Isolation and properties of lipoproteins from normal rat serum

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ABSTRACT Three major classes of lipoproteins (VLDL, d < 1.006; LDL, d 1.006–1.040; HDL, d 1.063–1.21) were isolated by ultracentrifugal flotation from the serum of normal male Sprague–Dawley rats. Their physical, chemical, and immunological properties were analyzed and compared with those of their water-soluble, essentially lipid-free derivatives. Studies were also carried out on the d > 1.21 fractions. Each product was found to have distinct characteristics, and this was also indicated by spectral analyses carried out by the techniques of circular dichroism and UV absorption spectroscopy. The results provided evidence for the mutual role of the protein and lipids in determining the structure, and perhaps the immunological specificity, of serum lipoproteins.

SUPPLEMENTARY KEY WORDS apoproteins · immunology · disc gel electrophoresis · circular dichroism · UV absorption · lipoprotein structure

In spite of the rather extensive use of rats in the study of lipid metabolism, little information is available on the structural properties of their serum lipoproteins except for isolated reports (1, 2). The present studies are part of a program aimed at the elucidation of structure and function of serum lipoproteins in the rat. The report describes methods of isolation and delipidation of these lipoproteins, some of their physical, chemical, and immunological properties, and their spectral behavior under a number of environmental conditions. A preliminary account of these findings has appeared (3).

MATERIALS AND METHODS

All chemicals were reagent grade. SDS was crystallized from ethanol before use. To minimize variations in results, only Sprague–Dawley male rats (300–350 g) about 16 wk of age were used in all the experiments. The animals were fed a standard Purina Chow laboratory diet for a minimum of 6 wk and fasted for 16 hr before bleeding. They were bled from the abdominal aorta under sodium pentobarbital anesthesia (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa). Pooled blood from 60 animals was allowed to clot at room temperature and the serum was separated by centrifugation (10,000 *g* for 10 min) at 4°C. 1 ml of 5% EDTA (disodium salt) solution neutralized to pH 7.0 with NaOH was added to each 100 ml of serum. Lipoprotein fractionation was started within 24 hr of the blood collection.

Preparation and Delipidation of Lipoproteins

Chylomicrons were removed by centrifugation at 12,000 *g* for 20 min at 4°C (Sorvall RC-2 centrifuge, SS-34 rotor). From these chylomicron-free sera, VLDL was isolated by ultracentrifugation in a Spinco model L ultracentrifuge, using a 30.2 rotor (30,000 rpm for 24 hr at 16°C). After removal of the top milliliter (containing all the VLDL), the tubes were sliced in the middle clear

Abbreviations: VLDL, very low density lipoproteins, d < 1.006; LDL, low density lipoprotein, d 1.006–1.040; HDL, high density lipoprotein, d 1.063–1.21; apo VLDL, apo LDL, apo HDL, apoprotein of VLDL, LDL, and HDL, respectively; apo VLDL (G) and apo LDL (G) refer to proteins solubilized in the presence of SDS (sodium dodecyl sulfate). Whenever in the text the terms apo VLDL or apo LDL are used without specification the results are equally applicable to either method of delipidation. VLDL (E), very low density lipoprotein extracted with ethyl ether.

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zone. The bottom fractions were pooled, adjusted to d 1.063 by addition of solid NaCl, and centrifuged again (30.2 rotor, 30,000 rpm, 24 hr, 16°C). The top 1 ml fractions were diluted to a final density of 1.040 and again ultracentrifuged under the above experimental conditions to obtain pure LDL (top 1 ml) while the d 1.040–1.063 fraction was discarded; the infranates were adjusted to d 1.21 with NaBr, and HDL were floated in a 40.3 rotor at 40,000 rpm for 24 hr, 16°C. VLDL and LDL were washed once, and HDL twice, at densities 1.006, 1.040, and 1.21, respectively, to remove possible contaminants. Repeated washings of HDL caused approximately 20% lipoprotein losses.

Isolated lipoproteins were dialyzed at 4°C against several changes of 0.15 M NaCl, containing 0.05% EDTA, adjusted to pH 7.0 with 0.1 N NaOH. When samples were stored for more than 5 days, 1/100,000 (w/v) merthiolate was added as a preservative.

HDL was delipidated with ethanol–ether 3:2 at −10°C (4). LDL was extracted with ethanol–ether 3:1 (5) in the presence of SDS (6), or without the detergent. Similar procedures were applied to VLDL.

Solubilization of Apoproteins

Apo HDL dissolved readily in a number of buffers between pH 8 and 11.5. Solubilization was facilitated in media of low ionic strength (0.01 M).

Apo VLDL and apo LDL extracted with ethanol–ether without SDS were made water-soluble by titration at pH 11.5 (7) and will be referred to as apo VLDL and apo LDL, respectively. We recently found that these products could be solubilized more quickly in 8 M urea. When the latter agent is removed by dialysis, the product is identical by all criteria employed (see Results) with material that has never been in contact with urea. Apo VLDL and apo LDL extracted with ethanol–ether 3:1 in the presence of 0.2 M SDS were solubilized in 0.2 M SDS according to Granda and Scanu (6), and such delipidated preparations will be referred to as apo VLDL (G) or apo LDL (G).

Purification of d 1.21 Infranate and Isolation of Albumin

After the separation of HDL, the tubes were sliced about one-third from the top, and the bottom fraction was purified by two ultracentrifugal steps in a 40.3 rotor at 40,000 rpm for 24 hr. After each run the tubes were sliced at the upper one-third to separate any floating lipoproteins. The purified d > 1.21 fractions were then dialyzed against 0.15 M NaCl, 0.05% EDTA.

The globulins from the d > 1.21 infranates were precipitated by 50% saturated ammonium sulfate, and the crude albumin fraction was precipitated by 5% (w/v) trichloroacetic acid (TCA). The precipitate was washed with 5% TCA and then suspended in 1% (w/v) TCA in 96% ethanol (8). Such preparations contained pure albumin, as indicated by either agarose or polyacrylamide gel electrophoresis, in which the observed single band had mobility equal to that of albumin of unfractionated rat serum. It sedimented as a single symmetrical component (4.5 S) in a Model E Spinco analytical ultracentrifuge and reacted only with antialbumin sera.

