# Apoprotein Composition of Very Low Density Lipoproteins of Human Serum

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ABSTRACT Methods for quantitation of the major apoproteins of human serum very low density lipoprotein have been developed employing tetramethylurea, which delipidates the lipoprotein and selectively precipitates apolipoprotein B. Six soluble apoproteins are separated by electrophoresis in polyacrylamide gel. One of these is a previously unrecognized species of R-alanine ($R_4$-alanine), more anionic than the $R_3$-alanine polypeptide. Conditions of staining have been found which yield reproducibly linear chromogenic response with native lipoprotein and with each purified apoprotein. Recovery of protein in the seven species measured accounts for over 97% of the total in the very low density lipoprotein of normolipidemic individuals and in most samples from individuals with endogenous hyperlipemia.

The mean content of apolipoprotein B in 43 samples from normolipidemic subjects was 36.9(±1.2 SEM)% of total protein. The distribution of the major soluble apoproteins as mean (±SEM) percentage of the soluble fraction was: R-serine, 5.3±0.5; arginine-rich, 20.6± 1.0; R-glutamic, 10.6±0.4; $R_2$-alanine, 28.3±0.7; $R_3$-alanine, 26.9±0.5; and $R_4$-alanine, 8.0±0.5.

Distribution of the apoproteins was a function of particle diameter of very low density lipoprotein in fractions separated by gel permeation chromatography and by density gradient ultracentrifugation. In fractions below 700-800 Å, apolipoprotein B comprised an increasing percentage of the total protein with decreasing particle diameter. Among the soluble proteins the percentage of the arginine-rich and R-serine polypeptides increased and that of the R-glutamic polypeptide declined progressively with decreasing particle size. Apoprotein distribution was similar in fractions of similar particle size from normolipidemic and hyperlipemic subjects with the exception that all fractions from the hyperlipemic subjects contained more R-serine and some, more arginine rich polypeptide. Even in the absence of chylomicrons, the distribution of soluble apoproteins in particles of diameters greater than 700-800 Å was usually similar to that of the smallest particles. This suggests that the largest particles may include products of the partial catabolism of chylomicrons.

## INTRODUCTION

The protein moiety of very low density lipoproteins (VLDL)[1] of human serum contains several discrete polypeptides. One of the predominant species, apolipoprotein B (apoB), which appears to be identical with the major apoprotein of low density lipoprotein (LDL) (1), is insoluble in aqueous buffers after delipidation. Several water-soluble polypeptides denoted by their carboxyl termini as the "R-serine," "R-glutamic acid," and "R-alanine" species (2–4) and a major apoprotein, rich in arginine (5) have, together with a number of minor elements, been isolated by ion exchange chromatography. A physiological role, that of activation of lipoprotein lipase, has already been assigned to one of the apoprotein species of VLDL, the R-glutamic polypeptide (6).

Until this time, only immunochemical techniques have been available for quantitation of some of the soluble apoproteins in VLDL. These techniques are subject to the criticism that immunoreactivity may be incomplete in both native and partially delipidated lipoproteins. This criticism also applies to the immunochemical determination of apoB. Measurement of apoB content by gel permeation chromatography (3) is complicated by

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[1] *Abbreviations used in this paper:* apoB, apolipoprotein B; LDL, low density lipoprotein(s); TMU, 1,1,3,3-tetramethylurea; VLDL, very low density lipoprotein(s).

poor resolution of apoB and the arginine-rich polypeptide, and gravimetric measurement (7) is too laborious for examination of large numbers of samples.

A method for determination of the content of apoB in LDL and VLDL using the denaturing solvent 1,1,3,3-tetramethylurea (TMU) has been described (8). In this report we describe improved conditions for the determination of apoB and a practical technique for the quantitative determination of the major soluble apoproteins of VLDL. TMU rapidly and quantitatively delipidates serum lipoproteins (8) and provides the basis for determination of apoB content by selective precipitation. Under carefully controlled conditions the soluble apoproteins, after delipidation by TMU, migrate quantitatively within reproducible $R_f$ zones upon electrophoresis in polyacrylamide gels. Conditions of staining and destaining have been found which provide linearity of photometric response over a wide range for all the major soluble apoproteins. Determination of the absolute chromogenicity of the purified apoproteins permits quantitation of each in the electrophoretic gels. The distribution of the major apoproteins is presented for whole human serum VLDL from normolipidemic subjects and for fractions of VLDL of different particle diameters.

## METHODS

### Materials

Reagents were as described previously (8) except that TMU was redistilled monthly in glass and stored under nitrogen in the dark at 3°C. TMU which is suitably purified has a pH of 6.0–7.0 when diluted fivefold wih water. Amidoschwarz 10B, lot no. 0766421, was from the Allied Chemical Corp., Morristown, N. J.

VLDL ($d < 1.006$) and LDL ($1.024 < d < 1.050$) were prepared from serum obtained from subjects who had fasted for at least 14 h by repetitive ultracentrifugation as described previously (8). All lipoprotein solutions contained EDTA (1 mM) and sodium azide (0.2 mg/ml).

All normolipidemic donors were healthy students and hospital personnel between 20 and 60 yr of age, with serum levels of cholesterol and triglyceride below 250 and 140 mg/dl, respectively. To avoid preselection bias, blood was drawn for the preparation of whole VLDL and VLDL subfractions from putative normal donors without pretesting. Preparations from all subjects with serum lipid levels below the limits given above were then studied. Subjects with hyperlipemia were free of known causes of secondary hyperlipidemia or serious disease other than atherosclerotic vascular disease or gout. Serum was examined for chylomicrons after standing overnight at 3°C (9) and by electrophoresis in agarose gel (10). Samples containing detectible chylomicrons were excluded.

### Determination of the content of apoB in lipoproteins

#### Procedure

ApoB content is determined as the difference between the soluble protein content of a lipoprotein solution treated with 4.2 M TMU and an untreated sample. The protein content of the untreated sample is measured by the method of Lowry et al. (11). To minimize light scattering, nonpolar lipids are extracted in chloroform before colorimetry.

To delipidate the lipoprotein and precipitate apoB, the lipoprotein solution containing 1 mM EDTA is diluted so as to contain approximately 600 $\mu$g of protein per ml. The ionic strength must be $\geq 0.05$ and the pH between 6.0 and 9.0. The lipoprotein solution and TMU are individually brought to 37°C. 250 $\mu$l of the sample is placed in a $10 \times 75$-mm glass tube and 250 $\mu$l of TMU is added with thorough, immediate mixing by a mechanical vibrator. The mixture is held at 37°C for 30 min. The tubes are then centrifuged for $2 \times 10^4$ $g \cdot$min at room temperature. The lipid-apoB pellicle at the top of the tube is carefully lifted aside and 100-$\mu$l aliquots of the clear TMU-water phase are removed and mixed with 400 $\mu$l of 0.15 M NaCl for determination of soluble protein content by the Lowry technique, omitting the extraction with chloroform. For the determination of protein in the TMU-water solution, a second set of protein standards is prepared containing 10% TMU.

Because of its ready solubility, hydrated bovine albumin is used for working standards. Its water content is determined accurately by gravimetry and confirmed by Kjeldahl analysis. It is stored in sealed containers. To correct to absolute mass of apoB, a factor relating the chromogenicity of albumin to that of purified apoB, determined gravimetrically and corrected for the mass of the carbohydrate moiety (taken as 6.4%) (12–14), is used. The value of the factor (albumin equivalent $\times$ factor = mass of apoB) for anhydrous albumin is 1.00 (mean of four preparations). The factor for salt-free anhydrous protein of the whole TMU-soluble fraction of VLDL, determined by assuming 5.3% carbohydrate (15), is 1.16 (mean of three preparations). The three values obtained all fell within 1% of the mean, including one from a patient with primary dysbetalipoproteinemia.

