Solubilization and Properties of the Apoproteins of the Very Low- and Low-Density Lipoproteins of Human Serum*

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ABSTRACT: Normal human serum lipoproteins of $\rho < 1.006$ g/ml (VLDL), $1.006 < \rho < 1.019$ g/ml (LDL_1), and $1.019 < \rho < 1.063$ g/ml (LDL_2) were subjected to delipidation by either diethyl ether (Et_2O) or a mixture of 1:3 ethanol–diethyl ether (EtOH–Et_2O) in the presence of 0.2 M sodium dodecyl sulfate (SDS). The products were soluble in alkaline aqueous buffers with protein recoveries of 98–100% (Et_2O treatment) and 85–90% (EtOH–Et_2O treatment). Et_2O removed essentially all cholesterol and triglycerides; EtOH–Et_2O removed all lipids except for 1–3% phospholipids and traces of triglycerides. Small amounts of SDS remained in the delipidated products in spite of extensive dialysis. By sedimentation analyses, the proteins from VLDL and LDL_2, after Et_2O extraction, showed two peaks in common with $s^0_{20,w}$ 10.9 and 15.5. A third peak with $s^0_{20,w}$ 4.5 was present in VLDL. The same components were noted after EtOH–Et_2O treatment, although they had slightly lower s values. Variations in pH (alkaline region) or ionic strength did not affect the patterns significantly. After succinylation, VLDL and LDL_2 (EtOH–Et_2O treatment) sedimented as a single peak of $s^0_{20,w}$ 3.6 and 7.2, respectively. The gel which formed during ultracentrifugation of LDL_2 succinylated was readily soluble in aqueous buffer and sedimented with the same s value of the supernatant. The proteins of VLDL and LDL_2 had the same amino acid composition and immunological behavior but differed in their amino acid terminals (serine and threonine for VLDL and glutamic acid for LDL_2). Both proteins exhibited affinity for micellar phospholipids. The apoprotein from LDL_1, which represents only 5% of the whole LDL class, had properties in common with VLDL and LDL.

The serum VLDL from two patients with carbohydrate-induced hyperlipemia showed a notable difference from the normal serum VLDL in that its protein moiety contained additional N-terminal amino acids (aspartic and glutamic acid) and cross reacted with antisera produced in the rabbit against either high-density lipoprotein or albumin. The above results indicate that differences exist between the protein moieties of VLDL and LDL. They do not answer, however, the question whether the number of N-terminal residues of VLDL are indeed part of the structural protein or due to nonlipoprotein contaminants. The second interpretation appears to be valid for VLDL from hyperlipemic sera.

An approach to the structure of serum lipoproteins, based on the disassembly–reassembly technique, has been recently introduced by Scanu *et al.* (1958, 1960, 1962) for the study of the high-density class, HDL.[1] This technique entails preparation of lipoprotein apoproteins in an essentially lipid-free form with sufficient solubility in aqueous media to permit assessment of their physical and chemical properties and their capacity to combine with lipid. Whereas these studies have been possible with human serum HDL, their application to serum VLDL[1] and LDL[1] has been hampered by the poor water solubility of each of these apoproteins following lipid removal by organic solvents (Scanu and Hughes, 1960; Banaszak and McDonald, 1960). In recent studies we have been able to resolve such difficulty. It is the purpose of this communication to describe: (1) techniques of preparation of delipidated, water-soluble apoproteins from human serum VLDL and LDL, (2) some of their physical, chemical, and immunochemical properties, and (3) their *in vitro* affinity for micellar phospholipids. A preliminary report of these findings has appeared (Granda and Scanu, 1965).

Experimental Procedures

Separation of Lipoproteins. Normal sera were obtained from freshly drawn blood of healthy human

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[1] Abbreviations used: VLDL, lipoprotein of density <1.006; LDL_1 = $1.006 < \rho < 1.019$; LDL_2 = $1.019 < \rho < 1.063$; HDL = $1.063 < \rho < 1.21$; VLD-Ppl = VLDL after ether treatment; LD_1-Ppl = LDL_1 after ether treatment; LD_2-Ppl = LDL_2 after ether treatment; VLD-P = VLDL after ethanol–ether treatment; LD_1-P = LDL_1 after ethanol–ether treatment; LD_2-P = LDL_2 after ethanol–ether treatment; SDS = sodium dodecyl sulfate.

