Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 700.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.
Weber, R. E., and Tanford, C. (1959), *J. Am. Chem. Soc. 81*, 3255.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.

Some Physical and Chemical Studies on the Protein Moiety of a High-Density (1.126–1.195 g/ml) Lipoprotein Fraction of Human Serum*

V. Shore and B. Shore

ABSTRACT: The protein moiety of a high-density (1.126–1.195 g/ml or HDL_3) lipoprotein fraction from human serum was obtained in greater than 95% yield in lipid-free, water-soluble form after removal of the lipids. Dissociation of the protein into subunits was facilitated by sodium dodecyl sulfate. Sedimentation equilibrium experiments with HDL_3 protein were conducted principally in systems consisting of protein, detergent, and buffer under conditions of essentially complete binding of the detergent by protein. These experiments indicated homogeneity with respect to the size of protein subunits and a molecular weight between 30 and 31 $\times 10^3$ for the subunit. The molecular weight calculated from the amino acid composition is in good agreement with this value. From the previously reported molecular weight and the per cent protein of the lipoprotein, it was concluded that there are three protein subunits per molecule in the intact lipoprotein.

The high-density (1.065–1.20 g/ml) lipoprotein fraction isolated from human serum shows in the analytical ultracentrifuge two major components, which have been designated HDL_2 and HDL_3 (DeLalla and Gofman, 1954). The lipoproteins of density 1.125–1.21 g/ml, or HDL_3, contain about 53% (present work) to 57% (Scanu and Granda, 1966) protein. From the reported molecular weight values of 175,000 (Hazelwood, 1958) and 170,000 (Scanu and Granda, 1966) for HDL_3 and the per cent protein, the protein moiety can be estimated to be about 95,000 g/mole of lipoprotein. Although the intact lipoprotein has not been dissociated into subunits, apparently the protein moiety is comprised of subunits whose dissociation in the absence of lipid is favored by the presence of detergent. After removal of lipids, the protein moiety in aqueous solution was found by Scanu *et al.* (1958) to have a molecular weight of 75,000, and by Shore and Shore (1962) to be comprised of a major and a minor component with $s_{20,w}$ values 2.3–2.6 and 4.2–4.6, respectively, as well as some highly aggregated material. On addition of SDS[1] to the protein solution, subunits of mol wt 36,000 (Shore and Shore, 1962) and 21,500 (Scanu and Granda, 1966) were found by the Archibald (1947) method of approach to sedimentation equilibrium.

In the present study, sedimentation equilibrium was used to obtain information on the molecular weight and homogeneity of the subunits of the protein moiety of density 1.125–1.195-g/ml lipoproteins of human serum. A complete amino acid analysis of the protein is also presented.

Materials and Methods

Lipoprotein Isolation. Lipoproteins of density 1.126–1.195 g/ml were isolated from human serum from individual donors, both male and female. Aliquots of 0.2 M Na-EDTA at pH 7.4 were added as needed to maintain a concentration of 8×10^{-4} M during all stages of isolation of the lipoprotein fraction. Less dense lipoproteins were removed by two centrifugations at a solvent density of 1.124 g/ml (20°). In the first, serum adjusted to this density with solid sodium chloride was centrifuged 48 hr at 39,000 rpm in a 40.3 rotor at 13–14° in a Spinco Model L centrifuge. The bottom 3-ml portions in the tubes were combined, diluted with an equal volume of salt solution of density 1.124 g/ml, and centrifuged as above. After the second centrifugation, the bottom 3-ml portions in the tubes were combined and adjusted to a solvent density of 1.195 g/ml by addition of a D_2O solution of sodium nitrate of density 1.450 and pH 7.4. The resulting

* From the Division of Biology and Medicine, Lawrence Radiation Laboratory, University of California, Livermore, California, 94550. *Received March 6, 1967.* This investigation was supported by the Division of Biology and Medicine, U. S. Atomic Energy Commission.

[1] Abbreviation used: SDS, sodium dodecyl sulfate.

solution was centrifuged 48 hr at 39,000 rpm in a 40.3 rotor. The top milliliter from each tube, which contained the lipoprotein of interest, was again centrifuged at a solution density of 1.195 g/ml for 48 hr at 39,000 rpm. In most cases, this fraction was further purified by centrifugation for 28 hr at 39,000 rpm in a 40.3 rotor after layering 3 ml of sodium chloride solution of density 1.126 g/ml above 3 ml of lipoprotein solution adjusted to a solvent density of 1.129 g/ml. The bottom 3 ml of each tube was used in the studies reported here. A plot of the sedimentation coefficient of the lipoprotein (corrected for the viscosity of the solution) *vs.* the density of the solution extrapolated to the solution density of zero sedimentation indicated a density of 1.142 g/ml for the lipoprotein.