#### Validation

The present method, modified from that published previously (8), was validated in three ways. First, the total amino acid composition of the TMU precipitate was compared with that of the TMU-insoluble apoprotein of LDL ($1.024 < d < 1.050$), which is assumed to be pure apoB, and of the whole protein moiety of VLDL. VLDL and LDL were delipidated at 37°C as above and the TMU-water phase was removed. The precipitate was dispersed with a glass rod, washed with 4.2 M TMU, and extracted overnight with 3:1 ethanol-diethyl ether. The ratio of ethanol to ether was then decreased to 2:3 to precipitate alcohol-soluble apoproteins (16). The precipitate was extracted again, washed with ether, dried, and hydrolyzed in 6 N HCl (17). Preliminary experiments showed that even trace amounts of TMU resulted in nearly total loss of tyrosine and partial loss of cysteine and methionine. With bovine albumin it was found that the addition of 1 mg of phenol to the hydrolysis tube gave quantitative recovery of tyrosine. The amino acid compositions of the whole apoprotein of VLDL obtained by precipitation with 3:1 ethanol: ether and those of the TMU precipitate from both LDL and VLDL were determined with a Beckman model 121-M amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (17).

Second, TMU-insoluble material from VLDL was prepared as above and extracted twice with the same organic solvents. The protein was solubilized with decyl sulfate and subjected to gel permeation chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (3).

Third, to determine the extent to which soluble apoproteins might become immured in the apoB-lipid precipitate, samples of VLDL, with protein contents varying from 1.0 to 1.5 mg/dl, were delipidated with TMU as in the technique for determination of apoB at 13, 23, 30, 37, and 45°C. The precipitates were washed with 4.2 M TMU, and the lipids were extracted with ethanol: ether as above. The protein was dispersed in 0.04 M Tris-glycine buffer, pH 8.91, containing 8 M urea, and the content of soluble apoproteins was determined by electrophoresis as described below.

## Quantitation of TMU-soluble apolipoproteins of VLDL

### Procedure

*Preparations.* VLDL solutions contain no more than 1.5 mg of protein per ml. Ionic strength must be at least 0.05 and the pH 6–9. 50–150 μg of total TMU-soluble protein is optimal for photodensitometry. Sample volumes should not be less than 50 μl because the dilution of TMU by buffer from the surface of the stacking gel will cause impaired delipidation. The maximum practical application volume is 400 μl.

*Gels.* Gel tubes are 6 × 135–150 mm. Running gels are 100–120 mm in length to permit longer migration and improved photometric resolution. Stacking gels are 10 mm in length.

*Delipidation.* Delipidation is carried out on top of the stacking gels, eliminating losses in transfer. VLDL samples are pipetted onto the gels and an equal volume of pure TMU is mixed rapidly with each sample immediately, with a Pasteur pipette. After delipidation, reducing solution and tracking dye are added to all specimens (8). $\frac{1}{10}$th vol of sucrose solution (80% wt/vol) is then added with mixing to increase the density of the TMU-water phase so that the upper tank buffer can be layered over it.

*Electrophoresis.* Upper tank buffer, pH 8.91, is carefully layered in the gel tubes over the mixture on the top of the stacking gels (8). The upper buffer tank is filled so as to avoid disturbing the layers in the gel tubes, and a constant current of 1.25 mA per gel is applied immediately. When the tracking dye has entered the stacking gel, the upper buffer is removed and replaced by upper tank buffer which has been titrated to pH 9.7,[2] and the current is increased to 2.5 mA per gel. Electrophoresis is stopped 30 min after the tracking bands have emerged from the bottoms of the running gels.

*Staining.* The precipitated apoB and lipid are carefully washed off the top of the gels with a jet of water from a needle. The gels are removed with a stream of water from a 10-cm, 18 gauge needle with a flat tip. The needle is advanced from the bottoms of the gels in order to avoid transferring any remaining apoB from the surface of the stacking gel onto the running gel. The gels are stained in a solution of 1% Amidoschwarz 10B dye (wt/vol) in 7% acetic acid (vol/vol) for 18 h in 16 × 150-mm tubes. They are then destained in a circulating destainer (Hoefer Scientific Instruments, San Francisco, Calif.) without the charcoal filter with 7% acetic acid solution which is changed after 5 h and again after an additional 18 h. The gels are then held in an equilibration solution of 7% acetic acid containing 0.5 mg/liter of Amidoschwarz dye in the destainer for 24 h. Staining and destaining are carried out at 23±2°C. Gels are stored in the equilibration solution protected from light. Densitometric scanning is performed with an integrating densitometer (model 445, Clifford Instruments, Inc., Natick, Mass.) using a beam width of 3 mm. The instrument is calibrated with a neutral density incremental filter.

[2] The increased pH is necessary to increase the mobility of the R-serine polypeptide so that it separates from the stacking gel for precise densitometry.

### Determination of absolute chromogenicities and ranges of linearity

Authentic polypeptide standards were prepared from VLDL separated from the serum of subjects with endogenous hyperlipemia (2–4, 8, 18). Identity and purity of apoprotein standards was established by electrophoresis in the polyacrylamide gel system and by amino acid analysis. The purified apoproteins were quantitated as aminoacyl mass from the amino acid analysis after correction for tryptophan content using published values (5).

Absolute chromogenicities were determined for at least three preparations of each major polypeptide species by using multiple points in the range of linear densitometric response. Ranges of linearity were established by analyses of both purified polypeptides and native lipoproteins in the gel system in dilution series of 20-fold or greater.

The identity of proteins in the bands separated from VLDL in the TMU-polyacrylamide gel system described above was determined in representative gels by analysis of the amino acid content of excised bands by the method of Houston (19). When this technique was applied, α-aminobutyric acid was used in place of glycine in the electrophoresis buffer (the $pK_a$ of the amino group of α-aminobutyric acid is identical to that of glycine, and it can be resolved from glycine in amino acid analysis).

An additional protein band preceding $R_3$-alanine by the same interval that separates the $R_3$-alanine and $R_2$-alanine bands is resolved by electrophoresis after delipidation with TMU. To measure the contribution of sialic acid to its charge, preparations rich in this band were incubated with *Vibrio cholerae* neuraminidase (20) (Calbiochem, San Diego, Calif.). VLDL containing 1 mg of soluble apoprotein in 0.2 N sodium acetate buffer, pH 6.5, containing 0.004 M $CaCl_2$ and 5 μg/ml chloramphenicol was incubated at 37°C with 20 U of the enzyme. Duplicate samples were removed for gel electrophoresis after 3, 6, and 24 h.

## Fractionation of VLDL on the basis of particle size

### Gel permeation chromatography

VLDL were applied to a column of 2% agarose beads (21) and eluted in 0.2 N NaCl containing 1 mM EDTA and 0.02% sodium azide at pH 7.0. After passage of the void volume, each succeeding group of five tubes was pooled. Five such pools of 41 ml were each concentrated in an ultrafiltration cell (UM 2 or 10 membrane, Amicon Corp., Lexington, Mass.) for delipidation and electrophoresis.

### Density gradient ultracentrifugation

VLDL were subjected to preliminary ultracentrifugation in a 40.3 rotor at 20,000 rpm and 12°C in a Beckman model 2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div.) for 15 min to remove any floating denatured or aggregated material or chylomicrons. After removal of the top 0.5 ml, the density of the VLDL solution was increased to 1.065 and it was placed in $\frac{9}{16}$ × 3½-inch thick-walled cellulose nitrate tubes (Beckman Instruments, Inc., Spinco Div.). The gradient was layered as described by

Lindgren et al. (22). The tubes were centrifuged at 35,000 rpm in a Beckman SW 41 rotor. Samples of 1 ml were removed from the top of the gradient after 90 min (fraction I) and 210 min (fraction II). A final sample of 2.0 ml, fraction III, was removed after an additional 10 h of ultracentrifugation.