donors (fasted overnight), 20–30 years of age, group A, Rh positive. Hyperlipemic serum was obtained from the blood of two patients with carbohydrate-induced hyperlipemia, class III according to Fredrickson and Lees (1965). EDTA solution (1 ml of 5%) neutralized to pH 7.0 with 0.1 M NaOH was added to each 100 ml of serum. Chylomicrons, VLDL, and LDL were separated by flotation in a Spinco Model L ultracentrifuge using a type 30.2 rotor. Following removal of chylomicrons (9500*g*, 10 min, 16°), the density of the serum was increased by addition of solid sodium chloride to allow flotation (70,488*g*, 18 hr, 16°) of the following classes: VLDL, $\rho < 1.006$ g/ml; LDL, $1.006 < \rho < 1.063$ g/ml; LDL_1, $1.006 < \rho < 1.019$ g/ml; and LDL_2, $1.019 < \rho < 1.063$ g/ml. They were centrifuged for an additional 24 hr in a medium of $\rho = 1.063$ g/ml to remove nonlipoprotein contaminants.

The procedure for separating lipoproteins from the hyperlipemic serum was the same as with normals except that VLDL was diluted 1:10 with 0.15 M NaCl containing 0.05% EDTA and was centrifuged twice at density 1.006 g/ml at 79,488*g* for 24 hr each time.

The lipoproteins were dialyzed against several changes of 0.15 M NaCl containing 0.05% EDTA. Their purity was tested by agar double-diffusion immunoprecipitin technique (Ouchterlony, 1949; Scheidegger, 1955) against antisera prepared in the rabbit against whole human serum, albumin, and HDL. No contaminants were noted. Lipoprotein preparations used for study were stored at 4° for less than 4 days.

Preparation of Protein–Phospholipid Complexes (VLD-Ppl, LD-Ppl, LD_1-Ppl, LD_2-Ppl). After dialysis, lipoprotein solutions containing from 2 to 5 mg of protein/ml were made 0.2 M in respect to SDS by the addition of solid detergent and incubated at 40° for 90 min. Aliquots (5 ml) were then placed in a 50-ml round-bottom centrifuge tube layered with peroxide-free diethyl ether, and allowed to rotate at 10–15 rpm in a Multi-Purpose Rotator, Model 150 V (Scientific Industries, Inc., Springfield, Ill.) at 4° for 16 hr. Thereafter, the water phase containing the lipoprotein residues, was separated by low-speed centrifugation (495 *g*) in a Sorvall refrigerated centrifuge, washed three times with peroxide-free diethyl ether, and dialyzed against two daily changes of Tris buffer, pH 8.6, ionic strength 0.1, for 10–12 days. Protein recoveries were from 98 to 100%.

Preparation of Nearly Lipid-Free Protein (VLD-P, LD-P, LD_1-P, LD_2-P). As in the previous technique, lipoprotein solutions were made 0.2 M with SDS and kept at 40° for 90 min. Aliquots (1 ml) containing not more than 7 mg of protein/ml (higher concentrations of protein resulted in the formation of large aggregates with poor water solubility), were added dropwise to a mixture of ethanol–diethyl ether, 1:3, v/v, at −10°, in a 50-ml centrifuge tube. The tube was filled with the solvent mixture, stoppered air tight, and allowed to rotate for 4 hr at 10–15 rpm at −10°. The white precipitate which formed was separated from the organic phase by 10 min of centrifugation in a Sorvall refrigerated centrifuge (495*g*) and extracted again with diethyl ether by rotation at 10–15 rpm at −10° for 16 hr. After a final wash with diethyl ether the precipitate was dried under nitrogen and stored at −10°. Storage, even for several weeks, did not affect the physical and chemical properties of the protein. Before use it was dissolved in 2 ml of Tris buffer, pH 8.6, ionic strength 0.1, made 0.2 M with SDS in 2–6 hr at 40°. The soluble product was dialyzed against Tris buffer, pH 8.6, ionic strength 0.1, for 10–12 days at room temperature, with two daily changes of buffer in the attempt to remove the detergent.

As in the previous case 0.2 M SDS proved to be the optimal concentration for good protein recoveries (85–90%) and solubilization. Neither nonionic (Triton WR-1339) or cationic (benzalkonium chloride) detergents could replace SDS in these procedures. Solubility was poor in the absence of the detergent.

Chemical Analysis. Protein was determined by the method of Lowry *et al.* (1951). In 2:1 chloroform–methanol (Folch *et al.*, 1957) extracts, lipid phosphorous was determined by the method of Stewart and Hendry (1935), total cholesterol according to Abell *et al.* (1952), and triglycerides according to Van Handel and Zilversmit (1957). The method of Karush and Sonnenberg (1950) was used for SDS determination. Presence of protein in concentration less than 2 mg/ml did not interfere with the analysis as determined by comparative studies between free and albumin-bound SDS. The sulfate content of twice-crystallized SDS (Fisher Scientific Co.), determined by the combustion method of Alicino (1958) was used as a standard in the colorimetric assay.