Immunological Studies

Antisera were prepared by injecting male albino rabbits (2.0–3.0 kg) in the foot pad with 1.0–1.5 mg of any of the lipoprotein preparations emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.). After 3 wk, the animals received a booster injection intramuscularly (2.0–4.0 mg of antigen suspended in the adjuvant) and 10 days later they were bled by heart puncture. The antisera, after determination of the titers, were stored at −20°C in the presence of 1/100,000 (w/v) merthiolate.

The immunological studies were carried out by the techniques of double diffusion (9) and immunoelectrophoresis (10) using 1% (w/v) agarose (Fisher Scientific Co., Chicago, Ill.) in 0.05 M Veronal buffer at pH 8.6.

Electrophoresis

Agarose gel electrophoresis was conducted in 1% agarose in 0.05 M Veronal buffer at pH 8.6. After electrophoresis at 220 v for 1 hr, gels were fixed with 3% acetic acid, then dried and stained with Amido Schwarz.

Polyacrylamide gel electrophoresis (11) used 7.5, 3.75, and 3.5% gels, pH 8.6, with and without 8 M urea. With urea systems, all samples were dialyzed against 0.01 M Tris (pH 8.6) containing 8 M urea before electrophoresis. The electrophoresis was conducted with a constant current of 3 ma per tube for 1.5 hr at 4°C. The gels were stained for protein with 1% Amido Schwarz in 7% acetic acid (11), and for lipid in Oil Red O (12).

Bands from polyacrylamide gel electrophoresis were immunologically identified in some cases. After electrophoresis, the unfixed gels were sliced into two identical portions and then aligned at the center of a microscopic slide. This was placed in a slide frame for immunoelectrophoresis (LKB Instruments, Inc., Washington, D.C.) and then filled with 1% agarose. The anisera were applied in a trough parallel to the polyacrylamide gel and allowed to diffuse for up to 3 days at 4°C.

Analytical Ultracentrifugation

Sedimentation velocity and flotation analysis were conducted in a Model E Spinco analytical ultracentrifuge using an An-D rotor and a 12 mm single-sector cell with an aluminum centerpiece. Runs were made at 52,640 rpm at 20°C. Sedimentation and flotation con-

stants were calculated from Schlieren patterns, with appropriate corrections for the viscosity and density of the solvent medium and for the apparent partial specific volume of the solute. Each sample was run in at least three different dilutions to permit extrapolation to zero protein concentration.

Molecular weights were determined by a high-speed sedimentation equilibrium technique (13) in a Model E Spinco analytical ultracentrifuge at 20°C equipped with Rayleigh interference optics. A 6-channel, high strength centerpiece of Epon allowed for the simultaneous interferometric observation of three solution-solvent pairs. Rotor speed was: 15,220 rpm (HDL); 12,570 rpm (LDL); 25,980 rpm (apo HDL); 12,590 rpm (apo LDL). In each determination the solutions analyzed had a protein concentration between 0.1 and 0.5 mg/ml. Calculated molecular weights were also extrapolated to zero protein concentration. Human serum albumin in 0.15 M NaCl served as a control and gave a mole wt of 67,000.

The partial specific volume, $\bar{v}$, of HDL was obtained from the equation: $\bar{v} = 1/d_0 - (d/d_0 - 1)/x$, where d = density of solution, d_0 = density of solvent, and x = lipoprotein concentration (g/ml). All density measurements were made in a calibrated 10-ml pycnometer at 20°C. The $\bar{v}$ values of apo HDL and apo LDL were calculated from the amino acid composition (14).

Circular Dichroism

Circular dichroism was studied at 27°C in a Cary Model 6001 Spectropolarimeter equipped with a circular dichroism attachment. Cells of 0.1 mm path length (Pyrocell, Shoreham Mfg. Co., Inc., Winfield, N.J.) and a protein concentration of 0.7–2.0 mg/ml in phosphate buffer (pH 8.6), 0.1 M were used. The value of molar ellipticity $[\theta]$ was obtained from the relation $[\theta] = \theta/10 \times MRW/lc$, where θ is observed ellipticity, MRW (mean residue weight) = 112, l = cell path length in cm, and c = concentration of solute in g/ml. The conditions for the thermal experiments were described previously (7).

UV Absorption Spectroscopy

UV absorption spectra were recorded in a Cary model 14 Spectrophotometer. Protein samples (0.5–0.7 mg/ml) were dialyzed against 0.1 M phosphate buffer, pH 8.6, and measured in cells with path lengths of 1.0 cm. Changes in pH from 8.6 to 11.6 were obtained by titration with 0.1 N NaOH. In the case of LDL, appropriate corrections for scattering were made according to Reddi (15) using the equation $K = C/\lambda^4$, where K is extinction due to scattering, C is the scattering constant, and λ is the wavelength in nanometers. The scattering constant C was calculated from the absorption at 370, 360, 350, and 340 nm by means of the relation $C = K\lambda^4$.

Analyses

Hydrolysates (6 N HCl, 110°C) of delipidated specimens of HDL and LDL were analyzed on a Beckman model 120 C amino acid analyzer (Spinco Division, Beckman Instruments, Palo Alto, Calif.). Cystine was determined as carboxymethyl cysteine. Losses due to hydrolysis were of the order reported by Moore and Stein (16). Tryptophan, destroyed during acid hydrolysis, was determined spectrophotometrically according to Goodwin and Morton (17).

Lipids contained in chloroform–methanol extracts (18) of each lipoprotein were separated by column chromatography on silicic acid (Unisil 100–200 mesh, Clarkson Chemical Co., Williamsport, Pa.). Chloroform eluates were used for the determination of total cholesterol (19) and triglyceride (20). Phospholipids were eluted with absolute methanol. The factor 25 was used to convert lipid P (21) into phospholipid.

Total protein determinations were carried out by the method of Lowry, Rosebrough, Farr, and Randall (22) with bovine serum albumin as a standard.

RESULTS

Whole Lipoproteins

VLDL and LDL were colorless and somewhat opalescent; HDL was slightly yellowish and clear.

Immunology. VLDL and LDL reacted against anti-LDL and anti-VLDL sera but not against anti-HDL sera. HDL reacted with anti-HDL and anti-VLDL sera (Fig. 1). The fraction separated between d 1.040 and 1.050, or 1.050 and 1.063, for which no further analysis was carried out, contained both LDL and HDL although the former was predominant. The d > 1.21 fraction reacted with anti-HDL or anti-apo HDL sera, but not with anti-LDL or anti-apo LDL sera.