Particle size was determined by electron microscopy. VLDL fractions were negatively stained with 2% potassium phosphotungstate (23) and photographed at 20,000 × magnification. Diameters of 200 particles from each fraction were measured on the negatives by using an optical microcomparator (Nikon model 6C, Nippon Kogaku, Tokyo, Japan). Lipid composition of the whole VLDL and VLDL fractions was determined as described previously (21). The distribution of each apoprotein species as a function of particle size was analyzed by the Kruskal-Wallis rank-sum test (24).

## RESULTS

### Determination of apoB by precipitation with TMU: purity of the precipitate

98% of the total protein of LDL was insoluble in TMU (SEM = 0.34, $n = 5$). Polypeptides with mobilities identical to those of the R-serine, R-glutamic, and R-alanine species in gel electrophoresis as described above account for virtually all the remaining protein. By contrast, the protein of all density fractions of high density lipoproteins is essentially completely recovered in the TMU-water phase.

The amino acid compositions of the TMU-insoluble fractions of LDL and VLDL are shown in Table I, together with the composition of whole apo-VLDL. Because of variable recovery of cysteine and methionine in the presence of traces of TMU, these amino acids have been omitted from the comparison. The composition of the protein precipitated by TMU from VLDL resembles very closely that of the TMU precipitate from LDL prepared between densities 1.024 and 1.050 (which is assumed to be virtually pure apoB).

ApoB and the non-B protein of VLDL differ most in their content of histidine, glutamic acid, proline, alanine, isoleucine, and tyrosine. Contamination of the precipitate with non-B proteins would therefore cause the content of at least some of these amino acids to differ from apoB. The difference in the content of arginine of the two preparations is such that contamination of B protein by arginine-rich apoprotein in the precipitate cannot exceed 1.5%.

Completeness of recovery of the soluble apoproteins in 4.2 M TMU appears to be thermally dependent. Values for the whole TMU-soluble protein fraction are maximal in the region of 37°C and are 1-2% higher than those obtained at 23°C. This difference is wholly attributable to retention of arginine-rich polypeptide in the lipid-apoB pellicle at lower temperatures. The mean content of arginine-rich polypeptide in the pellicles from two VLDL preparations delipidated at 13°C was 29% of that present in the original lipoprotein sample, whereas only 2.8% remained insoluble at 23°C and only 0.3% at 30°C and above.

TABLE I
*Mean Total Amino Acid Composition*

| | TMU precipitate | | Whole apo-VLDL‡ |
|---|---|---|---|
| | LDL* | VLDL* | |
| | *mol/10² mol* | | *mol/10²mol* |
| Lys | 82.5±1.0 | 81.7±0.6 | 80.6 |
| His | 24.3±0.4 | 23.7±0.4 | 12.9 |
| Arg | 33.0±0.5 | 34.3±1.1 | 37.3 |
| Asp | 102.0±1.2 | 102.0±1.0 | 105.3 |
| Thr | 70.6±1.1 | 67.5±0.5 | 70.5 |
| Ser | 99.9±1.9 | 97.2±2.0 | 101.8 |
| Glu | 130.2±0.9 | 133.0±1.4 | 150.4 |
| Pro | 39.2±0.1 | 38.8±0.2 | 50.1 |
| Gly | 50.2±0.2 | 52.6±0.5 | 47.6 |
| Ala | 59.5±0.8 | 62.8±0.3 | 87.8 |
| Val | 53.1±0.7 | 55.2±1.2 | 55.3 |
| Ile | 54.0±0.3 | 52.6±0.7 | 31.2 |
| Leu | 116.1±0.7 | 115.2±1.4 | 108.4 |
| Tyr | 34.6±0.6 | 34.3±0.2 | 19.9 |
| Phe | 50.9±0.4 | 49.1±0.5 | 42.7 |

* Mean of duplicate analyses on each of two hydrolysates (±SEM).
‡ Mean of triplicate analyses on a single hydrolysate.

Gel chromatography of the TMU-insoluble fraction from VLDL yielded a single peak of protein at the void volume corresponding to the behavior of apoB from LDL. Like apoB from LDL this fraction retained immunoprecipitability by anti-LDL serum despite the presence of decyl sulfate.

### Quantitation of soluble apoproteins

*Resolution and identification of the major apolipoproteins.* In Fig. 1 the gel electrophoretogram of a typical preparation of normal serum VLDL is compared with electrophoretograms of pure samples of the major soluble apoproteins. The amino acid composition of the protein in the major band near the top of the running gel determined by the method of Houston (19) agrees well with that of pure R-serine. Fainter bands of additional incompletely characterized apoprotein species often precede and follow the R-serine band. With the R2-alanine peak as a reference, pure R-serine is restricted to the $R_f$ zone of 0.08 to 0.21 with the buffer systems described above; hence, integration for R-serine area is carried out between these limits. Below R-serine the next major band, identified by its amino acid composition, is the arginine-rich polypeptide. When present, serum albumin and apoprotein A1 precede it. A second ultracentrifugation of VLDL eliminates the former. The latter

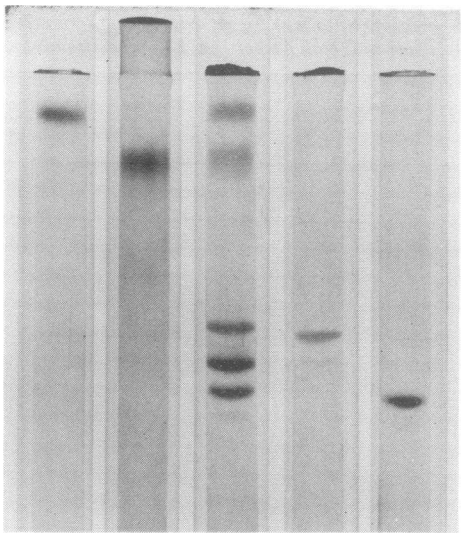

FIGURE 1 Polyacrylamide gel electrophoresis of pure VLDL apoproteins and whole apo-VLDL. Left to right: R-serine, arginine-rich polypeptides, whole apo-VLDL, R-glutamic, and $R_3$-alanine polypeptides. In the center gel (whole apoVLDL) the five major bands are, from top to bottom: R-serine, arginine-rich, R-glutamic, $R_2$-alanine, and $R_3$-alanine polypeptides.

is seldom observed in amounts exceeding a fraction of 1% of soluble apoprotein in VLDL uncontaminated by chylomicrons. The $R_f$ zone for pure arginine-rich polypeptide, with the $R_2$-alanine band as reference, is 0.30–0.43. Below the arginine-rich polypeptide is the R-glutamic polypeptide (the third major band from the top in the third gel, Fig. 1), and below it are the R-alanine species. The amino acid composition of a band below the $R_3$-alanine band agrees well with those of the R-alanine species. It is hereafter referred to as "$R_4$-alanine." (After incubation of VLDL with neuraminidase this band and the $R_3$-alanine band disappear entirely with the appearance of a large band just below the usual position of the R-glutamic polypeptide. However, a minor band with the mobility of $R_2$-alanine persists.) Two additional bands of faint intensity are sometimes encountered between the R-glutamic and $R_2$-alanine bands and one just above the R-glutamic band. Often a faint band occurs just below the $R_4$-alanine component as well. Sufficient protein for amino acid analysis has not been obtained from these minor bands. A typical densitometric scan of the apoproteins of VLDL is shown in Fig. 2.

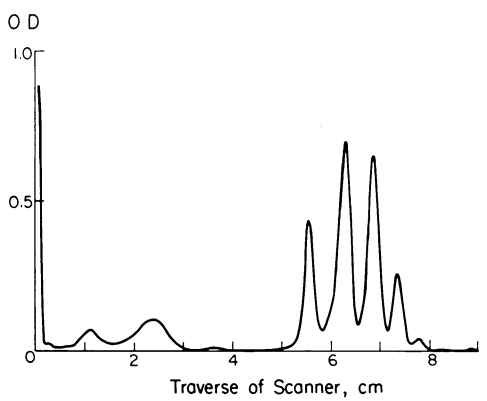


FIGURE 2 Photodensitometric scan of gel electrophoretogram, whole apo-VLDL from a normolipidemic subject. Ordinate: OD at 600 nm. Top of gel is at left, abscissa is the distance from the top of the running gel in centimeters. The major peaks, from left to right, are the R-serine, arginine-rich, R-glutamic acid, and $R_2$-, $R_3$-, and $R_4$-alanine species. Densitometric areas for the R-serine and arginine-rich species are delineated by the $R_f$ zones 0.08–0.21 and 0.30–0.43, respectively. Areas of the more anionic species are delineated at the minima between the peaks.