Amino Acid Analysis. Delipidated specimens of LDL were hydrolyzed by 6 M HCl according to Moore and Stein (1963). Separation of the amino acids was conducted by ion-exchange chromatography using a single column packed with Dowex 50-X8 (Chromobeads, type A, Technicon, Chauncey, N. Y.) in a Technicon automatic amino acid analyzer, according to Piez and Morris (1960). Cysteine and cystine were determined as cystic acid after performic acid oxidation according to Moore (1963). Tryptophan, destroyed by acid hydrolysis, was determined spectrophotometrically according to Goodwin and Morton (1946).

N-Terminal Amino Acid Analysis. The N-terminal amino acids were determined by the reaction of the nearly lipid-free proteins with fluorodinitrobenzene according to Levy and Li (1955). The DNP-amino acids were identified by paper chromatography according to Fraenkel-Conrat *et al.* (1955) using known DNP-amino acid derivative as markers.

Acetylation and Succinylation. The delipidated apoproteins were acetylated at 0° by the reaction with acetic anhydride (Fraenkel-Conrat, 1957). Succinylation was conducted at 25° according to Hass (1964) employing a molar ratio of succinic anhydride to free amino groups of lysine of 60:1. The extent of acetylation (or succinylation) was determined by the ninhydrin reaction.

Sedimentation Analysis. Sedimentation analyses were conducted in a Model E Spinco analytical ultracentri-

TABLE I: Per Cent Chemical Composition of VLDL and LDL_2 before and after Ethyl Ether or Ethanol–Ethyl Ether Treatment.

	Before Delipidation		After Ether		After Ethanol–Ether	
Constituents	VLDL	LDL	VLDL	LDL	VLDL	LDL
Protein	10.0 ± 1.0[a]	22.0 ± 1.0	38.5 ± 2.0	46.0 ± 2.0	96.5 ± 1.0	95.0 ± 2.0
Phospholipids	17.0 ± 1.0	25.0 ± 1.0	58.0 ± 2.0	51.0 ± 1.0	1.0 ± 0.5	2.5 ± 1.0
Total cholesterol	22.0 ± 1.0	46.0 ± 1.0	1.5 ± 0.5	1.2 ± 0.5	0.0	0.0
Triglycerides	51.0 ± 2.0	7.0 ± 1.0	1.5 ± 0.5	1.0 ± 0.5	0.5 ± 0.3	0.5 ± 0.3
SDS	—	—	0.5 ± 0.3	0.8 ± 0.3	2.0 ± 0.5	2.0 ± 0.5

[a] ± standard error of four determinations.

fuge using an An-D rotor and a 12-mm single-sector cell. Runs were made at 52,640 rpm at 20° with schlieren patterns photographed at 16-min intervals at a bar angle of 65°. The values of sedimentation coefficient were corrected for the viscosity and density of the solvent and were extrapolated to zero protein concentration (Schachman, 1957).

Electrophoretic Analyses. Conditions for agar gel, starch gel, and free-boundary electrophoresis have been described before (Scanu *et al.*, 1958; Scanu and Granda, 1966a).

Immunochemical Analysis. Antisera were prepared in rabbits by injecting intramuscularly, 5–10 mg of antigen mixed with 0.4 ml of complete Freund's adjuvant weekly for 4 weeks. One week after the last injection the animals were bled by cardiac puncture. The following antigens were used: LDL, VLD-P, LD_2-P, HDL, and human serum. Titers of antisera were determined by the dilution method of Kabat and Meyer (1961). The antisera selected for studies were those with titers higher than 1:50,000. Antialbumin serum was obtained commercially (Sylvana Co., N. J.). Antigen–antibody reactions were studied by agar double diffusion (Ouchterlony, 1949) and microimmunoelectrophoresis (Scheidegger, 1955).