Agarose Gel Electrophoresis. HDL showed a single band in the pre-albumin region, with some tailing. LDL and VLDL migrated also as a single band in the β- and pre-β regions, respectively. By *immunoelectrophoresis*, HDL showed two distinct arcs of precipitation against anti-HDL (not shown in Fig. 2), or anti-apo HDL sera (Fig. 2). One sharp arc was observed in the reaction between LDL and anti-LDL sera.

Polyacrylamide Gel Electrophoresis. In 3.75% gel with 8 M urea, HDL exhibited two main bands (*a*, *b*) and four to five small components (*c*, *d*, *e*, *f*) with Amido Schwarz staining (Fig. 3), while only bands *a*, *b*, and *d* were visible with Oil Red O. LDL showed one major band (*a*) and a few faint bands (*b*, *c*, *d*) detected by Amido Schwarz

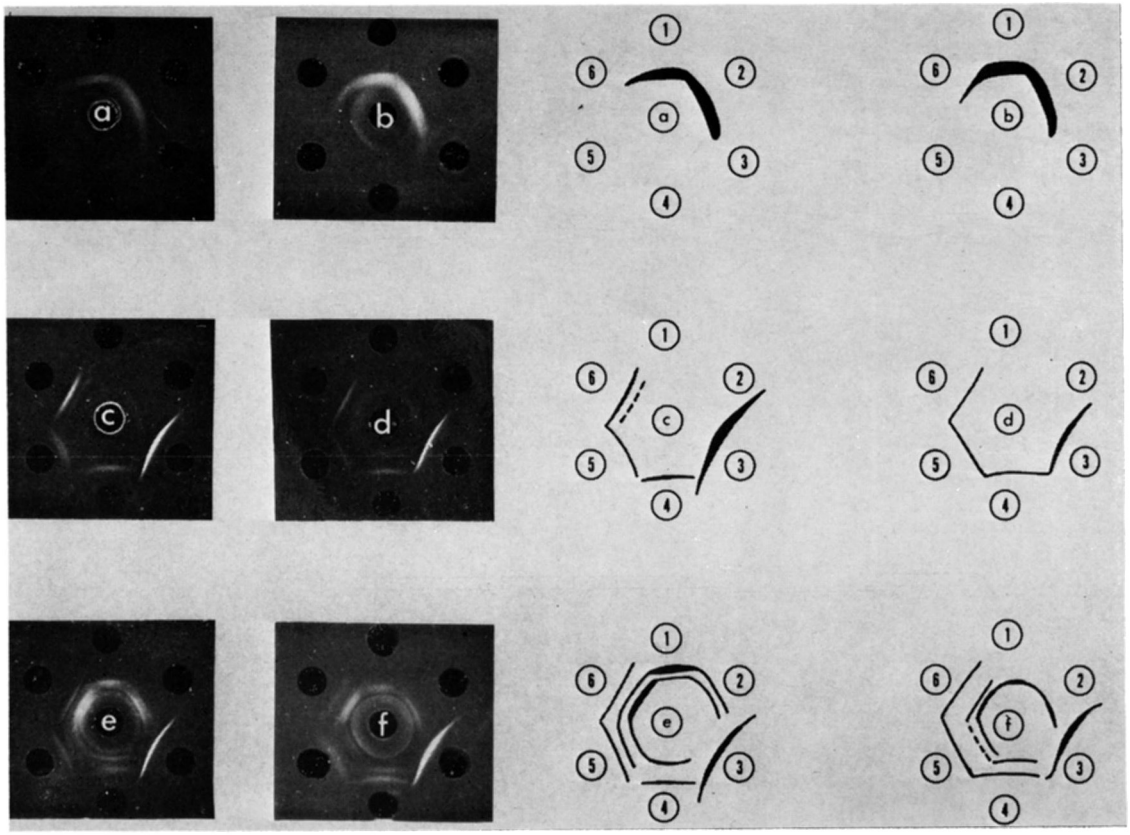

FIG. 1. Patterns of double immunodiffusion in agarose of rat serum lipoproteins and apoproteins. Antisera: *a*, anti-HDL; *b*, anti-apo HDL; *c*, anti-LDL; *d*, anti-apo LDL; *e*, anti-VLDL; *f*, anti-apo VLDL. Antigens: *1*, HDL; *2*, apo HDL; *3*, LDL; *4*, apo LDL; *5*, VLDL (E); *6*, apo VLDL.

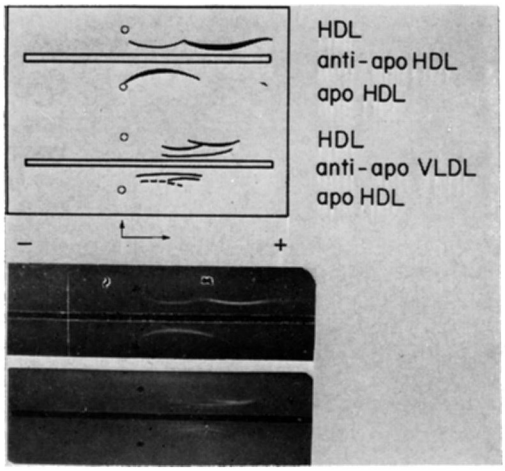

FIG. 2. Immunoelectrophoretic patterns of HDL and apo HDL (1% agarose).

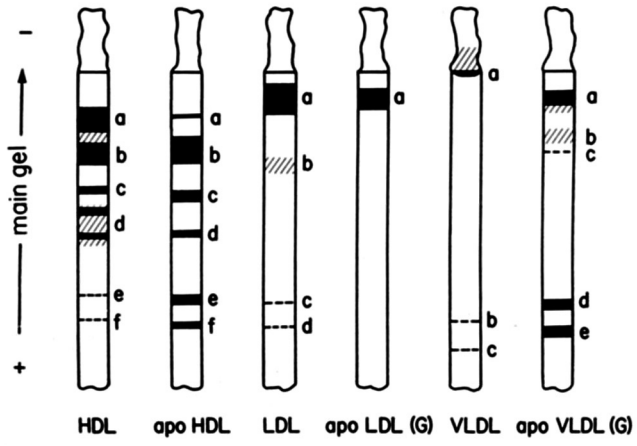

FIG. 3. Electrophoretic patterns of rat serum lipoproteins and apoproteins (polyacrylamide gel, 3.75% with 8 M urea).

staining. The minor components were not seen in all preparations studied. Oil Red O stained only bands *a* and *b*. The greatest portion of VLDL did not enter the 3.75% gel, but fast-migrating small components (*b*, *c*) were observed. Fig. 4 illustrates electrophoretic patterns of lipoproteins and their apoproteins in 3.5% polyacrylamide gel without urea. Under these conditions HDL and apo HDL showed a less sharp separation than with urea. Particularly remarkable was the difference between VLDL and apo VLDL.

