	9.0[b]	8.9	9.7[b]
Glu	13.6	14.2 ± 0.2	11.6	12.0 ± 0.1	10.9	11.3 ± 0.1
Gly	2.8	3.1 ± 0.2	4.5	5.1 ± 0.2	5.4	6.0 ± 0.1
Ala	7.9	7.8 ± 0.1	5.9	5.8 ± 0.1	6.5	6.0 ± 0.1
Cys	6.0	[c]	0.6	[c]	0.8	[c]
Val	6.2	5.9 ± 0.2	5.6	6.5 ± 0.1	5.3	6.6 ± 0.1
Met	0.7	[c]	1.7	[c]	2.1	[c]
Ile	2.4	2.5 ± 0.1	6.3	5.5 ± 0.1	4.9	4.5 ± 0.0
Leu	10.5	10.3 ± 0.1	11.6	11.4 ± 0.1	12.1	11.7 ± 0.0
Tyr	3.3	2.8 ± 0.0	3.3	3.3 ± 0.0	3.5	3.0 ± 0.0
Phe	4.7	4.7 ± 0.2	4.9	5.1 ± 0.1	4.1	4.5 ± 0.1
His	2.9	2.7 ± 0.1	2.5	2.2 ± 0.0	2.3	2.1 ± 0.0
Lys	10.2	9.6 ± 0.4	7.9	7.5 ± 0.3	7.7	7.1 ± 0.0
Arg	4.0	3.7 ± 0.1	3.3	3.6 ± 0.1	3.6	3.8 ± 0.1
Pro	4.8	[c]	3.7	[c]	3.8	[c]
Trp	0.3	[c]	0.8	[c]	0.6	[c]
			%(w/w)			
(Cys + Met + Pro + Trp)	10.9		7.0		7.5	

	BSA	B-100	B-48
ng Aminoacyl mass/nmol norleucine, corrected[d]	980 ± 23	781 ± 19	664 ± 15
ng Lowry protein/nmol norleucine	988 ± 15	968 ± 18	860 ± 23
Recovery, %[e]	99 ± 3	81 ± 3	77 ± 3

Samples of bovine serum albumin, rat B-100, and rat B-48, precisely measured by the Lowry procedure, were subject to quantitative amino acid analysis as described in Methods, using norleucine as internal standard. The top part of the table compares these results to the amino acid compositions predicted by the cDNA sequences of BSA and the human apoB peptides. The lower part shows recovery of amino acyl mass for the samples (corrected for their expected content of cysteine, methionine, proline, and tryptophan) and compares it to their original protein content as estimated by the Lowry method.

[a]SEM.

[b]Extrapolated values are averages of duplicates differing by less than 10%.

[c]Assumed equal to cloned values.

[d]ng Aminoacyl mass recovered/nanomole norleucine, corrected for (cys + met + pro + trp).

[e]Corrected aminoacyl mass recovered as % of original Lowry protein.

using interpolated values of molecular weight for the even-numbered fractions. The results were combined with the corresponding values for apoB to yield the experimentally determined number of apoB peptides per VLDL particle. As seen in **Table 5,** this ratio is close to unity for all fractions, with a trend towards increased ratios with increased particle size. Thus, VLDL particles in fractions 6 through 11, which account

TABLE 3. ApoB concentrations in rat VLDL zonal fractions

ApoB	Fraction										
	1	2	3	4	5	6	7	8	9	10	11
Total apoB (nM)	11.4 ± 0.5[a] (5)[b]	16.7 ± 0.6 (3)	19.1 ± 0.6 (4)	39.2 ± 1.6 (3)	54.4 ± 2.7 (7)	86.2 ± 1.7 (3)	90.0 ± 6.4 (8)	172 ± 4 (3)	202 ± 13 (11)	262 ± 9 (3)	238 ± 18 (8)
B-100 (nM)	0.7	3.6 ± 0.6 (4)	4.2	10	14 ± 1 (4)	27	30 ± 1 (3)	59 ± 5 (3)	95 ± 4 (6)	148	112 ± 2 (5)
B-100 (mol%)	7	18	19	25	26	32	33	34	47	56	47
ApoB (% of protein in fraction)	4	5	6	10	11	13	13	18	21	26	29
ApoB (% of total)[c]	1.0	1.4	1.6	3.3	4.6	7.2	7.6	14.4	17.0	22.0	19.9

Molar concentrations of total apoB and B-100 were determined by separate radioimmunoassays as described in Materials and Methods.