*Chromogenicity and linearity.* Absolute chromogenicities with Amidoschwarz dye differ significantly among the major apoproteins as shown in Table II. However, there are no significant differences among the R-alanine subspecies. Delipidation of increasing amounts of VLDL with TMU in the gel system yields linear recovery of R-serine, R-glutamic acid, and R-alanine species over a wide range of concentration (Fig. 3). A linear response is always observed with the R-alanine species over a 40-fold range or greater, from approximately 10 $\mu$g to over 400 $\mu$g. The R-glutamic polypeptide response is linear between 5 and 75 $\mu$g and the R-serine polypeptide between approximately 4 and 30 $\mu$g when VLDL is delipidated in the gel system. The upper limits for the latter two polypeptides reflect the maximal amounts that can be applied in whole VLDL because they comprise such a small fraction of the soluble apoprotein.

Because so little R-serine is usually present in VLDL, it is necessary to apply 200 $\mu$g or more of soluble protein to an additional gel in order to achieve a precision comparable to that obtained with the other soluble apoproteins. Pure delipidated arginine-rich polypeptide gives a linear photometric response over the range of 4–150

TABLE II

*Absolute Chromogenicities of the Principal Soluble Apoproteins of VLDL Stained in Polyacrylamide Gels with Amidoschwarz 10B*

| Apoprotein | Number of preparations | Chromogenicity |
|---|---|---|
| | | *OD·cm* $\times 10^{-5}/\mu g$ *protein* |
| R-Serine | 4 | 1.36±0.11* |
| Arginine-rich | 3 | 1.06±0.02 |
| R-Glutamic acid | 3 | 1.59±0.10 |
| R-Alanine (all) | 4 | 1.09±0.06 |

* ±SEM.

μg. However, when the total apoprotein content of the native lipoprotein delipidated in the gel system exceeds 350 μg, recovery of this protein begins to decrease and is 9% low at twice that level. Quantitative determination of this apoprotein is thus routinely carried out on samples of VLDL containing less than 350 μg of total protein. The photometric response at such levels is linear as shown in Fig. 3. Optimal quantitation of a preparation of VLDL with a high content of the arginine-rich polypeptide and poor in R-serine thus requires analysis at two levels of protein load.

*Replication.* Analysis of the percentage composition of the soluble apoproteins of two preparations of VLDL, determined in quadruplicate in four successive runs, gave the following average within-run coefficients of variation: $R_2$-alanine, 4.5%; $R_3$-alanine, 3.7%; $R_4$-alanine, 8.0%; R-glutamic acid, 8.2%; arginine-rich polypeptide, 8.1%; and R-serine, 8.0% (200 μg soluble protein loaded on gels). Within-run and between-run errors were partitioned out by analysis of variance. Between-run error was smaller in all cases except for $R_2$-alanine, in which it equalled within-run error. Absolute band absorbancies determined by rescanning gels after storage for 1 yr in the dark in the equilibration dye solution at 23°C were virtually identical with those of the original scans. With the exception of the partial conversion of $R_3$- and $R_4$-alanine to slowly migrating R-alanine species at 3°C (see below) no significant differences were found in recovery of the apoprotein species between samples frozen at −20°C and those held at 3°C for 3 days. Also, the gel patterns were unchanged by storage of VLDL at −20°C for at least 6 mo. No differences were observed in the distribution of apoproteins in VLDL from serum and from plasma (prepared with 6 mM EDTA) of individual donors, including that of a patient with primary dysbetalipoproteinemia whose VLDL contained approximately four times the normal amount of the arignine-rich apoprotein.

*Recovery.* The sum of the aminoacyl mass of the soluble apoproteins recovered in the six major bands in the gel system was compared in 20 consecutive preparations of VLDL from normolipidemic and hyperlipemic subjects with the TMU-soluble protein fraction determined directly on the VLDL. Mean recovery was 95.3% (±1.3 SEM). No soluble apoproteins were detected when the protein-lipid residue, extracted with ethanol: ether and dispersed in 8 M urea, was subjected to electrophoresis in the gel system without TMU.

## Apoprotein distribution in normal VLDL

Because chromogenicity factors are not available for most of the minor apoprotein elements and because the protein recovered in the six major bands accounts for over 95% of the total TMU-soluble apoprotein mass, the amount of each individual protein species is expressed as a percentage of the total of the aminoacyl

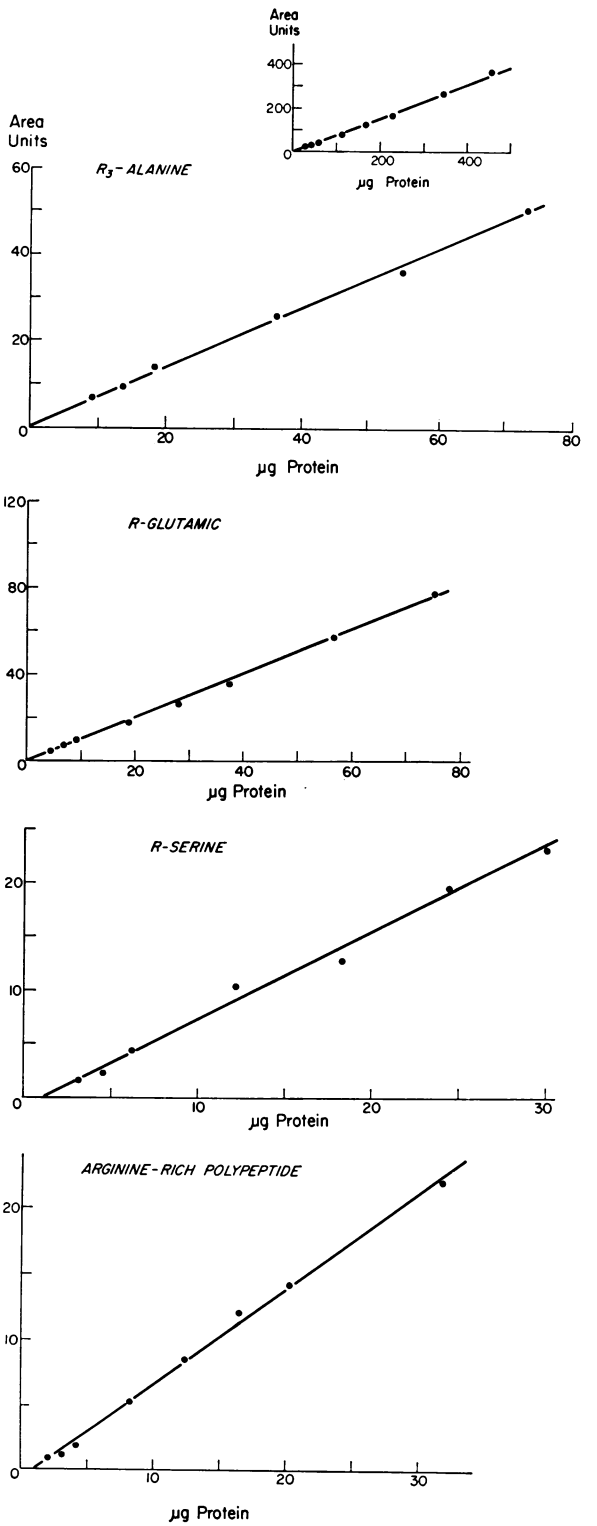


FIGURE 3 Densitometric response (peak areas) of individual apoproteins when increasing amounts of VLDL are delipidated with TMU and subjected to electrophoresis in the gel system. The greatest area obtained with the arginine-rich polypeptide corresponds to a load of apo-VLDL of 350 μg.