Preparation of the Protein Moiety. Before removal of lipids, the lipoprotein was dialyzed against deaerated 0.1 M NaCl at 2–4° and adjusted to a concentration of about 4 mg/ml. The lipoprotein solution was extracted first with an equal volume of ether–ethanol (3:2, v/v). The water phase was then extracted repeatedly (five or six times) with aliquots of ether–ethanol (3:1, v/v) until the volume of the water phase was 75–100% of the initial volume. In some experiments the pH was adjusted to 4 before the final extraction and neutralized immediately after the final extraction. At this point, ether and some of the ethanol were removed from the protein solution by blowing nitrogen (Matheson, prepurified) over it. Aliquots of the aqueous phase were taken for prompt thin layer chromatography of lipids on silica gel G plates with chloroform–methanol–water (65:25:4) and gas chromatography of cholesterol to estimate the completeness of lipid extraction. The protein solution was then dialyzed at room temperature either against redistilled water (CO_2 free) or against 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5). Any adjustment of volume required to obtain a suitable protein concentration for ultracentrifuge experiments (2–3 mg/ml) was accomplished by pervaporation or dilution early in the dialysis period.

Dialysis tubing was prepared for use by heating it to 80° in 1% $NaHCO_3$ for 1 hr after a preliminary soaking in distilled water. It was then transferred to a second bicarbonate solution, soaked overnight at room temperature, and rinsed several times with redistilled water. The tubing was kept in redistilled water until needed. Ether and ethanol were redistilled after stirring the ether with concentrated $FeSO_4$ and the ethanol with alkali.

For determination of per cent C, H, N, and P and for carboxyl-terminal and total amino acid analyses, an aliquot of the protein solution, dialyzed against water, was lyophilized and then desiccated over P_2O_5. In some cases the protein solution, after dialysis against water, was further extracted with mixtures of chloroform, methanol, and acetone which caused the protein to precipitate. The insoluble protein was washed with acetone and dried over P_2O_5.

Chemical Composition. The C, H, and N content of the protein after drying at room temperature *in vacuo* oven P_2O_5 for 2 days and after further drying in a vacuum oven at 50 or 110° for 24 hr was determined with an F & M (Division of Hewlett-Packard Co.) Model 185 analyzer. A Kjeldahl–Nessler procedure was also used for nitrogen determinations on the dried protein, on protein hydrolysates, and on solutions of the protein. The colorimetric method of Lowry *et al.* (1951) was used to determine protein concentration after a preliminary determination of the chromogenic value of the protein relative to that of a serum standard. Phosphorus was determined colorimetrically by the method of Chen *et al.* (1956) after acid digestion of a weighed sample of protein or an aliquot of protein solution. Total cholesterol (free and ester) was determined by the method of Abell *et al.* (1952).

End-Group Analysis. Carboxyl-terminal amino acids were determined by hydrazinolysis followed by quantitative amino acid determination on a Beckman Model 120 amino acid analyzer. Hydrazine (0.05 ml/mg) was added to weighed samples (2.5–3.6 mg) of dried, lipid-free protein. Commercial anhydrous hydrazine (97% hydrazine) was used directly or in some cases the hydrazine was dried further and distilled before use as described by Kusama (1957). The hydrazinolysis mixtures were frozen, sealed under vacuum, and then heated at 105° for 10 hr, except that reaction mixtures containing hydrazine sulfate were heated at 70 or 80° for 16–18 hr (Bradbury, 1958). After heating, excess hydrazine was removed *in vacuo* over H_2SO_4 and P_2O_5. The residue was taken up in water (0.5 ml/mg of protein) and extracted at room temperature three or four times with aliquots of isovaleraldehyde (0.2 ml/ml of H_2O), over a period of about 1.5 hr. Aldehyde remaining in the water phase was removed by extraction with hexane.

The solution was made slightly acid, concentrated to 0.2 ml on a rotary evaporator, and diluted with 0.2 N sodium citrate buffer at pH 2.2 for amino acid analysis by the method of Spackman *et al.* (1958). Aliquots were applied to both the long and short columns of an amino acid analyzer, which was equipped with a high-sensitivity cuvet and an expanded-range measuring circuit. Amino-terminal amino acids were determined as previously described (Shore, 1957).

Amino acid composition was determined by the procedure of Spackman *et al.* (1958) using a Beckman Model 120B automatic amino acid analyzer. Samples (3–8 mg) of lipid-free protein dried at room temperature *in vacuo* over P_2O_5 were weighed for hydrolysis. Constant-boiling HCl solution was added to the sample (1 ml/4 mg), which was then frozen, evacuated, thawed and degassed, and sealed under a vacuum of about 50 μ. After hydrolysis at 110° for 20, 40, or 70 hr, water and HCl were removed from the sample in a rotary evaporator. Several water rinses were used to assure quantitative transfer of the sample and removal of HCl in the evaporator. The residue was dissolved in 0.2 N sodium citrate at pH 2.2. Two aliquots each for the short and long columns of the amino acid analyzer, as well as aliquots for total nitrogen deter-

mination, were taken. Corrections for loss of serine and threonine during hydrolysis were determined by extrapolation to zero time of plots of the logarithm of the recovery *vs.* the time of hydrolysis (Hirs *et al.*, 1954).