Ultracentrifugation. (Tables 1 and 2). HDL and LDL each sedimented as a single peak with $s_{20,w}$ values of 8.0 and 8.6, respectively. The two products exhibited also a

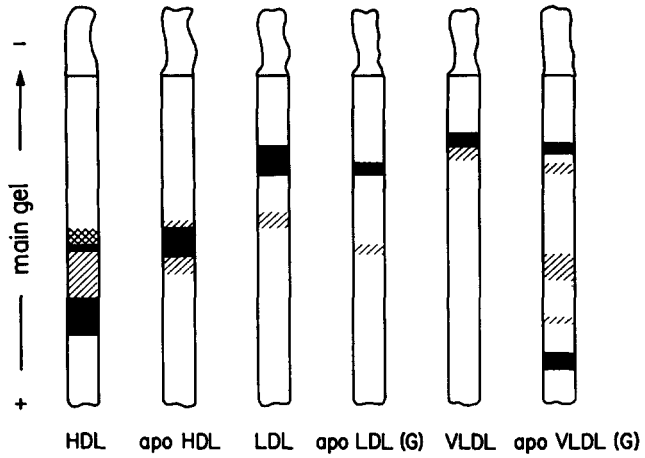

FIG. 4. Electrophoretic patterns of rat serum lipoproteins and apoproteins (polyacrylamide gel, 3.5%).

single flotation boundary with $S°_{f(1.063)}$ of 6.7 for LDL and $S°_{f(1.21)}$ of 7.1 for HDL. The mol wt of HDL and LDL calculated from the data of sedimentation equilibrium experiments were 6.0×10^5 and 2.58×10^6, respectively.

The *chemical compositions* of isolated lipoproteins are shown in Table 3.

Apoproteins

Apo VLDL. Apo VLDL demonstrated a single but rather broad band migrating in the β-region of agarose electrophoresis. Polyacrylamide gel electrophoresis (3.75 % gel with urea) revealed several bands (Fig. 3), some migrating in the same position as the major components of apo LDL and apo HDL, and two major fast-moving bands corresponding to the minor components *e* and *f* of apo HDL. By immunodiffusion, arcs corresponding to these fast bands were seen against anti-apo VLDL, but not against anti-apo HDL or anti-apo LDL sera.

TABLE 1 SEDIMENTATION PROPERTIES OF RAT SERUM LIPOPROTEINS AND APOPROTEINS

	Solvent	$s_{20,w}$	Peak
HDL	0.15 M NaCl + EDTA (0.05%)	8.0	Single, sharp
LDL	0.15 M NaCl + EDTA (0.05%)	8.6	Single, sharp
Apo HDL	0.1 M Tris (pH 8.6)	2.3	Single, broad
Apo HDL	0.1 M Tris + 0.1 M KCl (pH 10.4)	2.0	Single, broad
Apo HDL	0.1 M Sodium carbonate (pH 11.0)	2.3	Single, broad
Apo LDL (G)	0.1 M Tris (pH 8.6)	10.9	Broad, fast-sedimenting minor component observed

TABLE 2 MOLECULAR WEIGHTS OF RAT SERUM LIPOPROTEINS AND APOPROTEINS CALCULATED FROM THE DATA ON SEDIMENTATION EQUILIBRIUM

	Solvent	No. of Expts.	Mol wt* $\times 10^{-5}$	Partial Specific Volume Used for Calculation
HDL	0.15 M NaCl + EDTA (0.05%)	3	6.03 (5.89–6.10)	0.894†
LDL	0.15 M NaCl + EDTA (0.05%)	2	25.8 (24.3–27.3)	0.964‡
Apo HDL	0.1 M Tris–HCl (pH 8.6)	3	0.427 (0.425–0.431)	0.729§
Apo HDL	0.1 M Tris + 0.1 M KCl (pH 10.4)	1	0.317	0.729§
Apo HDL	0.1 M Carbonate (pH 11.0)	2	0.279 (0.263–0.295)	0.729§
Apo LDL (G)	0.1 M Tris–HCl (pH 8.6)	1	1.88	0.738§
Apo LDL	0.1 M Tris–HCl (pH 8.6)	1	0.927	0.738§

* Average of determinations. Values in the parentheses indicate the range of the observations.
† Measured by pycnometry.
‡ Data for corresponding human serum fraction (6).
§ Calculated from the amino acid composition.

TABLE 3 CHEMICAL COMPOSITION OF RAT SERUM LIPOPROTEINS AND APOPROTEINS

Lipoprotein Class	Density Range	Weight Relative to Protein = 100		
		Total Cholesterol*	Phospholipids†	Triglycerides†
VLDL	<1.006	63.3	92.5	425
LDL	1.006–1.040	102.1	82.6	130
HDL	1.063–1.21	56.1	36.0	9.1
	1.006–1.050	84.2	72.5	78.9
	1.040–1.050	99.7	70.9	67.2
	1.050–1.063	101.3	53.1	46.9
Apo VLDL‡		§	<1	§
Apo LDL‡		§	<1	§
Apo HDL‡		§	<1	§

* Mean of duplicate determinations.
† Mean of triplicate determinations.
‡ Lipids were extracted from apoproteins with chloroform–methanol 2:1 (18). Extracts were analyzed by thin-layer chromatography as previously described (4).
§, not detected.