mass in the six bands. This mode of expression facilitates comparison of the distribution of these apoproteins when the percent of total apoprotein that is TMU soluble changes. The mean distribution of these apoproteins in the soluble protein moiety and the percentage of apoprotein mass represented by apoB in VLDL from 43 normolipidemic fasting subjects (20 male, 23 female) are shown in Table III. Particle size distribution was examined by electron microscopy in 12 of these subjects. At least 98% of the particles were between 250 and 500 Å in diameter for each subject. The average mean and median diameters were 364 Å ±7 SEM and 343 Å ±8 SEM, respectively. The portion of total apoprotein represented by apoB in VLDL from normal individuals is relatively closely distributed about a mean of approximately 37%. VLDL from hyperlipemic individuals (even in the absence of chylomicronemia) often contain considerably less apoB relative to soluble protein and a percentage of polar constituents (21) consistent with a larger mean particle diameter. The R-alanine species together comprise over 60% of the soluble protein mass or about 40% of the total protein moiety. Freshly prepared VLDL may have a slight predominance of either the $R_2$-alanine or $R_3$-alanine apoprotein. With storage at 3°C the R-alanine subspecies of greater mobility are progressively lost with a concomitant increase in $R_2$-alanine. While the content of $R_4$-alanine decreases visibly within a day or two, the decrease in the area of $R_3$-alanine proceeds somewhat more slowly. This change in mobility, presumably reflecting spontaneous desialation, is not observed in VLDL frozen at −20°C. The R-glutamic and arginine-rich polypeptides comprise about 11 and 21% of the TMU-soluble protein and 7 and 13% of the total protein moiety, respectively. R-Serine is the most variable in its representation. It never exceeds 10% of the TMU-soluble protein and is almost undetectible in some preparations of normal VLDL.

## Distribution of apoproteins in VLDL of different mean particle diameters

A representative example of particle diameters of fractions of VLDL from a hyperlipemic donor, separated by gel permeation chromatography in agarose gel, is presented in Fig. 4. Electron photomicrographs of the preparations are shown on the right and particle size frequency distributions on the left. VLDL prepared from all normolipidemic subjects emerged mainly in the last two fractions. In several of them there was insufficient material for analysis in the first fraction or two even though the VLDL originally present in a unit of blood were applied to the column. Among the five hyperlipemic subjects two distinct patterns of distribution were observed. Two subjects had relatively small amounts of lipoprotein in the first two fractions whereas the other three had abundant material in the region of the void volume. However, the particle sizes of the VLDL appearing in those fractions did not differ significantly from those found in the corresponding fractions of the other two hyperlipemic subjects.

The distribution of the apolipoproteins in fractions obtained by gel permeation chromatography of VLDL from the five hyperlipemic subjects is presented in Table IV. Content of apoB increased uniformly and progressively from 13.5% to 44.0% of total protein with decreasing particle size from the void volume fraction (fraction 1) through to the smallest particles (fraction 5). The percentage of the R-glutamic polypeptide fell progressively in all subjects between fraction 2 and fraction 5 (11.2 to 6.8%), but the mean percentage was lower in fraction 1 than in fraction 2.

The percentage of the arginine-rich apoprotein also increased with decreasing particle diameter (fraction 2 to fraction 5) but it was appreciably higher in the void volume fraction (fraction 1) than in fraction 2. Of the four samples of the void volume (fraction 1) which were sufficient for analysis, three contained a higher percentage of this apoprotein than that found in the succeeding fraction. These were from the three subjects whose VLDL yielded a large amount of material in the void volume fraction.

The distribution of apoproteins in fractions of VLDL from seven normal donors separated on the same agarose column as the VLDL from the hyperlipemic subjects is shown in Table V. The mean percentage of apoB in-

TABLE III

*Apoprotein Distribution in Normal VLDL (43 Subjects)**

| | Percent of soluble protein‡ | | | | | | |
|---|---|---|---|---|---|---|---|
| | R-Ser | Arginine-rich | R-Glu | $R_2$-Ala | $R_3$-Ala | $R_4$-Ala | ApoB |
| | | | | | | | *% of total protein* |
| Mean±SEM | 5.3±0.5 | 20.6±1.0 | 10.6±0.4 | 28.3±0.7 | 26.9±0.5 | 8.0±0.5 | 36.9±1.2 |

* Duplicate or triplicate analyses were made on each preparation.

‡ Total soluble protein is taken as the sum of mass of the six soluble apoprotein species shown.

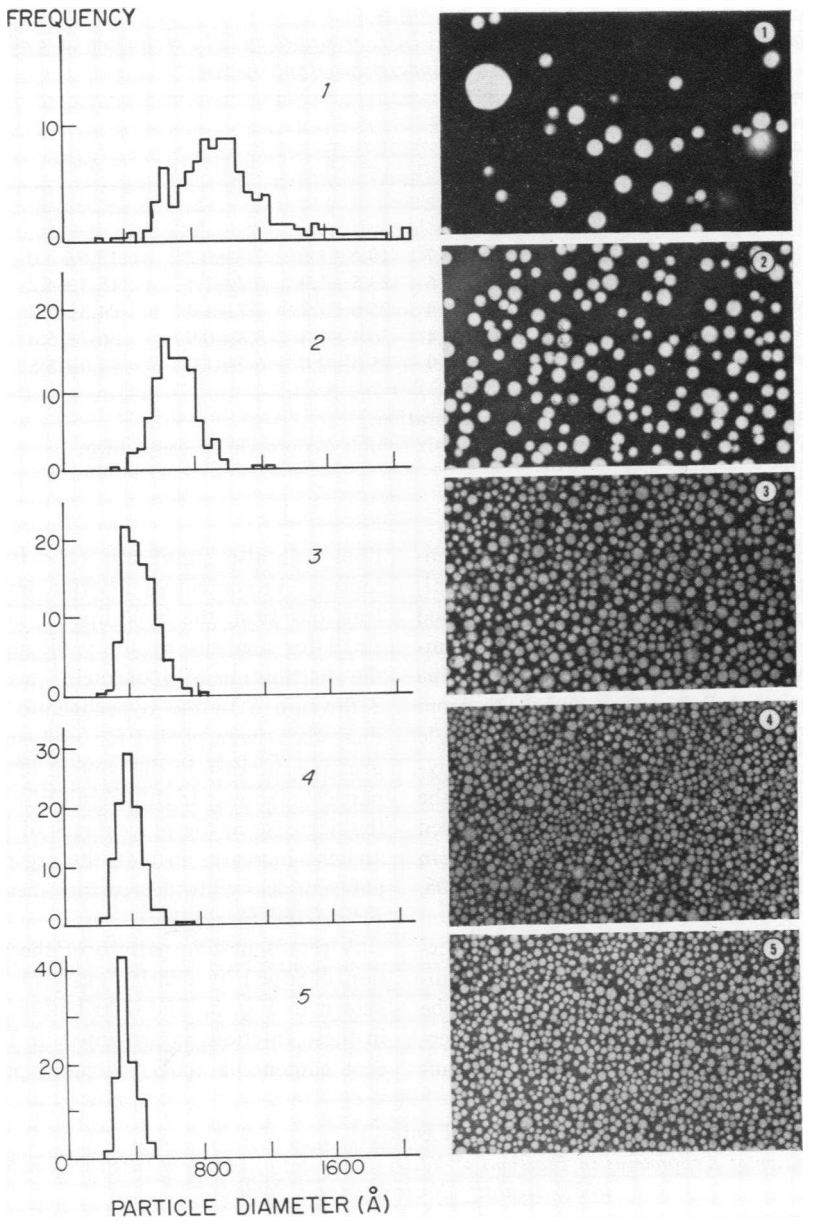


FIGURE 4 Distribution of particle diameters in fractions of a representative sample of VLDL separated by gel permeation chromatography in 2% agarose. First fraction (void volume fraction) is at top followed below by successive fractions denoted by arabic numerals. Left: frequency distribution of particles measured by electron microscopy. Right: corresponding electron photomicrographs of the preparations, negatively stained with 2% potassium phosphotungstate (× 20,000).