Cysteine was determined by amperometric titration of the intact lipoprotein and the lipid-free protein in 8 M urea with 0.002 M $AgNO_3$ as described by Benesch *et al.* (1955). Cystine was determined by amperometric titration at 37° of the lipid-free protein in 8 M urea solution at pH 8.2–8.3 after reaction with sodium sulfite (Carter, 1959). Both sulfhydryl and disulfide measurements were made in 50-ml beakers with a total volume of 15 ml instead of 30 ml as described by Benesch *et al.* and by Carter. The cysteic acid content of the performic acid oxidized protein, prepared as described by Moore (1963), was determined on the amino acid analyzer after acid hydrolysis for 20- and 40-hr intervals.

Tryptophan was determined spectrophotometrically on the unhydrolyzed protein by the method of Bencze and Schmid (1957). Spies and Chambers' (1948) colorimetric "Procedure B," in which *p*-dimethylaminobenzaldehyde is used as the chromogenic agent, was also used.

Amide nitrogen was determined by the modification of Hirs *et al.* (1954) of the procedure of Laki *et al.* (1954). Ammonia was determined colorimetrically with ninhydrin reagent, with Nessler's reagent, and on the short column of the amino acid analyzer.

Preparation of Detergent–Protein Complexes. For some ultracentrifuge experiments, the SDS–protein complexes were prepared by adding detergent, equivalent to 32–34% of the protein by weight, to a protein solution that had been dialyzed against 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5). The solution of SDS–protein was mixed occasionally over a period of 3–4 hr at 21–22° before carrying out the centrifuge experiments. In other experiments, the SDS–protein solution was dialyzed after addition of SDS to protein (1:1, w/w) in water solution. The solution of SDS–protein in water was mixed occasionally over a period of 3–4 hr at 21–22°, then adjusted to 0.1 M in NaCl and 0.02 M in Tris-HCl by addition of 0.5 M NaCl–0.10 M Tris-HCl (pH 8.5), and dialyzed against 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5) at 21–22° for 24–62 hr. The SDS remaining after dialysis was determined colorimetrically (Karush and Sonenberg, 1950). The SDS was recrystallized three times before use and gave one spot in thin layer chromatography on silicic acid plates with chloroform–methanol–water (65:25:4).

The partial specific volume, $\bar{V}$, of the lipid-free protein was calculated from its composition as described by Cohn and Edsall (1943) and that of the SDS–protein complex was calculated according to Hersh and Schachman (1958). It is assumed that the molar volumes of protein and bound SDS can be considered additive. Rosenberg and Klotz (1955) have provided evidence for the approximate validity of this assumption for small amounts of SDS; Aoki (1959) has validated the assumption for larger amounts of bound SDS.

Ultracentrifuge Experiments. Molecular weights of the SDS–protein complexes were measured by the method of equilibrium sedimentation (Richards and Schachman, 1959) in the Beckman-Spinco Model E ultracentrifuge with Rayleigh interference optics. Standard 12-mm, filled Kel F double-sector centerpieces were used in equilibrium and velocity experiments. Sapphire windows were used in sedimentation velocity and equilibrium runs, and quartz ones in synthetic boundary runs. A small volume (10–12 μl) of a fluorochemical FC43 (Spinco Division, Beckman Instrument Co., Palo Alto, Calif.) was added to the solution side of the double-sector cell for equilibrium sedimentation. In experiments in which the sample was prepared by dialyzing the lipid-free protein in water against 5 M guanidine-HCl (recrystallized from methanol–water), the volume of FC43, and also the volume of solvent and solution, in each sector of the cell was the same. 1,3-Butanediol was added to the solvent so that its refractive index matched that of the protein solution in equilibrium runs and was intermediate between those of solvent and solution in velocity runs.

Sedimentation velocity patterns were obtained with the interference optics. The samples were those used in sedimentation equilibrium experiments. The boundary position ($\bar{x}$) and the concentration in fringes (c) were determined as functions of time as described by Richards and Schachman (1959). Sedimentation coefficients were corrected to standard conditions in the usual manner (Svedberg and Pedersen, 1940). All velocity measurements were carried out at 52,640 rpm at 20.0°.

Synthetic boundary runs for the determination of initial protein concentration (c_0) were done in triplicate except in the first experiment, for which only one was done. Double-sector cells of the capillary type were used. The rotor speed was 9945 rpm and pictures were taken with monochromatic light and interference optics immediately after full speed was attained.

The method of Van Holde and Baldwin (1958) was used to estimate the time required for equilibrium. Photographs were made at this time and again about 10 hr later; identical fringe patterns indicated that equilibrium had been reached.