[a]SEM.

[b]Number of determinations in parentheses.

[c]ApoB in each fraction as percent of apoB recovered in all fractions.

TABLE 4. Composition of rat VLDL zonal fractions

Composition	Fraction										
	1	2	3	4	5	6	7	8	9	10	11
	% (w/w)										
TG	78.8 ± 11.0[a]	75.8 ± 4.4	72.5 ± 6.3	73.2 ± 3.3	71.3 ± 4.3	71.8 ± 3.5	71.9 ± 6.2	67.7 ± 3.4	69.1 ± 3.1	66.8 ± 7.4	64.0 ± 4.2
PL	11.9	13.3	14.2	15.4	16.0	16.1	15.8	19.0	17.1	19.7	19.2
FC	2.3	3.0	4.4	3.6	4.1	3.7	3.3	4.1	4.2	3.8	5.3
CE	1.6	2.1	3.1	2.6	2.9	2.6	2.4	3.0	3.1	2.8	3.8
Protein	5.3	5.8 ± 0.9	5.8	5.3 ± 0.4	5.6 ± 0.5	5.8 ± 0.7	6.6	6.2 ± 0.8	6.5 ± 0.6	7.0 ± 0.7	7.2 ± 0.9
Total mass (mg/ml)	1.32 ± 0.12	1.54 ± 0.06	1.56 ± 0.1	2.26 ± 0.07	2.60 ± 0.11	3.54 ± 0.12	3.48 ± 0.20	4.92 ± 0.18	5.38 ± 0.17	5.62 ± 0.39	4.10 ± 0.18

Triglyceride values are means ± SEM of five to eight separate determinations, each in duplicate. Phospholipids and total cholesterol values are averages of two concentrations, each in duplicate, differing by < 5%. Free and esterified cholesterol (as cholesteryl oleate) were calculated as described in Materials and Methods using a 2.3:1 molar ratio. Lowry protein values are averages of single, or averages ± SEM of three, determinations, each in duplicate differing by < 5%.

[a]SEM.

for 90% of the total, contain on the average a single apoB peptide per particle, be it B-100 or B-48. Values for the larger particles in fractions 1–5, which account for about one-tenth of the apoB on the zonal gradient, are marginally greater; the significance of this is unclear.

The particle densities in the zonal fractions were also calculated from their composition, assuming additivity of partial specific volumes for the components. For this purpose phospholipid density was taken to equal that of aqueous lecithin bilayers (36). Cholesterol density was taken to be that in benzene corrected to water (37). As seen in **Table 5,** assuming a protein partial specific volume of 0.725 ml/g, calculated and observed VLDL densities are brought into reasonable agreement when the density of VLDL triglycerides is taken as 0.901 g/ml, a value about one-half percent lower than that given for triolein (38). The density of cholesteryl esters (39) contributes little, due to their low estimated abundance.

The compositional data in Table 4 were also analyzed in terms of the surface/core relationship for spherical lipoprotein particles (40,41), with protein and phospholipids as obligate surface components, and a partition coefficient of 11 for the distribution of free cholesterol between phospholipid surface and triglyceride plus cholesteryl ester core phases (42). The average diameter of the VLDL particles in each zonal fraction was calculated from the average particle molecular weight and density, and the thickness of the unhydrated surface layer was obtained from the volume fraction of surface components. As seen in Table 5, the values so obtained are close to the expected 20 Å (41), with a trend towards increasing thickness with increasing particle size.