By Ouchterlony's double diffusion technique, whole apo VLDL reacted with anti-VLDL or anti-apo VLDL sera, with the appearance of two or three precipitin lines (Fig. 1, *e*, *f*). The outside precipitin lines corresponded to apo LDL and the inside line(s) to apo HDL. Only the outside line was shown by anti-LDL or anti-apo LDL (Fig. 1, *c*, *d*). Apo VLDL reacted slightly against either

TABLE 4 IMMUNOLOGICAL PROPERTIES OF RAT SERUM LIPOPROTEINS AND THEIR APOPROTEINS

Antigen	Anti-HDL	Anti-apo HDL	Anti-LDL	Anti-apo LDL	Anti-apo VLDL*		Anti-Alb
					precipitin line identical to (*apo HDL*)	(*apo LDL*)	
HDL	++	++	−	−	++	−	−
Apo HDL	++	++	−	−	++	−	−
LDL	−	−	+++	+++	−	++	−
Apo LDL	−	−	+	++	−	+	−
VLDL (E)	−	−	++	++	++	++	−
Apo VLDL	+	+	++	++	++	++	−
Serum protein d > 1.21	+	+	−	−	+	−	++
Lipoprotein 1.040 < d < 1.063	+	+	++	+++	+	+++	−
Albumin	−	−	−	−	−	−	+++

* Anti-VLDL sera showed same reactivity as anti-apo VLDL against each of the antigens.

anti-HDL or anti-apo HDL sera (Fig. 1, *a*, *b*). When SDS alone was employed, or was present, as in undialyzed apo VLDL (G) specimens, it produced a nonspecific white line between antigen and antiserum. This was not present with apo VLDL (G) after prolonged dialysis against Tris buffer (0.1 M, pH 8.6). The SDS line was not stained by Amido Schwarz. A summary of the results is given in Table 4.

The amino acid composition is shown in Table 5.

Apo LDL. On agarose gel electrophoresis, apo LDL migrated in the β-region as a single band. Apo LDL (G) exhibited a greater anodic mobility. The same preparation showed a single slowly migrating band by polyacrylamide gel electrophoresis (Fig. 3).

TABLE 5 AMINO ACID COMPOSITION OF RAT SERUM LIPOPROTEINS

	HDL	LDL	VLDL
	moles/100 moles of amino acids		
Aspartic acid	14.2	10.0	8.81
Threonine	5.52	5.83	5.48
Serine	5.56	7.72	6.79
Glutamic acid	21.0	13.0	18.7
Proline	3.08	3.78	4.04
Glycine	5.49	4.91	4.92
Alanine	7.83	6.26	7.70
Half-cystine	0.16	tr.	—
Valine	4.27	5.56	5.13
Methionine	2.59	2.33	2.75
Isoleucine	2.14	4.52	3.68
Leucine	9.64	11.8	10.0
Tyrosine	2.21	2.61	2.15
Phenylalanine	2.53	4.54	3.34
Lysine	5.93	8.85	5.43
Histidine	1.47	1.94	1.38
Arginine	4.79	5.40	7.75
Tryptophan	1.39	0.85	1.96

Results are averages of duplicate analyses of a sample fractionated from pooled serum of 60 animals.

Apo LDL reacted against anti-LDL, anti-apo LDL, anti-VLDL, and anti-apo VLDL sera. LDL and apo LDL demonstrated partial identity against anti LDL sera (Fig. 1, *c*) and total identity against anti-apo LDL sera (Fig. 1, *d*).

Apo LDL (G) showed heterogeneity by sedimentation velocity analysis. The mean peak had an $s_{20,w}$ value of 10.9 (Table 1). By sedimentation equilibrium the plot of ln y against r^2 was nonlinear (at least three slopes could be fitted). An average mol wt calculated from the minor slope of the plot gave values of 188,000 and 92,700 for apo LDL (G) and apo LDL, respectively (Table 2).

The amino acid composition of apo LDL is shown in Table 5.

Apo HDL. Apo HDL migrated as a major band in the β–α_2 region in agarose gel and showed a small faster-moving component. In polyacrylamide gel electrophoresis (3.75% gel with urea) apo HDL was separated into six bands (Fig. 3), one of these being particularly prominent. The components of apo HDL separated by polyacrylamide gel electrophoresis were also checked immunologically. Only the precipitin arc corresponding to the main component (*b*) was detected by anti-apo HDL sera. On the other hand, the arc corresponding to the fast migrating minor components (*e*, *f*), as well as the major component near the origin, was demonstrated by anti-apo VLDL sera. The distribution of immunologically identified apoproteins in rat serum lipoproteins is shown in Table 6. By agarose immunoelectrophoresis, apo HDL reacted against anti-apo HDL sera, showing one main and one variable arc of precipitation (Fig. 2). Two other components were clearly shown by anti-apo VLDL sera in HDL and apo HDL (Fig. 2).

In the Ouchterlony plates, apo HDL reacted both against anti-HDL and anti-apo HDL sera. The arc of precipitation was rather broad and was sometimes seen

TABLE 6 IMMUNOLOGICAL IDENTIFICATION OF APOPROTEINS IN RAT SERUM LIPOPROTEINS

	VLDL	LDL	HDL
Apo LDL	++	++	−
Apo HDL (S)*	+	−	++
Apo HDL (F)†	++	±	+

++, major component; +, minor component; ±, variable; −, nil.
* Slow-migrating component of apo HDL in polyacrylamide gel electrophoresis in 8 M urea.
† Faster-migrating component of apo HDL.

as a double line (Fig. 1, *a*, *b*). This was also seen with anti-VLDL or anti-apo VLDL sera (Fig. 1, *e*, *f*).

Calculated values of $s_{20,w}$ at several different pH's were in the range between 2.0 and 2.3 (Table 1). The sedimentation Schlieren pattern showed a single but rather broad peak suggesting heterogeneity. The molecular weights were calculated from the data on sedimentation equilibrium. The plot of ln y against r^2 deviated from linear near the cell bottom. The molecular weight calculated from the linear portion of the plot was pH-dependent and showed wide variations (Table 2), probably an expression of the degree of association of the system.

The amino acid composition of apo HDL is shown in Table 5.

Spectroscopic Studies

UV Spectroscopy. The UV spectra of HDL, apo HDL, LDL, and apo LDL are shown in Figs. 5 and 6. At pH 8.6, each spectrum showed absorption maxima at approximately 280 nm and at 290 nm. At pH 11.6, there was a shift of the 280 nm peak toward the red, and the 290 nm component was more distinct than at pH 8.6. The spectral changes around 290 nm were more prominent in both of the delipidated materials.