Results

Essentially all (>95%) of the protein from the HDL_3 lipoprotein fraction was recovered in soluble form in the water phase after removal of lipids. The lipids extracted accounted for 47–48% of the starting lipoprotein. Thin layer chromatography showed no lipids remaining in the protein preparation. Cholesterol plus cholesterol ester was <0.02%, cholesterol <0.006%, and phosphorus 0.004–0.007% of the protein after extraction. The protein contained 16.2% nitrogen, 52.5% carbon, and 6.9% hydrogen. The protein samples dried at room temperature over P_2O_5 for amino acid analysis contained 5% water, which could be removed by heating in a vacuum oven.

TABLE I: Amino Acids Released by Hydrazinolysis of HDL_3 Protein.

Expt	Hydrazinolysis Conditions		Moles of Amino Acid/Mole of Protein[a]			
	Time (hr)	Temp (°C)	Threonine	Serine	Glycine	Alanine
1	10	105	0.31	0.12	0.09	0.04
2	16	70	0.30	0.07	0.01	0.04
3	16	80	0.30	0.15	0.15	0.08
4	18	80	0.33	0.08	0.08	0.07

[a] Uncorrected for losses during the experimental procedure.

TABLE II: Amino Acid Composition of HDL_3 Protein.

Amino Acid	Moles/100 Moles of Amino Acids			Moles of Amino Acid Residue/Mole of Subunit[d]
	Present Study[a]	Scanu and Granda[b]	Levy and Fredrickson[c]	
Aspartic acid	7.72 ± 0.14	7.56 ± 0.16	8.00	20.9
Threonine	5.13 ± 0.07	4.82 ± 0.12	4.80	13.9
Serine	6.76 ± 0.18	6.29 ± 0.08	6.49	18.3
Glutamic acid	18.50 ± 0.28	18.1 ± 0.17	19.1	50.0
Proline	4.72 ± 0.07	4.77 ± 0.09	4.75	12.8
Glycine	4.36 ± 0.05	4.25 ± 0.16	4.17	11.8
Alanine	7.46 ± 0.05	7.30 ± 0.16	7.45	20.2
Cysteine[e]	0 ± 0.00			0
Cystine[e]	0.37 ± 0.01			1.0
Cystine[f]	0.36 ± 0.03	0.45 ± 0.04	0.48	1.0
Valine	5.95 ± 0.13	6.01 ± 0.18	5.21	16.1
Methionine	1.50 ± 0.04	0.91 ± 0.08	0.80	4.0
Isoleucine	0.76 ± 0.04	0.84 ± 0.09	0.48	2.0
Leucine	13.36 ± 0.05	13.42 ± 0.28	12.8	36.1
Tyrosine	3.77 ± 0.06	3.96 ± 0.19	3.21	9.9
Phenylalanine	3.37 ± 0.04	3.52 ± 0.08	3.21	9.1
Tryptophan	1.45 ± 0.06	2.97 ± 0.13		3.9
Lysine	9.17 ± 0.30	8.97 + 0.29	10.5	24.8
Histidine	1.48 ± 0.03	1.48 ± 0.05	1.76	4.0
Arginine	4.55 ± 0.20	4.33 ± 0.09	5.29	12.3
Amide ammonia	8.70 ± 0.14	13.29 ± 0.47		22.9

[a] Average of duplicate determinations on five protein preparations plus and minus standard error. [b] Calculated from data presented as moles of amino acids/100,000 g of protein (Scanu and Granda, 1966). The total number of amino acid residues/10^5 g of protein was 772 as compared with 840 in the present study. [c] Calculated from data presented as moles relative to aspartic acid taken as 100 (Levy and Fredrickson, 1965; Levy *et al.*, 1966). [d] From present study. [e] Obtained from amperometric titration of the protein with silver nitrate. [f] Determined as cysteic acid after treatment of the protein with performic acid.

The absorption spectrum of the protein moiety obtained after removal of the lipids from serum lipoproteins of density 1.126–1.195 g/ml is shown in Figure 1. The extinction coefficient $E_{cm}^{1\%}$ at 278 mμ and pH 8.5 is 10.8.

Terminal Amino Acid Composition. The amino-terminal amino acid of the lipid-free protein preparations of this study was found to be aspartic acid as reported previously for HDL_3 lipoproteins (Shore, 1957). The highest yield obtained was 1 mole/33,000 g of protein and the average yield was 1 mole/38,000 g of protein after correction for 74% recovery.