Fractionation of human VLDL

We have also performed a similar investigation of the number of apoB per particle for human VLDL, incorporating the $S_f°$ values and compositional data previously reported by Patsch et al. (21). For this purpose human VLDL was fractionated by zonal centrifugation into fractions with concentrations and schli-

TABLE 5. Number of apoB peptides per rat VLDL particle

Parameter	Fraction										
	1	2	3	4	5	6	7	8	9	10	11
VLDL mol mass (MDa)	146	115	90.0	72.4	58.2	47.3	38.5	31.0	24.9	20.6	17.0
VLDL concentration (nM)	9 ± 1[a]	13 ± 1	17 ± 1	31 ± 1	45 ± 2	75 ± 3	90 ± 5	159 ± 6	216 ± 7	273 ± 19	241 ± 10
ApoB VLDL (mol/mol)	1.26 ± 0.13	1.24 ± 0.07	1.10 ± 0.08	1.26 ± 0.07	1.22 ± 0.08	1.15 ± 0.05	1.01 ± 0.09	1.08 ± 0.05	0.94 ± 0.07	0.96 ± 0.08	0.99 ± 0.09
VLDL measured density (g/ml)	0.934	0.936	0.938	0.940	0.942	0.945	0.948	0.950	0.953	0.957	0.960
VLDL calculated density (g/ml)	0.936	0.940	0.944	0.942	0.945	0.945	0.947	0.951	0.950	0.954	0.957
Surface shell thickness (Å)	21.8 ± 2.4	23.1 ± 1.7	23.5 ± 2.0	22.2 ± 1.0	22.1 ± 1.3	20.5 ± 1.1	19.2 ± 1.2	21.0 ± 1.2	18.2 ± 0.9	19.2 ± 1.7	18.7 ± 1.2

Molar particle concentration in each fraction was calculated from the VLDL mass concentrations and molecular weights given in Tables 4 and 1, respectively, with interpolation of molecular weights for the even fractions. VLDL particle densities and surface shell thickness were calculated from compositional data (Table 4) as described in Materials and Methods. Measured densities are reciprocals of $\bar{v}$ values in Table 1, with interpolation for even fractions.

[a]SEM.

eren patterns similar to those for the rat VLDL subfractions, and the $S_f°$ and $\bar{v}$ values determined for eleven fractions covering the range corresponding to fractions 2 to 7 described by Patsch et al. (**Fig. 5**). This experimentally determined relationship was then applied to the $S_f°$ values reported by Patsch et al., to obtain the $\bar{v}$ and molecular weights for their fractions. The average number of moles of 512 kDa B-100 peptide per mole VLDL particle in each fraction could then be calculated from the tetramethylurea-insoluble Lowry protein values reported by Patsch et al., corrected for apoB hyperchromicity. As seen in **Table 6,** this number again comes out close to one, consistent with the presence of a single B-100 peptide per human VLDL particle. Table 6 also shows good agreement between densities calculated for the fractions is also very close to the expected 20 Å (41) (Table 6), providing additional evidence for the internal consistency of the combined data.

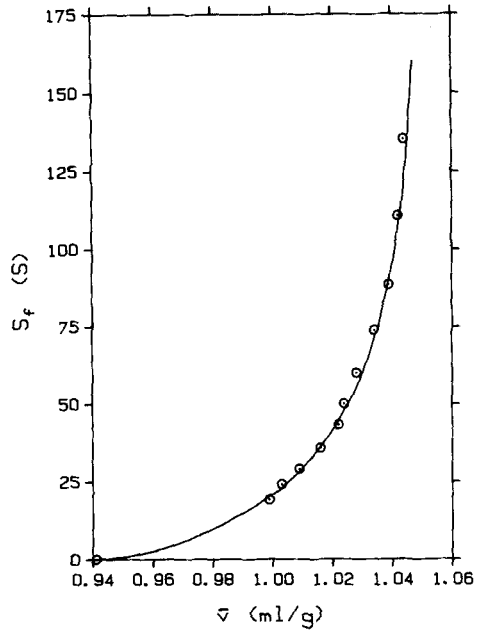

Fig. 5. Estimation of partial specific volume for human VLDL c a given flotation coefficient. Flotation coefficient, $S_f°$, was plotted a a function of partial specific volume, $\bar{v}$, for 11 human VLD subfractions, and the best smooth curve was fitted to the data. Value of $\bar{v}$ for VLDL of a given $S_f°$ were then predicted from this plot.