Combination of the Delipidated Lipoprotein Protein with Lipid. The nearly lipid-free protein was labeled with ^{125}I according to a modification (Scanu and Granda, 1966a) of the method of McFarlane (1958) and incubated for 24 hr at 24° with phospholipids separated from 1:3, v/v, ethanol–diethyl ether extracts of LDL by column chromatography (Phillips, 1958) and rendered in micellar form either according to Fleischer and Klouwen (1961) or by sonication (20 kcycles/sec) 10 min at 0°. Defatted lipoprotein protein incubated in a buffer medium (Tris, pH 8.6, ionic strength 0.1) served as a control. After incubation, the density of the medium was increased to 1.21 g/ml with solid NaCl–NaBr and then centrifuged at 108,000*g* for 24 hr at 16°. Serial 1-ml fractions from top to bottom were removed from each ultracentrifuge tube and 0.1-ml aliquots dissolved in 15 ml of Bray's solution (1960) and counted in a Packard Tri-Carb liquid scintillation spectrometer. The recombined product could be separated from the phospholipid micelles by centrifugation at $\rho = 1.063$. Under these conditions the micelles were collected in the top milliliter and the newly formed protein–phospholipid complex sedimented.

Results

A. Normal Sera

1. Studies on VLDL and LDL_2. PROTEIN–LIPID COMPOSITION OF VLDL AND LDL_2 FOLLOWING DELIPIDATION. Treatment of either VLDL or LDL_2 with diethyl ether in the presence of SDS resulted in almost complete removal of cholesterol and triglycerides. After ethanol–diethyl ether treatment, the final products contained from 1 to 3% phospholipids and traces of triglycerides. Small amounts of SDS were present in all specimens (Table I).

SOLUBILITY PROPERTIES. To assess the effect of changes in pH on their solubility, solutions in Tris buffer (pH 8.6, ionic strength 0.1) of delipidated proteins containing from 3 to 5 mg/ml of protein were dialyzed at 4° against buffers having an ionic strength of 0.1: HCl–KCl (pH 1), glycine–NaOH (pH 2), formic–formate (pH 3), acetic acid–acetate (pH 4 and 5), phosphate buffer (pH 6 and 7), and Tris (pH 8 and 9). The solubility of the ether-treated lipoproteins was similar to that observed in the ethanol–diethyl ether treated products, *i.e.*, formation of a precipitate between pH 1 and 5, partial solubility between pH 5 and 7, and aggregation of the insoluble portion as a gel, true solutions above pH 8. The changes in solubility were found dependent on the H^+ concentration rather than on the nature of the buffer.

Addition of Ca^{2+} reduced the solubility of the delipidated proteins in Tris buffer. This was also true for other cations: Cu^{2+}, Cd^{2+}, and Mg^{2+} (Table II).

SEDIMENTATION ANALYSES. Representative patterns of sedimentation of the Tris-soluble defatted lipoproteins are shown in Figure 1. VLD-Ppl and VLD-P each exhibited three components, one major and two minor ones (Figure 1A and C). Only two components were seen with either LD_2-Ppl or LD_2-P (Figure 1B and D). The corrected sedimentation values are re-

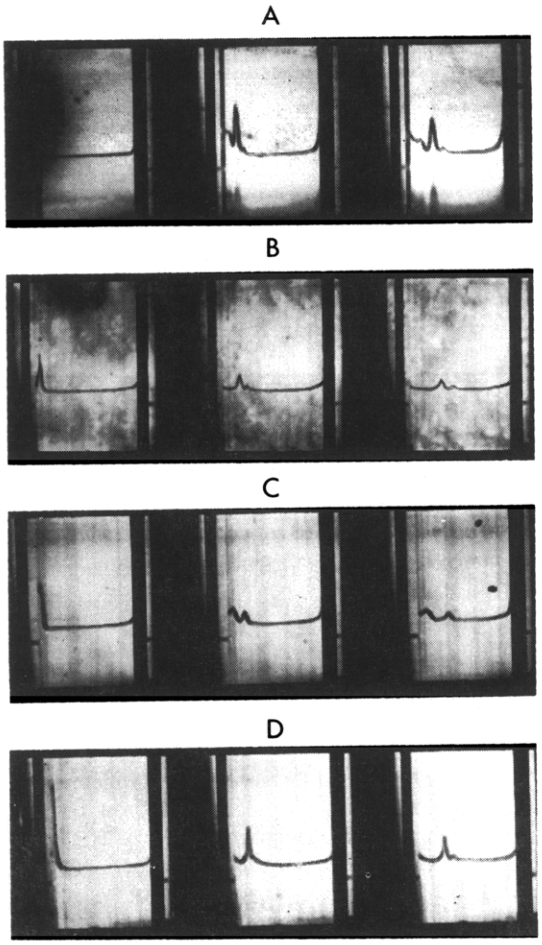

FIGURE 1: Ultracentrifugal schlieren patterns of VLD-Ppl (A), LD_2-Ppl (B), VLD-P (C), and LD_2-P (D). Experimental conditions: 52,604 rpm, 20°, solvent: Tris buffer, pH 8.6, ionic strength 0.1. Pictures taken at 16-min intervals after full speed was reached.