creased steadily with increasing elution volume. The higher percentage of apoB found especially in the last two fractions of the normal VLDL compared to those from the lipemic subjects may be attributable to a preponderance of smaller particles. In no fraction was the difference in percentage of apoB between normal and hyperlipemic VLDL significant by *t* test. The mean percentage of the R-glutamic polypeptide declined with decreasing particle diameter (between fraction 2 and fraction 4) in all subjects although absolute percentages varied substantially. In no fraction was the difference in content of this apoprotein between normal and hyperlipemic VLDL significant. The percentage of arginine-rich apoprotein in fractions 2, 3, and 4 of VLDL from

TABLE IV

*Distribution of Apoproteins in Fractions of VLDL Separated by Gel Permeation Chromatography in 2% Agarose (Endogenous Hyperlipemia)**

| Fraction | Percent of soluble protein‡ | | | | | | | Particle diameter | |
|---|---|---|---|---|---|---|---|---|---|
| | R-Ser | Arg-rich | R-Glu | R2-Ala | R3-Ala | R4-Ala | ApoB | Median | Mean |
| | | | | | | | *% of total protein* | *Å* | |
| 1 (4)§ | 10.1±1.1‖ | 26.7±3.2 | 8.8±1.8 | 27.0±2.7 | 19.6±1.0 | 7.3±0.2 | 13.5±3.0 | 899±34 | 917±39 |
| 2 (5) | 9.6±1.3 | 17.4±1.1 | 11.2±2.0 | 31.6±1.6 | 23.5±1.9 | 6.7±1.4 | 15.3±4.2 | 682±31 | 684±32 |
| 3 (5) | 8.4±1.5 | 21.3±2.3 | 8.8±1.7 | 28.8±1.4 | 25.0±1.7 | 7.7±1.4 | 24.8±3.3 | 523±23 | 538±25 |
| 4 (5) | 9.5±1.8 | 20.2±3.5 | 7.4±1.5 | 29.1±2.4 | 26.4±1.3 | 8.3±0.9 | 36.7±5.0 | 423±24 | 437±25 |
| 5 (5) | 10.4±1.5 | 22.0±2.4 | 6.8±1.2 | 29.0±1.9 | 25.6±2.0 | 6.2±1.0 | 44.0±5.5 | 360±17 | 367±31 |

* Duplicate or triplicate analyses were made on each preparation.
‡ Total soluble protein is taken as the sum of mass of the six soluble apoprotein species shown.
§ Number of individuals.
‖ ±SEM.

the hyperlipemic subjects exceeded that of the normolipidemic subjects ($t$ test: $P < 0.1$, $< 0.005$, and $< 0.1$, respectively). The mean content of R-serine increased from about 2.5% to around 6% with decreasing diameter in the normal VLDL. The percentage of R-serine was appreciably higher in all fractions of VLDL from the hyperlipemic subjects. With the exception of a single fraction in one subject, all the fractions from each hyperlipemic subject contained more R-serine than the mean of normals for that fraction. $t$ test analysis yielded the following $P$ values for the difference of R-serine in fractions 2 through 5, respectively: $P < 0.005$, $< 0.005$, $< 0.2$, and $< 0.05$.

Rank-sum analysis showed the increases in the percentage of apoB in the total protein in gel chromatographic fractions 2 through 5 for normal VLDL to be highly significant ($P < 0.005$). The decline in the percent content of the R-glutamic species in the same fractions was also significant ($P < 0.05$), but that for the increase in content of R-serine was not. The sample of fractions from the hyperlipemic subjects alone was too small for analysis by the rank-sum technique. Because the distribution of apoproteins was similar in fractions 2 through 5 in the hyperlipemic and normal subjects, these data were pooled for rank-sum analysis. The progressive increase in the content of apoB with decreasing particle size was highly significant ($P < 0.005$) as were the decrease in the percentages of the R-glutamic species and the increases in both the arginine-rich and R-serine polypeptides with decreasing mean particle diameters ($P < 0.005$ for all three).

A representative pattern of the particle size distribution obtained by density gradient ultracentrifugation is shown in Fig. 5. The distribution of apoprotein species in these fractions is presented in Table VI. Again, percent content of apoB increased progressively with de-

TABLE V

*Distribution of Apoproteins in Fractions of VLDL Separated by Gel Permeation Chromatography in 2% Agarose (Normolipidemic Subjects)**

| Fraction | Percent of soluble protein‡ | | | | | | |
|---|---|---|---|---|---|---|---|
| | R-Ser | Arg-rich | R-Glu | R2-Ala | R3-Ala | R4-Ala | ApoB |
| | | | | | | | *% of total protein* |
| 1 (2)§ | 2.5 | 20.0 | 15.2 | 30.5 | 24.3 | 7.3 | 21.2 |
| 2 (5) | 2.4±1.0‖ | 11.9±2.5 | 15.0±1.3 | 36.0±3.9 | 28.1±2.9 | 6.4±1.2 | 21.9±2.8 |
| 3 (7) | 4.3±1.1 | 10.8±1.8 | 10.9±0.6 | 39.0±2.9 | 28.1±1.9 | 6.8±1.0 | 32.4±4.0 |
| 4 (7) | 6.6±0.7 | 11.5±2.6 | 9.9±0.8 | 38.1±2.9 | 27.7±2.0 | 6.5±0.7 | 53.9±6.0 |
| 5 (6) | 5.6±1.3 | 18.5±4.5 | 10.1±1.3 | 34.5±2.5 | 26.4±3.1 | 4.8±0.8 | 59.9±5.1 |

* Duplicate analyses.
‡ Total soluble protein is taken as the sum of mass of the six soluble apoprotein species shown.
§ Number of samples.
‖ ±SEM.

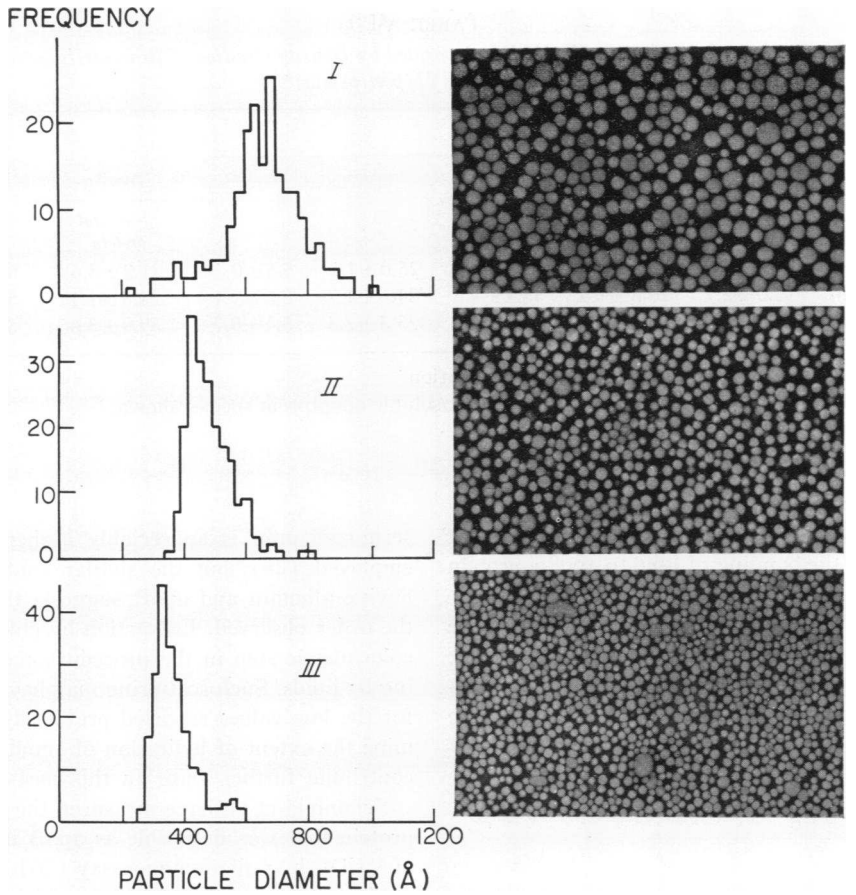


FIGURE 5 Distribution of particle diameters in fractions of VLDL separated by density gradient ultracentrifugation. Top: fraction I, initial supernatant fraction (90 min of centrifugation at 35,000 rpm). Middle: fraction II, second supernatant fraction (210 min). Bottom: fraction III, final supernatant fraction (10 h). Left: frequency distributions of particle diameters. Right: corresponding electron photomicrographs of preparations negatively stained with 2% potassium phosphotungstate (× 20,000).