DISCUSSION

The results presented here show that both rat and human plasma VLDL contain an average of one apoB peptide per particle. The possibility that a substantial portion of the particles may have more than one apoB and others none seems remote, since all evidence suggests that TG-rich lipoproteins require at least one apoB for assembly and secretion (1), and since apoB, alone among apolipoproteins, never has been found to dissociate during subsequent lipolytic remodeling of the lipoprotein particle (1,15). Furthermore, in preliminary experiments we find that over 85% of the triglycerides in unfractionated preparations of rat plasma VLDL are precipitable with our anti-apoB antibodies (J. Elovson and G. T. Bell, unpublished results), providing direct evidence that the averag value indeed reflects the fact that the vast majority o rat VLDL particles contain a single apoB peptide.

Although straightforward in principle, determina tion of the number of apoB peptides per VLDL par ticle presents some problems. Unlike LDL, VLDL con tain large amounts of other apoproteins. For the humai VLDL fractions analyzed by Patsch et al. (21), apoI can be estimated with adequate precision as the dif ference between total and tetramethylurea-solubl Lowry protein, since it still constitutes between 25 anc

TABLE 6. Molecular and compositional parameters for human VLDL zonal fractions

Parameter	Fraction 2	3	4	5	6	7
$S_f°$ (S)	138.9	96.2	67.7	50.3	41.0	27.0
$\bar{v}$ (ml/g)	1.046	1.040	1.033	1.025	1.020	1.008
f/f_o	1.020	1.024	1.027	1.030	1.033	1.037
Mol wt (MDa)	36.3	22.9	15.1	11.1	8.9	6.1
ApoB (% (w/w))	2.012	3.422	3.638	4.778	6.724	8.738
ApoB/particle	1.14	1.22	0.86	0.82	0.94	0.84
Calculated density (g/ml)	0.955	0.965	0.969	0.978	0.986	0.996
Measured density (g/ml)	0.956	0.962	0.968	0.976	0.980	0.992
Particle diameter (nm)	49.4	42.3	36.7	33.0	30.7	26.9
Surface shell thickness (Å)	19.9	20.4	19.0	19.6	19.5	19.2

Numbering and compositional data for the VLDL fractions are from Patsch et al. (21). $S_f°$, $\bar{v}$, f/f_o, and molecular weight were measured on fractions from a separate VLDL zonal gradient, and the two data sets were aligned by S_f values as described in Materials and Methods. The concentration of apoB is taken as tetramethylurea-insoluble protein, multiplied by 0.80 to correct for hyperchromicity in the Lowry values given by Patsch et al. Densities and surface layer thickness values were calculated as in Table 5, using compositional data from Patsch et al. (21).

65% of the total. However, this conventional approach is inherently less reliable for rat VLDL, which contain on the average much larger particles, with a correspondingly smaller proportion of apoB, in the range of 5 to 30% of total protein (Table 3). More importantly, since rat VLDL contain comparable amounts of B-48 and B-100 (12), their protein mass must be determined separately to get correct molar amounts of total apoB. As discussed elsewhere (25), using SDS-PAGE for this purpose is complicated by the variable but ubiquitous proteolysis of these large peptides. Furthermore, particularly for the larger particles, the amounts of rat apoB available were borderline for Coomassie-stained cylindrical gels, and our attempts to quantitate the apoB peptides on silver-stained slab-gels have been unsuccessful, with both standards and samples showing large and unpredictable fluctuations in color yields, both within and between gels. The radioimmunoassay employed here avoids these problems: since the anti-B-48 antiserum used recognizes B-48 and B-100 equally on a molar basis, it measures total apoB directly. This is of course the expected result, since B-48 now is firmly established to be coextensive with the N-terminal half of B-100 (19,20). The higher-affinity anti-B-48 antibodies used here have also improved the sensitivity of the assay compared to our previously published version, allowing reliable assay of samples containing 0.1 μg B-48/ml. In addition, rat B-100 is determined independently, using an antiserum specific for epitopes on its COOH-terminal moiety, which are absent from B-48. It should be emphasized that both assays were performed after completely denaturing all antigens by boiling in SDS. Thus, unlike the many immunoassays now used to detect subtle differences in antigen presentation on particles of different size and composition (43), our assays are designed to measure differences in actual apoB peptide mass.