The carboxyl-terminal procedure used on the protein preparations in this study was considerably improved by use of the amino acid analyzer instead of the fluorodinitrobenzene method used previously on this protein

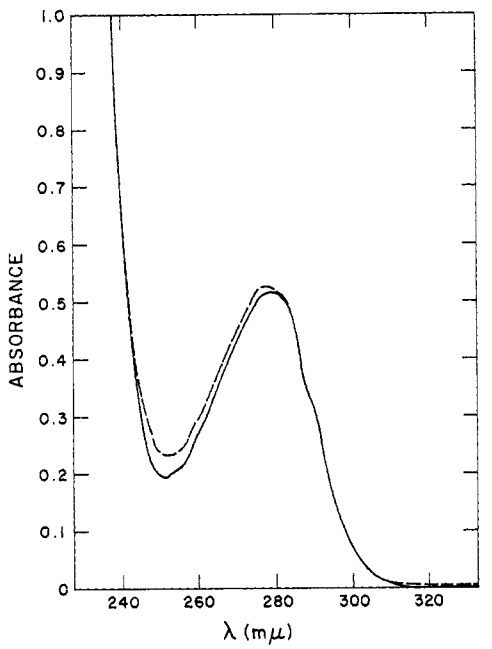

FIGURE 1: Absorption spectra of HDL_3 protein (– – –) and an SDS complex of the protein (———) containing 25% SDS. Solvent, 0.05 M Tris-HCl (pH 8.5), protein concentration, 0.480 mg/ml.

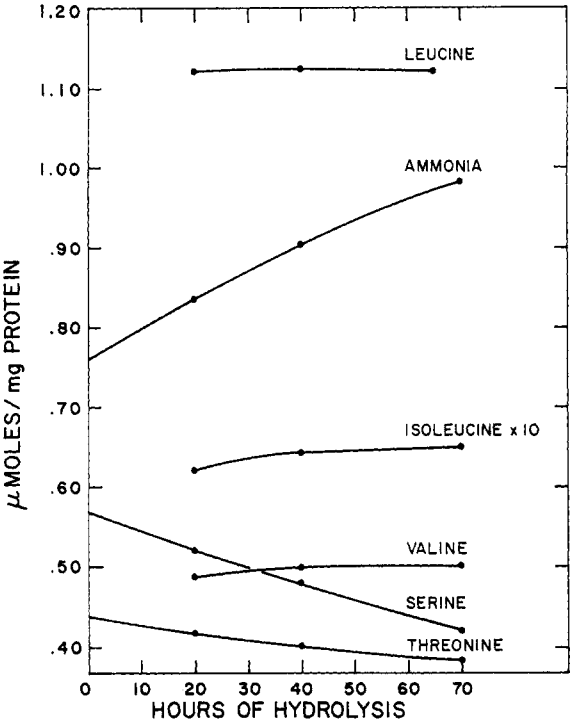

FIGURE 2: Amino acid recoveries from HDL_3 protein as a function of hydrolysis time.

(Shore, 1957). However, the results (Table I) are similar to previously reported data. If threonine is the only C-terminal group and if the molecular weight of the protein subunit is about 30,000 as indicated by sedimentation equilibrium experiments presented in this paper, the recovery in hydrazinolysis must be about 30%. Such low recoveries are not uncommon (Bradbury, 1958). The amino acids other than threonine in Table I are thought to be non-C terminal, since they have been observed frequently as non-C-terminal amino acids produced during or after hydrazinolysis of proteins (Bradbury, 1958).

The amino acid composition of HDL_3 protein is given in Table II. The minimal molecular weights calculated from the least abundant amino acids cystine and isoleucine are about 33,000 and 15,700, respectively. The molecular weight calculated from isoleucine content is approximately one-half the molecular weight found by sedimentation equilibrium and from the cystine content. The values in the right-hand column were obtained by fitting the data to give multiples of cystine content set equal to 1 mole/mole of protein. The molecular weight calculated by summing the products of the nearest integral number of residues of each amino acid (from the right-hand column of Table II) and the molecular weight of the respective residue gives a value of 31,160 for the minimum molecular weight of the protein. An average value of 31,150 ± 290 was calculated from the relationship (amino acid residue molecular weight) × 100/percentage of amino acid residue in the protein multiplied by the nearest integral number of amino acid residues consistent with one cystine and two isoleucine residues per molecule. The partial specific volume of the protein moiety of HDL_3 calculated from its composition is 0.737.

The amino acid values that changed significantly after 20-hr hydrolysis are shown in Figure 2. Extrapolation to zero time of the ammonia values from short-column chromatograms gives a value of 0.76 μmole/mg of protein or 23.6 amide residues/31,000 g of protein. A value of 0.74 μmole/mg of protein, or 22.9 amide residues/31,000 g of protein, was measured by a modification (Hirs *et al.*, 1954) of the method of Laki *et al.* (1954).

The number of residues of tryptophan obtained by two methods were in good agreement (12.4 residues/10^5 g of protein by the Bencze and Schmid (1957) and 12.7 by the Spies and Chambers (1948) methods). However, the correct number may be lower; as Noltmann *et al.* (1962) point out, the absorbancy indices for free tryptophan used in colorimetric and spectrophotometric procedures may not be applicable to tryptophan residues in intact proteins.