creasing particle diameter (fraction I to fraction III). The percentage of soluble protein represented by the R-glutamic polypeptide fell and that of the arginine-rich apoprotein in the soluble protein fraction rose in stepwise fashion with decreasing particle size. Both the mean particle size and the apoprotein composition of the largest fraction obtained by density gradient ultracentrifugation suggest that the larger particles which eluted in the void volume fraction on gel permeation chromatography were excluded by the preliminary ultracentrifugation before the gradient separation was carried out.

The relative changes in the ratio of each of the four major TMU-soluble apoproteins to apoB in a representative preparation of VLDL from a hyperlipemic subject in fractions varying in mean particle diameter from 671 to 340 Å is shown in Fig. 6.

The content of all the soluble apoproteins relative to apoB fell with decreasing particle diameter, with the R-glutamic polypeptide falling most rapidly. However, content of the arginine-rich apoprotein fell at an appreciably different rate. The increase in the relative content of R-serine in the smallest particle fraction over that in the next larger particle size fraction as shown in this figure was observed in three of five samples from hyperlipemic subjects.

## DISCUSSION

The methods presented here permit the quantitative determination of five major apoproteins in VLDL of human serum that account for slightly more than 97% of the total apoprotein of normal VLDL and of most specimens of VLDL from subjects with endogenous hyperlipemia. Under the modified conditions described above, 4.2 M TMU is a selective precipitant for apoB, providing a facile method for its determination in fractions of VLDL or LDL. The requirement for ions in delipida-

TABLE VI

*Distribution of Apoproteins in VLDL Separated by Density Gradient Ultracentrifugation (Endogenous Hyperlipemia)**

| Fraction | Percent of soluble protein‡ | | | | | | Particle diameter | |
|---|---|---|---|---|---|---|---|---|
| | R-Ser | Arg-rich | R-Glu | $R_{E}$-Ala | $R_{3}$-Ala | $R_{4}$-Ala | ApoB | Median | Mean |
| | | | | | | | *% of total protein* | | |
| I (4) | 7.9±1.5‖ | 10.4±2.6 | 12.6±1.9 | 38.5±2.1 | 25.0±1.3 | 5.5±0.3 | 21.2±3.6 | 661±86 | 663±85 |
| II (4) | 8.8±1.6 | 12.0±2.7 | 10.8±1.4 | 38.1±4.3 | 24.6±1.7 | 5.6±1.1 | 24.8±1.1 | 525±41 | 539±41 |
| III (4) | 8.4±0.6 | 20.5±4.3 | 8.2±1.5 | 34.2±4.3 | 23.3±2.3 | 5.4±0.7 | 39.9±3.6 | 392±27 | 400±24 |

* Duplicate or triplicate analyses were made on each preparation.
‡ Total soluble protein is taken as the sum of mass of the six soluble apoprotein species shown.
§ Number of samples.
‖ ±SEM.

tion by this solvent is consistent with the hypothesis that a significant part of the bonding of lipid to apolipoprotein involves ionic interaction.

The precision of this technique is equal to that of the Lowry method if redistilled TMU is employed and the temperature of delipidation is controlled. The factor determined for chromogenicity of apoB relative to bovine serum albumin is appreciably higher than others have employed (25), but the similar content of tyrosine in bovine albumin and apoB suggests that it should be of the order observed. Extraction by chloroform before the colorimetric step in the procedure reduces light scattering by lipids. Such scattering may have accounted in part for the low values reported previously. Failure to determine the extent of hydration of standard albumin would contribute further error in this measurement.

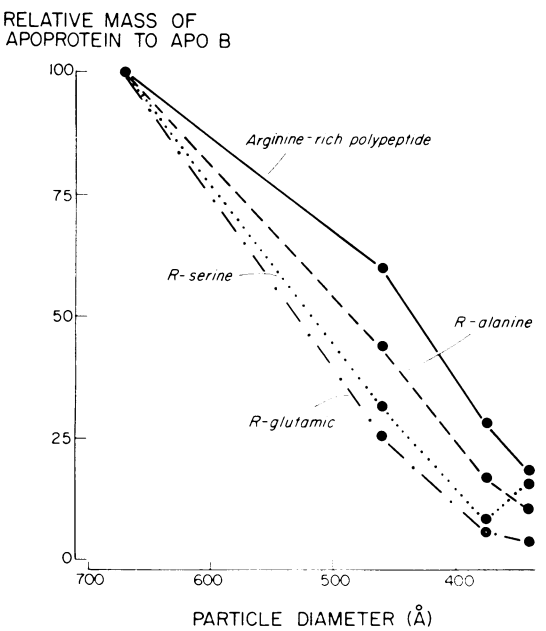


FIGURE 6 Ratio of four apoproteins relative to apoB as a function of particle size. For comparability, the ratio of each apoprotein to apoB in each particle fraction is expressed as a percentage of the ratio of that individual apoprotein to apoB in the largest particles (mean diameter, 671 Å) taken as 100 (ordinate). The abscissa gives the mean particle diameter of the fraction. Each point is the mean of duplicate determinations.

Schonfeld et al. have measured the percentage of total protein that was detectible as apoB in five preparations of VLDL by radioimmunoassay (26). They found 32.8% apoB with a range of 29–42%, which agrees rather well with the mean of 36.9% found in the 43 normal samples of this study. On the other hand Lee and Alaupović found apoB to comprise 55% of total protein in six specimens of VLDL by a technique of gravimetry and nitrogen determination (7). Correction for protein-bound carbohydrate yields a mean of 51.5%, still appreciably higher than found by the TMU and radioimmunoassay techniques.

Determination of the major TMU-soluble apoproteins by quantitative densitometric scanning of polyacrylamide gels is likewise a reproducible technique when delipidation, staining, and destaining are strictly controlled. The staining period of 18 h followed by destaining in the presence of an equilibrating stain solution within a relatively narrow range of temperature is required for consistent chromogenicity of the protein bands. Because of variation in scanner geometry and in stains, standardization with purified apoproteins or with VLDL of known composition is required. The relative migration of the bands is influenced by buffer pH and by gel concentration so that $R_f$ zones for authentic R-serine and arginine-rich apoprotein must be determined under the precise conditions employed. Some specimens of VLDL from hyperlipemic individuals contain bands of unusual elec-

trophoretic mobility. These bands can be identified by coelectrophoresis with purified apoproteins in electrophoretic systems of different pH or by excision of the bands followed by determination of the amino acid composition by the technique of Houston (19). This combination of techniques holds promise for the identification of polymorphism among the apolipoproteins.

The quantitative measurement of apoprotein composition in this study establishes certain general characteristics of whole normal VLDL. With the exception of the R-serine species all specimens contain all the major VLDL apoproteins. ApoB and the R-alanine species comprise nearly 80% of the total protein mass. Although R-serine was one of the first soluble apoproteins to be purified (3), it is a relatively minor component of normal VLDL. By contrast, the arginine-rich apoprotein emerges as a major component of the soluble protein fraction.