TABLE II: Solubility of LD_2-P in the Presence of Divalent Cations.[a]

Cation (10^{-2} M)	Protein in Soln (mg/ml)
None	5.40
$Ca^{2+}SO_4^{2-}$	0.36
$Cu^{2+}SO_4^{2-}$	0.71
$Cd^{2+}SO_4^{2-}$	0.40
$Mg^{2+}Cl_2^{2-}$	2.30

[a] The experiments were conducted at 4°. The protein was first dissolved in Tris buffer pH 8.6, ionic strength 0.1, made 0.2 M with SDS and the excess SDS was removed by extensive dialysis. Cations were then added to the desired molarity. Changes in solubility occurred immediately. The precipitate formed could be redissolved by the addition of EDTA (final concentration 1%).

TABLE III: $s^0_{20,w}$ Values of VLDL and LDL after Treatment with Ether and Ethanol–Ether.

Lipoprotein Fraction	Ultracentrifugal Components		
	Peak 1	Peak 2	Peak 3
VLD-Ppl	15.5	10.9	4.5
VLD-P	13.6	9.8	4.1
LD_2-Ppl	15.9	10.9	—
LD_2-P	13.6	9.8	—
VLD-P (acetylated)	3.6		—
LD_2-P (acetylated)	13.6	9.8	—
VLD-P (succinylated)	3.6	—	—
LD_2-P (succinylated)	7.2	—	—

ported in Table III. At pH 9–11, the patterns of sedimentation were similar to those observed at pH 8.6. After acetylation, only the pattern of VLD-P was changed in that a major and a minor component were observed (Figure 2 and Table III). After succinylation, VLD-P sedimented as a single peak. An homogeneous pattern was also observed with LD_2-P (Figure 3 and Table III). However, during ultracentrifugation, a gel formed at the bottom of the cell. This gel was readily dissolved in Tris buffer pH 8.6, ionic strength 0.1, or solutions of SDS (0.2 M) or urea (8 M). When studies of the succinylated LD_2-P were conducted in buffers of pH 8–11 progressively smaller quantities of gel formed.

ELECTROPHORETIC AND IMMUNOLOGICAL STUDIES. By free-boundary electrophoresis either VLDL or LDL_2 before delipidation exhibited a single symmetrical boundary. After treatment with either Et_2O or EtOH–Et_2O, 1:3, two components were observed with migration rates of 1.5 and 0.9 $\times$ 10^{-5} cm^2 sec^{-1} v^{-1}, respectively. By starch gel electrophoresis, VLDL, LDL_2, and VLD-Ppl, and LD_2-Ppl penetrated the gel poorly. The apoproteins moved at a single band in the β-globulin region. After succinylation the apoproteins gave a narrow band with mobility of albumin and a very faint band in the β-globulin region (Figure 4). By agar gel electrophoresis, VLDL or LDL_2 gave a single band close to the origin and a single immunoprecipitin line when tested against anti-VLDL or anti-LDL sera. After delipidation (Figure 5) a major and a minor component were observed giving a reaction of partial identity by immunoelectrophoresis. By the double-diffusion technique of Ouchterlony, VLDL or LDL_2, before and after delipidation, exhibited a single common antigenic determinant (Figure 6). The results were identical whether anti-VLDL or anti-LDL sera were used.

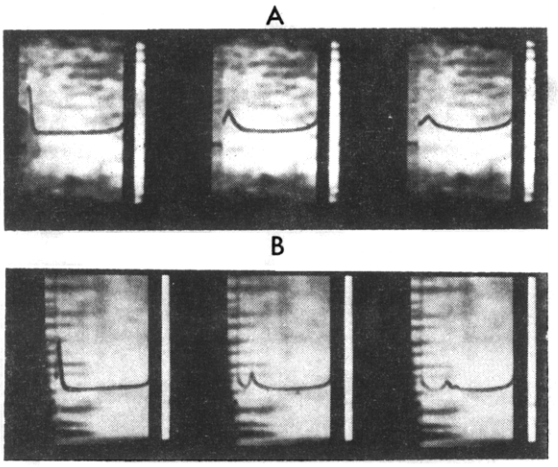

FIGURE 2: Ultracentrifugal schlieren patterns of VLD-P (A) and LD_2-P (B) after acetylation. Experimental conditions are the same as in Figure 1.