Ultracentrifuge Studies. Sedimentation velocity patterns for SDS–protein complexes subsequently used in sedimentation equilibrium expt 2–4 (Table III) yielded $s_{20,w}$ values of 2.6, 2.7, and 2.6, respectively. Plots of log $\bar{x}$ *vs.* time were linear. For these, $\bar{x}$, the distance between the boundary and the center of rotation, was obtained as described by Richards and Schachman (1959). In expt 1, 2, and 4 (Table III), SDS was added

TABLE III: Molecular Weight of HDL_3 Protein from Sedimentation Equilibrium.[a]

Expt	Concentration (%)		c_0 (fringes)	$(c_b - c_m)$ (fringes)	Column Ht (mm)	$\bar{M}_w$ of Protein[b]	
	Protein	SDS				A	B
1	0.188	0.060	9.36	8.00	2.36	30,300	30,300
2	0.214	0.072	10.96	11.50	2.76	31,300	30,800
3	0.240	0.081	12.20	12.18	2.78	30,000	30,400
4	0.190	0.062	9.62	8.55	2.39	30,800	30,400
5	0.276	0.070	13.80	15.71	2.77	31,000	31,000

[a] Temperature, 20.0°; rotor speed, 11,272 rpm in expt 1–4 and 12,020 rpm in expt 5. [b] Weight-average molecular weight values A and B were calculated according to Lansing and Kraemer (1935) and from the slopes of log c *vs.* x^2 plots, respectively. Molecular weight values from the slope of log c *vs.* x^2 were constant throughout the solution column in expt 1, 3, 4; they varied in expt 2 from 30,000 at x_m to 32,500 at x_b and in expt 5 from 30,700 at x_m to 31,800 at x_b.

to the dialyzed protein to give a solution which was not further dialyzed; in expt 3 and 5 (Table III), SDS was added to protein (1:1, w/w) in water solutions which were subsequently dialyzed 24 and 62 hr, respectively (see Materials and Methods). These preparations gave one protein band in polyacrylamide gel disk electrophoresis in the Canalco Model 12 apparatus (Canal Industrial Corp., Bethesda, Md.) according to the procedure for standard gels recommended by the manufacturer.

In the sedimentation velocity experiments, if all the detergent and protein are sedimenting as a single macromolecular species, $c_t(\bar{x}_t/x_m)^2$ will be equal to c_0 found in the synthetic boundary experiment for the solution (Cecil and Ogston, 1948); if unbound SDS is present it will sediment much more slowly so that $c_t(\bar{x}_t/x_m)^2$ will be less than c_0. The subscripts t and 0 refer to times t and zero, respectively, c is concentration, x_m is the distance of the meniscus from the center of rotation, and $\bar{x}$ is the boundary position. For sedimentation at 52,640 rpm of the SDS–protein complex of mol wt ~40,000, evaluation of the fractional fringe component of the total number of fringes c_t across the boundary proved to be somewhat less precise than that for c_0 because of diffusion of the macromolecule. However, in no case was $c_t(\bar{x}_t/x_m)^2$ different from c_0 by more than 0.2 fringe; in eleven measurements, the average deviation from c_0 was 0.07 fringe. Since the amount of SDS present in the solutions under investigation corresponded to about two fringes, it can be estimated that 90% or more of the SDS is protein bound in these experiments. These data are plotted as described by Richards and Schachman (1959) in Figure 3.

Data from sedimentation equilibrium patterns in conjunction with initial solute concentration (c_0) values obtained from synthetic boundary runs were used to calculate molecular weights of the detergent–protein complexes. From these values, the molecular weight of the protein was obtained from the expression $M_c(1 - \bar{V}_c\rho) = M_p[1 - \bar{V}_p\rho + x(1 - \bar{V}_{SDS}\rho)]$, where x is the number of grams of bound SDS per gram of protein and the subscripts c and p refer to complex and protein, respectively (Hersh and Schachman, 1958). Weight-average molecular weight values presented in Table III were obtained from the expression

$$\bar{M}_w = \frac{2RT}{(1 - \bar{V}\rho)\omega^2(x_b^2 - x_m^2)} \frac{c_b - c_m}{c_0}$$

(Lansing and Kraemer, 1935). The subscripts b and m refer to the bottom and to the meniscus of the solution column, respectively. R is the gas constant, T the absolute temperature, ω the angular velocity in radians per second, ρ the solution density, $\bar{V}$ the

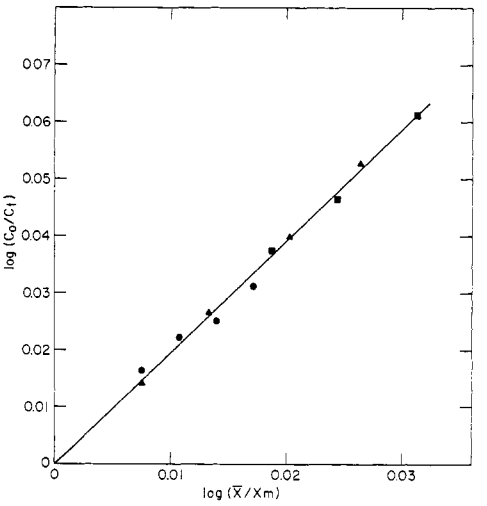

FIGURE 3: Application of the radial dilution equation to data from sedimentation velocity of SDS complexes of HDL_3 protein in 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5). (▲) 0.214% protein and 0.072% SDS. (●) 0.190% protein and 0.062% SDS. (■) 0.240% protein and 0.0081% SDS.