The $R_4$-alanine species resolved by TMU delipidation represents a previously unrecognized entity. Its electrophoretic mobility is compatible with an increment of one formal unit of charge over that of $R_3$-alanine. Whether the minor band with electrophoretic mobility characteristic of $R_2$-alanine which remains after VLDL or pure $R_3$-alanine has been subjected to hydrolysis by neuraminidase is due to incomplete desialation or instead reflects an additional variation in charge such as a desamido species is indeterminate. Quantitative measurement of the sialic acid content of purified $R_4$-alanine will be required to establish the basis of its increased mobility. The $R_4$-alanine apolipoprotein has been found in virtually all specimens of freshly prepared normal VLDL. Its absence in previous studies of VLDL apoproteins is probably due to the rate of its spontaneous disappearance relative to the time required for delipidation by techniques previously in use. Appreciable conversion to slower migrating species may also occur during ultracentrifugal preparation of VLDL.

Apoprotein composition changes systematically with diameter in particles below 700-800 Å. Content of apoB increases uniformly with decreasing particle diameter in normolipidemic and hyperlipemic subjects alike, in agreement with the observations of Eisenberg et al. (27) in hyperlipemic subjects. Systematic changes also occur in the composition of the soluble protein fraction. In both normolipidemic and hyperlipemic subjects, content of the R-glutamic apoprotein falls and that of the arginine-rich apoprotein increases with decreasing particle diameter, whereas content of R-serine appears to increase only in normolipidemic subjects. Also, content of R-serine appears to be lower for all particle sizes of VLDL in normolipidemic subjects. These observations indicate the importance of careful evaluation of particle diameter in the interpretation of apoprotein composition of VLDL in various physiological and pathological states.

The higher relative content of the arginine-rich apoprotein and, for hyperlipemic subjects, the lower relative content of the R-glutamic species in the largest particle fraction resemble the pattern of apoproteins in the VLDL of the smallest particle diameter. This similarity of soluble apoprotein composition between the largest and smallest VLDL particles may reflect the presence of partially catabolized lipoproteins in each. The larger particles may include remnants of chylomicrons and the smaller ones remnants of VLDL (28, 29). If the progressive changes observed with decreasing particle diameter below 700 Å represent the continuing process of catabolism of VLDL, it is evident the soluble apoprotein species dissociate from the complex at individually characteristic rates.

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## REFERENCES

1. Gotto, A. M., W. V. Brown, R. I. Levy, M. E. Birnbaumer, and D. S. Fredrickson. 1972. Evidence for the identity of the major apoprotein in low density and very low density lipoproteins in normal subjects and patients with familial hyperlipoproteinemia. *J. Clin. Invest.* **51**: 1486–1494.
2. Shore, B., and V. Shore. 1969. Isolation and characterization of polypeptides of human serum lipoproteins. *Biochemistry.* **8**: 4510–4516.
3. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244**: 5687–5694.
4. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1970. Further separation of the apoproteins of the human plasma very low density lipoproteins. *Biochim. Biophys. Acta.* **200**: 573–575.
5. Shore, B., and V. Shore. 1972. The apolipoproteins: their structure and functional roles in human serum lipoproteins. *In* Blood Lipids and Lipoproteins, Quantitation, Composition and Metabolism. G. J. Nelson, editor. Interscience Pubs., Inc., John Wiley and Sons, Inc., New York. 789–824.
6. Havel, R. J., V. G. Shore, B. Shore, and D. M. Bier. 1970. Role of specific glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase. *Circ. Res.* **27**: 595–600.
7. Lee, D. M., and P. Alaupović. 1974. Composition and concentration of apolipoproteins in very-low- and low-density lipoproteins of normal human plasma. *Atherosclerosis.* **19**: 501–520.
8. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53**: 350–364.
9. Havel, R. J. 1969. Pathogenesis, differentiation and management of hypertriglyceridemia. *Adv. Intern. Med.* **15**: 117–154.

10. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9**: 693–700.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
12. Schultze, H. E., and K. Heide. 1960. *In* Medizinische Grundlagenforschung. K. F. Bauer, editor. Georg Thieme Verlag KG Stuttgart. Vol. 3. 357.
13. Marshall, W. E., and F. A. Kummerow. 1962. The carbohydrate constituents of human serum β-lipoprotein: galactose, mannose, glucosamine, and sialic acid. *Arch. Biochem. Biophys.* **98**: 271–273.
14. Ayrault-Jarrier, M., R. I. Cheftel, and J. Polonovski. 1961. Les glucides de la β-lipoprotéine $S_{f1.063}$ O-12 du sérum sanquin humain. *Bull. Soc. Chim. Biol.* **43**: 811–816.
15. Brewer, H. B., Jr., R. Shulman, P. Herbert, R. Ronan, and K. Wehrly. 1974. The complete amino acid sequence of alanine apolipoprotein (apoC-III), an apolipoprotein from human plasma very low density lipoproteins. *J. Biol. Chem.* **249**: 4975–4984.
16. Scanu, A. M., and C. Edelstein. 1971. Solubility in aqueous solutions of ethanol of the small molecular weight peptides of the serum very low density and high density lipoproteins: relevance to the recovery problem during delipidation of serum lipoproteins. *Anal. Biochem.* **44**: 576–588.
17. Kane, J. P., E. G. Richards, and R. J. Havel. 1970. Subunit heterogeneity in human serum beta lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **66**: 1075–1082.
18. Shore, V. G., and B. Shore. 1973. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry.* **12**: 502–507.
19. Houston, L. L. 1971. Amino acid analysis of stained bands from polyacrylamide gels. *Anal. Biochem.* **44**: 81–88.
20. Morell, A. G., C. J. A. Van Den Hamer, I. H. Scheinberg, and G. Ashwell. 1966. Physical and chemical studies on ceruloplasmin. IV. Preparation of radioactive, sialic acid-free ceruloplasmin labeled with tritium on terminal D-galactose residues. *J. Biol. Chem.* **241**: 3745–3749.
21. Sata, T., R. J. Havel, and A. L. Jones. 1972. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipidemic humans. *J. Lipid Res.* **13**: 757–768.
22. Lindgren, F. T., A. V. Nichols, F. T. Upham, and R. D. Wills. 1962. Subfractionation of the $S_f$ 20-$10^5$ lipoproteins in a swinging bucket rotor. *J. Phys. Chem.* **66**: 2007–2011.
23. Hamilton, R. L., R. J. Havel, J. P. Kane, A. E. Blaurock, and T. Sata. 1971. Cholestasis: lamellar structure of the abnormal human serum lipoprotein. *Science (Wash. D. C.).* **172**: 475–478.
24. Walker, H. M., and J. Lev. 1953. *In* Statistical Inference. Holt, Rinehart and Winston, Inc., New York. 436–438.
25. Margolis, S., and R. G. Langdon. 1966. Studies on human serum $\beta_2$-lipoprotein. I. Amino acid composition. *J. Biol. Chem.* **241**: 469–476.
26. Schonfeld, G., R. S. Lees, P. K. George, and B. Pfleger. 1974. Assay of total plasma apolipoprotein B concentration in human subjects. *J. Clin. Invest.* **53**: 1458–1467.
27. Eisenberg, S., D. Bilheimer, F. Lindgren, and R. I. Levy. 1972. On the apoprotein composition of human plasma very low density lipoprotein subfractions. *Biochim. Biophys. Acta.* **260**: 329–333.
28. Havel, R. J., and J. P. Kane. 1973. Primary dysbetalipoproteinemia: predominance of a specific apoprotein species in triglyceride-rich lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2015–2019.
29. Mjøs, O. D., O. Faergeman, R. L. Hamilton, and R. J. Havel. 1975. Characterization of remnants produced during the metabolism of triglyceride-rich lipoproteins of blood plasma and intestinal lymph in the rat. *J. Clin. Invest.* **56**: 603–615.