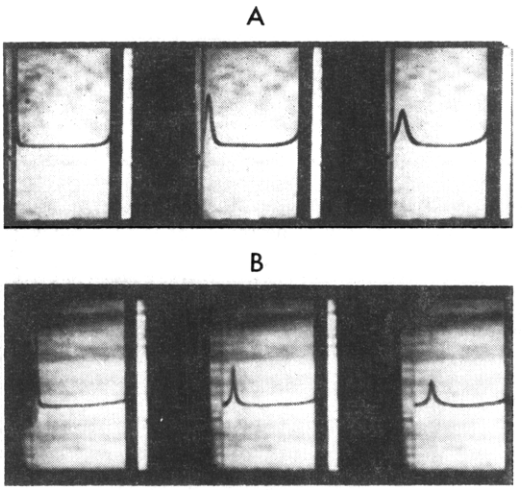

FIGURE 3: Ultracentrifugal schlieren patterns of VLD-P (A) and LD_2-P (B) after succinylation. Experimental conditions are the same as in Figure 1.

TABLE IV: Amino Acid Analysis (Residues/100,000 g) of VLD-P and LD_2-P.

Amino Acid	VLD-P	LD_2-P
Aspartic acid	92.5	94.9
Threonine	54.1	57.4
Serine	63.6	76.2
Glutamic acid	100.7	101.9
Proline	23.5	28.8
Glycine	37.6	38.2
Alanine	48.2	53.9
Valine	47.4	43.2
Cystine	4.0	3.0
Methionine	12.7	11.9
Isoleucine	31.3	46.5
Leucine	92.9	97.5
Tyrosine	26.8	27.9
Phenylalanine	41.1	42.4
Lysine	72.9	70.3
Histidine	13.4	18.5
Arginine	28.1	25.5
Tryptophan	15.8	12.8

TABLE V: N-Terminal Amino Acid Analysis of VLD-P and LD_2-P.

Protein	Amino Acid	Millimoles/g of Protein
VLD-P	Threonine	7.6
	Serine	6.0
	Glutamic acid	0.7
LD_2-P	Glutamic acid	2.5

AMINO ACID ANALYSIS (Table IV). The proteins from VLDL and LDL_2 had similar amino acid composition. The small difference between the two sets of values was most probably related to variations of the analytical technique employed.

N-TERMINAL AMINO ACID ANALYSIS (Table V). Serine and threonine were the main N-terminal amino acids of VLD-P in contrast to LD_2-P, which had glutamic acid as the single N-terminal. Estimates of molecular weight for VLD-P and LD_2-P from the data in Table V, assuming the existence of a single chain, gave 400,000 and 70,000, respectively. Because of the number of assumptions to be made, these values should be considered only tentative.

AFFINITY OF THE LABELED PROTEIN FOR MICELLAR PHOSPHOLIPIDS. After incubation with phospholipid micelles (1:5 protein:phospholipid ratio, 4 hr, 25°) LD_2-P-^{125}I sedimented at ρ 1.006 and 1.063 and floated at ρ 1.21. The labeled protein alone failed to float at any of these densities. The newly formed protein–phospholipid complexes gave a phospholipid protein ratio of 1.0 ± 0.15. They showed a marked tendency to aggregate under the high salt concentration but could be almost completely redissolved by dialysis against Tris buffer pH 8.6, ionic strength 0.1.

2. Studies on LDL_1. After delipidation, the protein moiety showed the same solubility, amino acid composition, and immunological properties as LDL_2 protein. The LD_1 protein, however, showed an additional 4S component in the ultracentrifuge and had in addition to glutamic acid, serine, and threonine as N-terminal amino acids. The results were taken to indicate that LDL_1 is a mixture of VLDL and LDL_2 and no further studies of the hybrid product were conducted.

FIGURE 4: Starch gel electrophoretic patterns. Conditions of electrophoresis vertical gel. Tris–borate buffer 8.6 (0.9 M Tris–0.5 M boric acid stock solution, diluted 1:20 prior to use) was the buffer of the gel block and Tris–boric acid (0.54 M Tris–0.2 M boric acid final dilution 1:4) was the buffer of the electrodes; A, LDL_2; B, LD_2-P; C, LD_2-P after succinylation.

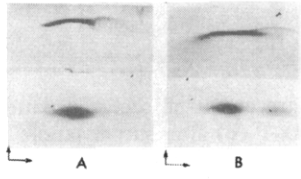

FIGURE 5: Agar gel electrophoretic and immunoelectrophoretic patterns of LD_2-Ppl (A) and LD_2-P (B). Antiserum, anti-LD_2-P.