CD Studies. The CD spectrum of HDL (Fig. 7) was characterized by two negative bands with minima at 222 and 208 nm. Apo HDL exhibited a similar pattern (Fig. 7), but differed from HDL in the relative intensity of the $[\theta]_{222}$ and $[\theta]_{208}$ bands, and the position of the crossover point. The differences between the two products were accentuated by changes in temperature (Fig. 7), which indicates a greater thermal sensitivity of apo HDL than of HDL. This is also shown in Fig. 8, in which the course of denaturation is expressed as a function of the percentage change of the 222 nm band. The thermal changes were totally reversible in both HDL and apo HDL although the spectrum of the latter was restored only after 24–48 hr.

The LDL spectrum (Fig. 9) exhibited a negative band with a minimum at 218–220 nm and a clear shoulder at 208 nm. The presence of this band was accentuated in apo LDL, in which a significant decrease of the 218–220 nm band was observed. As in the case of the high density compounds, the spectral difference between LDL and apo LDL was rendered more significant by changes in temperature, LDL being the less sensitive (Fig. 10). Apo LDL further showed a greater degree of thermal instability: a precipitate formed at 72°C, and the remaining soluble product did not give the original spectrum upon cooling. Because of this thermal instability, the data for apo LDL at high temperatures were corrected for the observed protein losses.

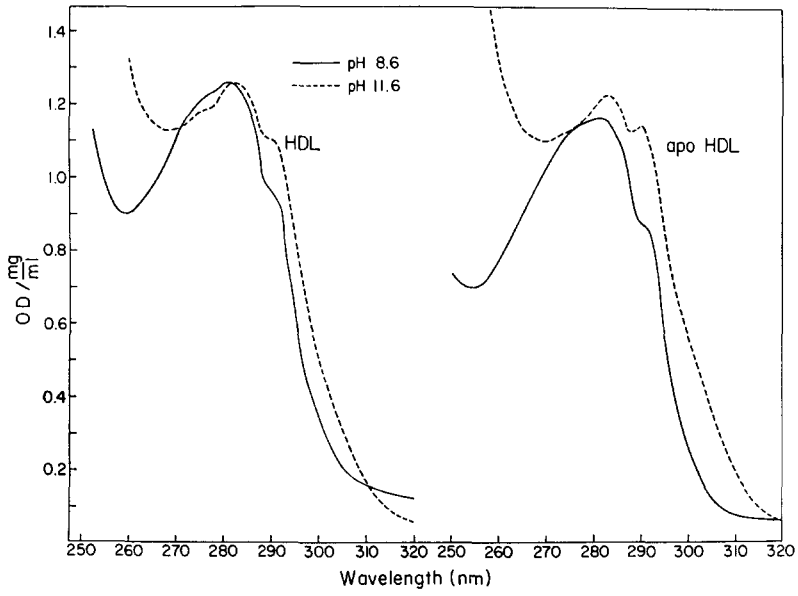

FIG. 5. Effect of pH on UV absorption of HDL and apo HDL.

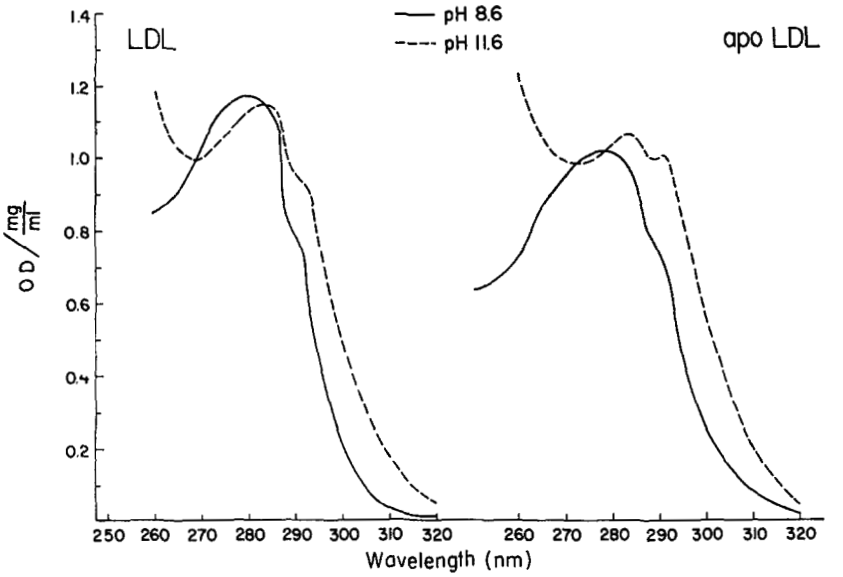

FIG. 6. Effect of pH on UV absorption of LDL and apo LDL.

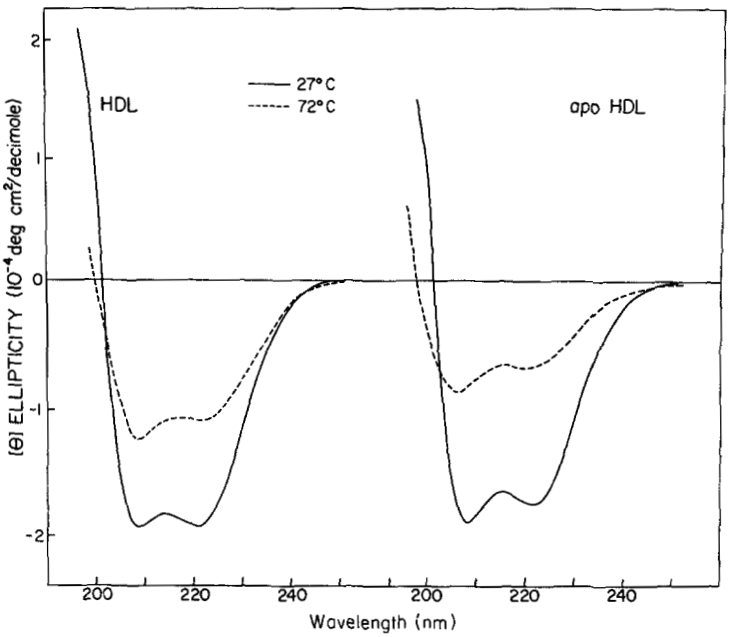

FIG. 7. Effect of temperature on circular dichroic spectra of HDL and apo HDL.