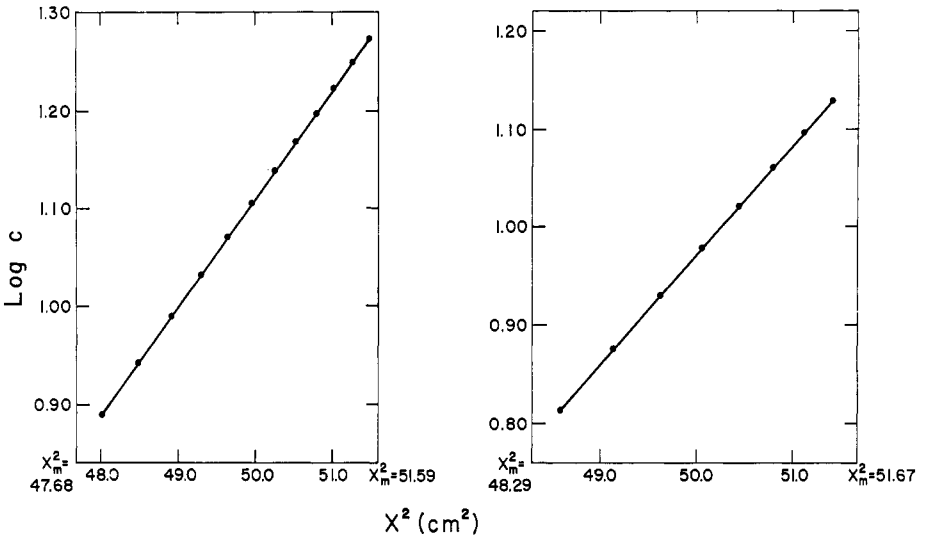

FIGURE 4: Concentration distribution at sedimentation equilibrium of SDS–HDL_3 protein samples 3 and 4 from Table I. (A) (left) The sample in 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5) was dialyzed 24 hr at 20° after addition of SDS to protein (1:1, w/w). (B) (right) The sample in 0.1 M NaCl–0.02 M Tris-HCl (pH 8.5) was not dialyzed after addition of SDS to protein.

partial specific volume of the solute, and x is the distance from the center of rotation. Values of $(c_b - c_m)$ were obtained as described by LaBar and Baldwin (1962). Molecular weights were also obtained from the relationship

$$M = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}x^2}$$

(Svedberg and Pedersen, 1940). The terms have the same meaning as above. Log c *vs.* x^2 is plotted as shown in Figure 4A,B. The values for c and x are from microcomparator readings of photographs taken at equilibrium in sedimentation equilibrium experiments; c was set equal to c_0 at the hinge point (Archibald, 1947). Molecular weight values calculated from the slopes of log c *vs.* x^2 plots are presented in Table III.

Plots of log c *vs.* x^2 for some SDS–protein preparations were concave upward over most of the solution column; others were straight over most of the solution column with increasing slope near the bottom of the cell. Whether these data indicate more than one kind of protein or the presence of aggregates in these preparations was not established with certainty. Sedimentation equilibrium experiments on two preparations of protein in 5 M guanidine-HCl gave nonlinear plots of log c *vs.* x^2.

Discussion

A molecular weight of 30–31 $\times$ 10^3 can now be assigned with some confidence to the protein subunit of HDL_3 lipoproteins of human serum. The value obtained by sedimentation equilibrium measurements is in good agreement with the results of amino acid analysis. Even without the corroboration of the latter, the present ultracentrifugal determination of 30 $\times$ 10^3 is more reliable than our previous estimate of the molecular weight in two respects. The present determination is based upon sedimentation equilibrium experiments for which homogeneity throughout the cell was indicated. In the previous study (Shore and Shore, 1962) molecular weights of protein–SDS complexes (37–39 $\times$ 10^3) were determined by the Archibald method, which is not as sensitive as sedimentation equilibrium methods for determining molecular weight and homogeneity. Subsequent experience with sedimentation equilibrium has shown that small amounts of aggregates or higher molecular weight species are not infrequently found in these preparations. Also, in the previous study the estimate of 36 $\times$ 10^3 for the molecular weight of the subunit was made without a reliable assessment of the contribution of the SDS to the measured molecular weights of the protein–SDS complexes.