Correct estimation of VLDL apoB stoichiometry depends on correct estimates of the molecular weights and absolute concentrations of the apoB standards used in the radioimmunoassay. Human B-100 cDNA encodes a 512 kDa mature peptide, and human B-48 has recently been shown to represent the 241 kDa 2152-residue NH_2-terminal moiety of B-100 (19,20). Although the full rat cDNA sequence is unavailable, the molecular weights of the rat peptides should also be very close to these values since the rat and human peptides comigrate on SDS-PAGE (12) and since the sequence of the COOH-terminal 20% of rat B-100 in fact is very similar to the human (M.A. Reuben, K. Swenson, and J. Elovson, unpublished observation). Such interspecies similarity is further supported by the fact that the same chain-termination site is responsible for the generation of B-48 in rabbit and human intestine (19). Thus, there is little reason to expect the molecular weight of rat B-48 to differ significantly from that of the human peptide.

The hyperchromicity of apoB relative to bovine serum albumin in the Lowry assay has been previously noted for human apoB (34, and references therein), although estimates have varied, presumably due to the different procedures used to measure absolute apoB protein mass. As already discussed in the Results section, the approximately 25% hyperchromicity for the rat peptides, compared to quantitative amino acid analysis, assumes a total cysteine plus methionine, proline, and tryptophan content equal to the 7% found in the human apoB peptides. However, this assumption is of little concern, since even doubling the estimated content of these amino acids in the rat peptides would only increase their estimated total amino acids mass by 7%. Thus, we feel confident that our radioimmunoassay measures correctly standardized molar amounts of apoB.

Measurements of particle concentrations in preparations as polydisperse as unfractionated VLDL present their own problems. One approach is to determine the particle size frequency distribution for negatively stained preparations in the electron microscope, and thus obtain a number average molecular weight for the sample. However, conversion of measured particle diameters into actual molecular volumes, i.e., weights, is complicated by variable particle flattening and other staining artifacts, which are aggravated by increased particle size and polydispersity. We have, therefore, chosen to approach this problem directly, by separating the VLDL into fractions with a sufficiently narrow size distribution to be able to directly determine the molecular weights of their component particles.

In order to estimate molecular weights from sedimentation coefficients, a spherical shape has been assumed for the VLDL; however, even if the actual shape were that of an oblate or prolate ellipsoid of revolution with an axial ratio of 2, the f/f_o value would only increase from 1.00 (for a sphere) to 1.04. The actual values for f/f_o utilized, which range from 1.02 to 1.05, could, of course, be due to either asymmetry and/or an increase in the hydrodynamic volume (hydration). The values which we have selected seem very reasonable, and the error here is likely to be on the order of, at most, a few percent.

A more sophisticated correction for the effects of preferential solvation has been ignored. Preferential solvation affects only the buoyant density of a macromolecule; its physical basis is the preferential binding on one component of the solvent (either the water or the salt) to alter the buoyant density of the particle. In fact, for most proteins in most solvents, water is preferentially bound, resulting in a slightly lowered buoyant density. If an experimental buoyant

density is utilized in the calculations, as we have done here, then the resulting molecular weight for the macromolecule includes the weight of the preferentially bound solvent. For LDL the preferential solvation has been determined by two groups of workers and found either to be negligible (23) or less than 5% (44). Like hydrodynamic hydration, preferential solvation is a surface effect, and the percentage correction should be proportional to the ratio of surface to volume. Thus, for VLDL, it probably would be less than for LDL. We have chosen to ignore preferential solvation which, in any case, is probably very small. If a correction were to be included for preferential solvation, however, it would be in the opposite direction to the small correction applied for asymmetry and hydration, making the total correction even smaller.