DISCUSSION

This report presents studies on some of the physical, chemical, and immunological properties of rat serum VLDL, LDL, and HDL separated from other serum components by ultracentrifugal flotation. The analysis was also extended to their protein moieties which had been freed of lipids and dissolved in aqueous media by techniques similar to those developed in this laboratory for the corresponding human products (4–7). It should be stated that since the results were obtained in a rather selected group of animals, they may not be extended to all strains of rats. The first significant observation was that the density criteria commonly adopted to separate human LDL and HDL were not applicable to our rat sera. The results indicated the presence of both LDL and HDL species in the fractions isolated between 1.040 and 1.063, and this agrees well with the electrophoretic and immunological data by Windmueller and Levy (23) and the electrophoretic observations by Narayan, Dudacek, and Kummerow (24). On the other hand Camejo and

coworkers (1, 2), who also used sensitive electrophoretic and immunological techniques, reported obtaining satisfactory lipoprotein fractionation essentially by the method of Havel, Eder, and Bragdon (25), which utilizes 1.063 as the limiting density between LDL and HDL. At the moment these findings are difficult to reconcile, although they may simply represent lipoprotein variability among different strains of rats or be dependent on the diet.

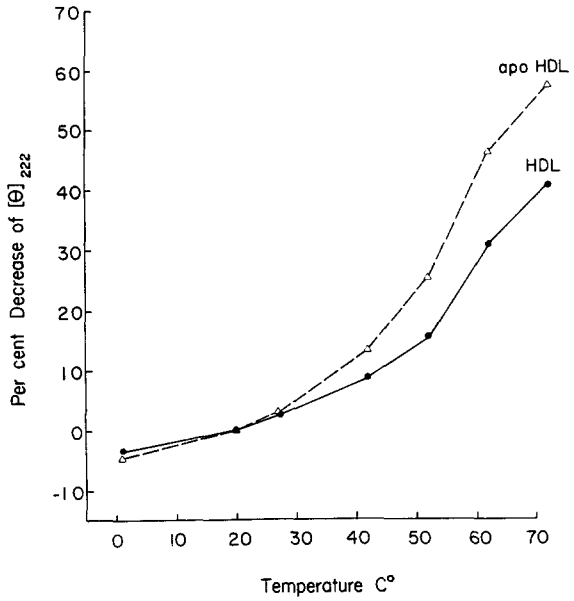

FIG. 8. Course of thermal denaturation of HDL and apo HDL based on percentage changes of the 222 nm band.

Another significant finding was the demonstration that the protein moieties of the three lipoprotein classes studied—VLDL, LDL, and HDL—could be deprived of essentially all their lipid complement and solubilized in aqueous media. As already observed with human products, the preparation of aqueous solutions of both apo VLDL and apo LDL proved more difficult than with apo HDL. In the latter instance, solubilization was achieved in mild alkaline media of low ionic strength, whereas both apo VLDL and apo LDL required more drastic pH conditions, or the use of a dissociating agent such as SDS. However, in spite of these treatments, the final products proved suitable for investigation in terms of the physical, chemical, and immunological techniques employed. Analogous observations have already been made for human products in this laboratory (6) and by others (26–29).

The lipoprotein classes of rat serum differed markedly from each other, and their properties will be separately analyzed.

HDL

This lipoprotein, which appeared physically homogeneous by the analytical ultracentrifuge (flotation and sedimentation studies, sedimentation equilibrium), seemed in contrast to be heterogeneous by the immunological and electrophoretic techniques employed. One reason for the heterogeneity could be that we are dealing with lipoprotein species having identical protein moieties but with varying amounts of bound lipids, which account for the observed differences in electrophoretic mobility

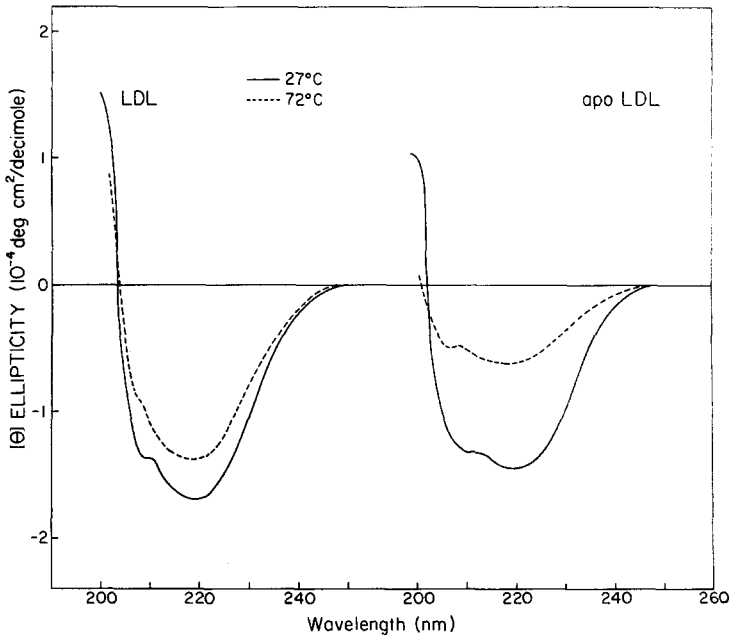

FIG. 9. Effect of temperature on circular dichroic spectra of LDL and apo LDL.

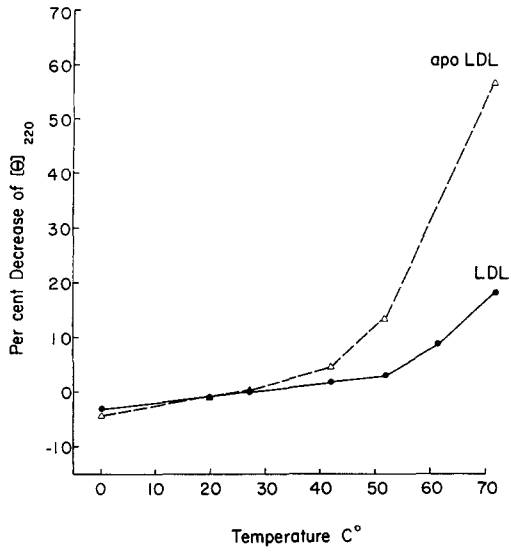

FIG. 10. Course of thermal denaturation of LDL and apo LDL based on percentage changes of the 220 nm band.

and immunological reactivity against anti-HDL sera. Alternatively, HDL could be composed of distinct lipoprotein species of similar density, in which the protein moieties differed both chemically and immunologically.