The relative abundance of each amino acid of HDL_3 protein, except in the cases of methionine, tryptophan, and amide nitrogen, was found in the present study to be very similar to data obtained by either Scanu and Granda (1966) or by Levy and Fredrickson (1965) or by both groups. The data, except for the proline, histidine, and methionine content, are also very similar to the amino acid composition reported previously (Shore and Shore, 1962). In the latter communication half-cystine was erroneously reported as cystine. A somewhat higher value for cystine was obtained subsequently by titrating the protein amperometrically at 37°, as suggested by Carter (1959), instead of at room temperature. The recoveries of methionine from hydrolysates of protein and of

methionine sulfone from hydrolysates of performic acid treated protein were considerably greater than previously reported. In the present study, the amide nitrogen content is in good agreement with the ammonia content of the protein hydrolysate less the amount arising from destruction of serine and threonine. The ammonia in protein hydrolysates of the present study was less than the amount of amide nitrogen reported by Scanu and Granda (1966).

If the subunits are identical (there is no evidence that they are not), the cystine content of the protein indicates that the 30–31 $\times$ 10^3 units is the smallest molecular species possible in the protein solutions used in the present study and also in the intact lipoprotein. Whether rupture of the disulfide group can give rise to smaller units remains to be seen. With one protein preparation, not included in this report, treatment with sodium borohydride followed by iodoacetamide before SDS addition did not change the sedimentation rate of the SDS–protein complex. Without such a possibility, it is difficult to reconcile the present data with the value of 22 $\times$ 10^3 reported by Scanu (1966) and by Scanu and Granda (1966) for the molecular weight of the protein subunit. That value, calculated from meniscus data in Archibald experiments, in conjunction with the molecular weight and per cent protein of the lipoprotein, led those authors to conclude that the parent lipoprotein contains five protein subunits. From the present data, it appears that there are three protein subunits, as was concluded previously by Shore and Shore (1962).

Acknowledgment

We acknowledge the capable assistance of Mr. Stanley Krotz, Mrs. Alice Montoya, and Miss Marie Laskaris. We are grateful to Drs. Roger Hart, Thomas Smith, and Valeska Evertsbusch for valuable suggestions on the preparation of the manuscript.

References

Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. (1952), *J. Biol. Chem. 195*, 357.

Aoki, K. (1959), *J. Phys. Chem. 63*, 1336.

Archibald, W. J. (1947), *J. Phys. Chem. 51*, 1204.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Benesch, R. E., Lardy, H. A., and Benesch, R. (1955), *J. Biol. Chem. 216*, 663.

Bradbury, J. H. (1958), *Biochem. J. 68*, 482.

Carter, J. R. (1959), *J. Biol. Chem. 234*, 1705.

Cecil, R., and Ogston, A. G. (1948), *Biochem. J. 43*, 592.

Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 375.

DeLalla, O. F., and Gofman, J. W. (1954), *Methods Biochem. Anal. 1*, 459.

Hazelwood, R. N. (1958), *J. Am. Chem. Soc. 80*, 2152.

Hersh, R. T., and Schachman, H. K. (1958), *Virology 6*, 234.

Hirs, C. H. W., Stein, W. H., and Moore, S. (1954), *J. Biol. Chem. 211*, 941.

Karush, F., and Sonenberg, M. (1950), *Anal. Chem. 22*, 175.

Kusama, K. (1957), *J. Biochem. (Tokyo) 44*, 375.

LaBar, F. E., and Baldwin, R. L. (1962), *J. Phys. Chem. 66*, 1952.

Laki, K., Kominz, D. R., Symonds, P., Lorand, L., and Seegers, W. H. (1954), *Arch. Biochem. Biophys. 49*, 276.

Lansing, W. D., and Kraemer, E. O. (1935), *J. Am. Chem. Soc. 57*, 1369.

Levy, R. I., and Fredrickson, D. S. (1965), *J. Clin. Invest. 44*, 426.

Levy, R. I., Fredrickson, D. S., and Laster, L. (1966), *J. Clin. Invest. 45*, 531.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Noltmann, E. A., Mahowald, T. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1146.

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Rosenberg, R. M., and Klotz, I. M. (1955), *J. Am. Chem. Soc. 77*, 2590.

Scanu, A. (1966), *J. Lipid Res. 7*, 295.

Scanu, A., and Granda, J. L. (1966), *Biochemistry 5*, 446.

Scanu, A., Lewis, L. A., and Bumpus, M. (1958), *Arch. Biochem. Biophys. 74*, 390.

Shore, B. (1957), *Arch. Biochem. Biophys. 71*, 1.

Shore, V., and Shore, B. (1962), *Biochem. Biophys. Res. Commun. 9*, 455.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spies, J. R., and Chambers, D. C. (1948), *Anal. Chem. 20*, 30.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, New York, N. Y., Oxford.

Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.