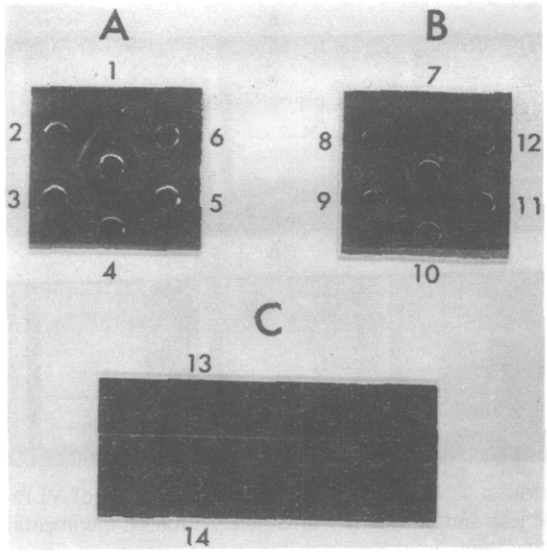

FIGURE 6: Agar gel double immunodiffusion patterns. (A) Central well; anti-LD_2-P serum; 1. HDL; 2. LD_2-P; 3. VLD-P (from normal sera); 4. VLD-P from hyperlipemic sera; 5. LD_1-P; 6. LD_2-P (B) Central well, antialbumin serum; 7. HDL; 8. VLD-P (from hyperlipemic serum); 9. serum albumin; 10. VLD-P (normal serum); 11. LD_2-P; 12. VLD-P (hyperlipemic serum) (C) Immunoelectrophoretic patterns: top, VLD; bottom, VLD-P; antiserum: antialbumin.

B. Hyperlipemic Sera

These were characterized by a severalfold increase in VLDL with decreased levels of LDL and HDL. The same delipidation techniques used for lipoproteins of normal sera were found applicable for VLDL and LDL_2. LDL_1 was not studied. No differences were noted between LD_2-P of normal and hyperlipemic sera. VLD-P from hyperlipemic sera differed from the normal product, in that it showed, aside from a major antigenic determinant reacting with anti-VLDL-LDL sera, two additional antigens one reacting against antialbumin and the other against anti-HDL sera. It should be noted that the presence of these two additional antigenic determinants were detected only after VLDL was delipidated (Figure 6). The N-terminal amino acids of hyperlipemic serum VLD-P were aspartic acid, glutamic acid, serine, and threonine.

Discussion

Attempts to extract totally the lipid from human serum low-density lipoproteins with mixtures of polar and nonpolar organic solvents (Scanu and Hughes, 1960; Banaszak and McDonald, 1960), or by freeze drying procedures (Gurd *et al.*, 1949; Oncley *et al.*, 1950) have resulted in products insoluble in water. Nonpolar solvents alone such as diethyl ether (McFarlane, 1942) or heptane (Gustafson, 1965) yield protein–phospholipid residues which partially dissolve in water. The solubility is enhanced if the extraction procedure occurs in the presence of starch which would increase the surface area between lipoprotein and solvent (Gustafson, 1965).

Our present studies have shown that the anionic detergent sodium dodecyl sulfate protects both VLD and LD lipoproteins against the denaturing effect of organic solvents (diethyl ether or a mixture of ethanol–diethyl ether) and allows preparation in high yields of soluble VLDL or LDL apoproteins varying in lipid content. The mechanism whereby SDS favors solubilization of VLDL or LDL apoproteins is not clear. It is possible that when the lipid of either VLDL or LDL is removed, nonpolar areas of the protein normally bound to lipid interact with each other and form insoluble aggregates. Addition of SDS would prevent such aggregation by favoring nonpolar interactions between the protein and the detergent. Such a process has been shown to occur between SDS and serum albumin by Karush and Sonnenberg (1950) and is probably partially responsible for the protective action of SDS against denaturation of albumin by urea (Markus *et al.*, 1964). In our studies either nonionic (Triton) or cationic (benzalkonium chloride) detergents failed to exhibit the solubilizing effect of the anionic SDS. This seems to suggest that the nature of the charge is essential for detergent–protein interaction, a concept which finds support in the studies on albumin–SDS complex by Markus *et al.* (1964). It cannot be ruled out that the nonpolar portions of Triton or benzalko-

nium chloride may not be sterically fitted for interaction with the nonpolar regions of the lipoprotein apoprotein.