The composition of our rat VLDL subfractions conforms reasonably well to that expected for lipoprotein particles with a 20 Å thick surface layer of protein, phospholipid, and free cholesterol surrounding a core of triglycerides and cholesteryl esters (40,41). Since the 2.3:1 ratio of free/esterified cholesterol was determined for only a single fraction, its application to all fractions, as done here, undoubtedly introduces some error. However, our fractions contain much less total cholesterol than phospholipids and triglycerides; thus, even large changes in the free/esterified cholesterol ratio produce little change in either the calculated number of apoB per VLDL particle or the surface/core relationships.

When summed over the 11 fractions, total protein and apoB constitute 6.3 and 1.1% (w/w), respectively, of the total VLDL mass. The corresponding values for total and tetramethylurea insoluble protein for rat total plasma VLDL given by Mjøs et al. (45), are 9.4 and 1.0%, respectively. Thus, our apoB values are identical to those of Mjøs et al. as percent of total VLDL mass. Our somewhat lower value for total VLDL protein is consistent with some additional loss of C-proteins caused by the repeated ultracentrifugations used in the preparation of the subfractions. Our other compositional data are also consistent with those given by Mjøs et al. (45), as well as with those given by Faergeman and Havel (46) for rat VLDL fractions separated by gel exclusion chromatography.

Our estimate of one B-100 peptide per human VLDL particle rests on the combined compositional data from Patsch et al. (21) and our molecular weight measurements, using S_f values to align the two sets of observations. As mentioned above, the validity of this approach is supported by the good agreement between the densities and surface/core relationships calculated from Patsch's data and those obtained from our hydrodynamic measurements. Thus, although less direct than the analysis for rat VLDL, this approach clearly establishes the presence of a single apoB per human VLDL. This is, of course, the expected result, since the molecular weight of cloned human B-100, combined with the protein content and molecular weight of human LDL, unequivocally establishes the presence of a single B-100 peptide on the major lipolytic endproduct of human VLDL.

Since the range of particles examined here presumably is almost exclusively of hepatic origin, our results do not provide direct information on the number of apoB peptides involved in the assembly of the intestinal triglyceride-rich particles. In fact, Bhattacharya and Redgrave (47) estimated the average apoB mass (as tetramethylurea-insoluble protein) per rat chylomicron particle to be 480 kDa, suggesting the presence of two copies of B-48. However, chylomicron apoB is difficult to measure accurately by the tetramethylurea procedure, since it constitutes only about 1% of the total protein. Furthermore, although the authors performed a detailed correction of their electron microscopic estimates of particle sizes and abundance, the problems referred to above may still have caused an overestimate of the average particle molecular weight, with a corresponding underestimate of particle concentration. Thus, the number of B-48 peptides per chylomicron particle needs to be reexamined by more direct methods.

The role of apoB in the assembly of the triglyceride-rich lipoproteins remains elusive. The primary sequence of B-100 offers few clues to regions specifically involved in assembly, other than a variety of hydrophobic and amphipathic motifs scattered throughout the molecule (5–9). Specifically, although there is little direct sequence homology between the NH_2-terminal and COOH-terminal halves of B-100, there is no obvious reason why the former, i.e., that common to B-100 and B-48, should determine competence in assembly, or conversely to exclude the possibility that two B-48 would be required to substitute for one B-100. Although the stoichiometry of apoB on the nascent VLDL particles themselves remains to be determined directly, our results now show that rat plasma VLDL contain a single apoB peptide per particle, be it B-48 or B-100. Thus, unless the nascent particles contain additional apoB peptides that are uniformly eliminated from the plasma particles, it appears that a single B-48 sequence can provide all the structural information required for the unique ability of apoB to assemble triglyceride-rich lipoproteins.

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