The interpretation of the mechanisms of action of SDS lends support to the concept earlier proposed for HDL (Scanu, 1965), that in either VLDL or LDL there are a number of protein units which are held together by lipid bridges. This appears corroborated by the solubility and sedimentation properties of the defatted lipoproteins in various solvent media and after chemical modification by succinic anhydride (Table III) and also by the recent findings on optical rotatory dispersion showing no difference between lipidated and delipidated products (Scanu and Granda, 1966b). This concept will fit with the model E discussed by Margolis and Langdon (1966b) provided some modifications are introduced; namely, that a globular protein has relatively low helix content (Scanu and Granda, 1966b) and therefore gives a possibility of larger areas of interaction. At this time it is not possible to determine the number of subunits involved since we have been unable so far to determine the molecular weight of the succinylated component due to its tendency to aggregate.

The relative stability of the apoprotein structure following lipid removal, may in part account for the conservation of their capacity to elicit an active immunological response in the rabbit and also for their property to associate with phospholipid. The latter observation is of significance in that it opens the way for studying *in vitro* the lipidation processes involved in lipoprotein formation.

The relationship between the apoproteins of VLDL and LDL has not been clearly established. Our present data (Table IV) and those of others on amino acid composition (Shore and Shore, 1962; Levy *et al.*, 1966; Scanu and Hughes, 1962; Levy and Frederickson, 1965; Margolis and Langdon, 1966a) and immunological behavior (Aladjem *et al.*, 1957; Briner *et al.*, 1959) tend to suggest that these apoproteins are made of closely related peptide chains. It should be noted that the difference between the amino acid analysis from different laboratories is limited to a small number of amino acids. The difference in the tryptophan content could be traced to the optical method used, since it is likely that a more accurate value would be obtained when a protein deprived of lipids rather than the whole lipoprotein is used in the analysis. Such conclusion, however, is not supported by the difference in solubility of these lipoproteins and by the results on their sedimentation and N-terminal amino acid analysis summarized in Table V, all pointing to the chemical dissimilarity between VLDL and LDL apoproteins. The peak maxima of one of the three sedimenting fractions obtained after partial delipidation of VLDL varied from author to author (Hayashi *et al.*, 1959; Gustafson *et al.*, 1964; Bobbitt and Levy, 1965). However, strict comparison of these data is not possible since the hyperlipemic plasma used in each experiment was likely derived from a nonhomogeneous patient population. The choice of hyperlipemic sera for defining the characteristics of its structural apoprotein may prove to be a poor one. The immunological studies by Levy *et al.* (1966) and Gustafson *et al.* (1964) have shown that HDL is present in VLDL and that it can be readily detected by submitting VLDL to partial lipid extraction. Our data agree with these conclusions but add albumin to the list of contaminants. Contamination of VLDL by HDL and/or albumin may well account for the reported presence of N-terminal aspartic acid in these preparations. It would, thus, appear that structural studies on VLDL should place more emphasis on products from normal serum, where according to our data, contaminants are either not present or are too small to be detected. As to the exact nature of VLDL apoprotein, Gustafson *et al.* (1964) have reported the isolation from partially delipidated VLDL, of a fraction, apoprotein C, which did not have immunological properties or peptide pattern of the apoproteins of either HDL or LDL and gave serine and threonine as N-terminal amino acids. It would thus appear that apoprotein C is specific for VLDL and exists (see data on N-terminal amino acid analysis in Table V) in pure form only in the VLDL obtained from normal sera. This is not corroborated by the immunological findings. Since, according to Gustafson *et al.* (1964) apoprotein C is antigenically unrelated to the apoprotein of LDL (which in normal sera has only glutamic acid as N terminal) one would expect a distinct immunological behavior between normal VLDL and LDL. Our present results and those previously reported in literature (Scanu, 1965) are at variance with this expectation. Thus this point remains open for clarification.

Concerning LDL_2 ($1.019 < \rho < 1.063$), our N-terminal amino acid analyses are in agreement with the data by Shore (1957) and Rodbell (1957) but not with those of Bobbitt and Levy (1965). Further, ultracentrifugation of partially delipidated LDL showed two components, 10 and 15 S, whereas Avigan (1957) and Bobbitt and Levy (1965) reported a single fraction having s values of 13 and 15, respectively. Differences in the source of serum (normal *vs.* hyperlipemic), techniques of delipidation or solvent medium employed for the sedimentation analyses, may have accounted for the results.

In terms of LDL_1 ($1.006 < \rho < 1.019$) the lipoprotein was shown to represent but a small percentage (5%) of the whole LDL class and to have an apoprotein with properties of both VLDL and LDL_2. Such heterogeneity limits the interest for further structural studies on this lipoprotein. On the basis of this information, however, it should be possible to find an intermediate solvent density between 1.006 and 1.019 g/ml where the two lipoprotein species may be separated by ultracentrifugal flotation.

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