This latter possibility appears to be supported by the persistence of heterogeneity in the HDL protein after removal of its lipid moiety (ultracentrifugal, electrophoretic, and immunological data), and also by the clear evidence now obtained in this laboratory that apo HDL can be fractionated into at least three chemically distinct classes of components by chromatographic procedures carried out in 8 M urea (Koga and Scanu, unpublished). The heterogeneity of human apo HDL has also been reported (30–33). In view of the above, our estimation of a mol wt of 27,900 for apo HDL, which agrees with Camejo's data (1), should not be considered definitive, but probably dependent upon the state of aggregation of the apoprotein under the experimental conditions employed. The molecular weight of HDL was about twice that of human HDL (30), porcine HDL (34), or rat HDL reported by Camejo (1). Although the reasons for these discrepancies are not apparent, it must be pointed out that in polyacrylamide gel electrophoresis (7.5% gel) some of our rat HDL did not enter the medium, whereas human HDL under comparable conditions entered it completely.

LDL

In contrast to HDL, LDL appeared homogeneous by the various techniques employed, with the exception of a few specimens in which unexplained small, fast electrophoretic components were seen. Upon delipidation, regardless of the method of solubilization employed (SDS or titration method), the immunological properties of the product were similar although not indentical with those of the parent lipoprotein, and its hydrodynamic properties (sedimentation velocity and equilibrium sedimentation studies) were suggestive of an aggregating system. This interpretation was supported by the results of disc electrophoresis, which indicated that heterogeneous bands were formed in gel containing 8 M urea. On the other hand, true chemical dissimilarities among protein components or even among LDL species cannot be ruled out by the results of our current experiments, and such a possibility is under inquiry in this laboratory. One is reminded at this point of the recent results obtained by Windmueller and Levy (35), which indicate that circulating β-lipoprotein protein may come from two sources—hepatic and intestinal—and the lipoproteins built on these proteins may have different physical, chemical, and immunological properties.

VLDL

Our immunological studies reveal three distinct antigenic determinants, two reacting with anti-HDL and anti-LDL sera, the third one unidentified. Immunological evidence of apo HDL and apo LDL in rat serum VLDL has already been reported by Camejo (1), and in humans by a number of authors (36, 37). However, the true chemical relationship between these protein components of VLDL and those of LDL and HDL is not known. That in fact some dissimilarities may exist between the components of apo HDL and VLDL–apo HDL is suggested by the observed differences in reactivity of apo HDL and apo VLDL against either anti-apo HDL or anti-apo VLDL sera (see Figs. 1 and 2), and by the electrophoretic data (disc gel in 8 M urea) showing that the two fast anodic bands, rather minor in apo HDL, were major components in VLDL–apo HDL (see Fig. 3). This observation was consistently made in all products analyzed. Definition of the chemical nature of these components is likely to provide a better understanding of the structural and functional relationship between VLDL and HDL and work along these lines is under way in this laboratory. In terms of VLDL–apo LDL our data do not go beyond its qualitative detection by both immunological and electrophoretic techniques, although others have suggested (38) that it represents about 20% of the whole VLDL protein. It should be stressed, however, that these estimates may vary according to the physiological condition of the animal or to any pathological state leading to an increase of circulating VLDL.

The identification of the third antigenic determinant of VLDL protein remains open to inquiry. In human products, a protein component distinct from apo HDL and apo LDL, having serine and threonine as *N*-terminal groups, has been observed (39) and its isolation has recently been reported (37, 40). Whether such observations

may be applicable to the rat remains to be established. It is clear that the definition of the nature and function of the "third" protein component of VLDL represents a challenging problem for investigation. These studies should not neglect the interesting observation by Ockner, Bloch, and Isselbacher (41), which indicates that VLDL, aside from its accepted hepatic origin (42–44), may also arise from the intestinal tract. We are presently exploring the possibility that the two fast electrophoretic bands seen as major components of apo VLDL, with peculiar immunolgical reactivity, are responsible for the unidentified arc of immunoprecipitation seen when apo VLDL reacts with either anti-VLDL or anti-apo VLDL sera.

d > 1.21 Infranate

Since this fraction is composed of a mixture of serum proteins, only immunological studies were possible. With the antisera employed, evidence was obtained only for a component reacting with both anti-HDL and anti-apo HDL sera, and this agrees well with data on human products (44, 45). The d > 1.21 fraction also reacted against anti-VLDL and anti-apo VLDL sera. However, in the case of the last two antisera, their reaction against d > 1.21 fraction was attributable to their anti-HDL component (see Table 4). Other lipoprotein components of the type described by Roheim, Miller, and Eder (46) and Lees (47) were not detected in this work. Obviously their antigenic, and probably their structural, makeup is distinct from the lipoprotein proteins analyzed in these studies, and thus no direct comparisons are permissible.

Structural Consideration Based upon Spectroscopic Studies

The circular dichroic spectra of both LDL and HDL (spectra of VLDL were not obtained because of high absorbancy) were remarkably similar to those recorded in this laboratory for the corresponding human products, and the reader is referred to those publications (7, 48, 49) for interpretation of the data. What we should like to stress here is that the spectral characteristics of both LDL and HDL protein are to a large extent dependent on their primary structure, and that lipid moiety contributes to secondary structure only minimally, if at all. On the other hand, lipids appear to stabilize the protein conformation of both apo LDL and apo HDL through protein–lipid interactions known to occur in the corresponding lipoproteins. The different ways in which the lipoprotein species under study and their delipidated derivatives respond to environmental changes support such a contention. The temperature curves for LDL and HDL (Figs. 8 and 10) impress one with the stability of the lipoproteins and the enhanced instability of the lipid-free products.

Structural Considerations Based upon Immunological Studies

If protein and lipids are mutually dependent for the structural stability of serum lipoproteins, the question arises as to whether the mutual dependence also determines the immunological specificity of these macromolecules. The results of our studies clearly indicate that all of the three apoproteins studied (apo VLDL, apo LDL, and apo HDL) retained, in their lipid-free state, antigenic activity that was similar to, although not identical with, that of the parent complexes. For apo HDL the difference from lipoprotein was clearly shown only by immunoelectrophoresis (Fig. 2), whereas with apo LDL and apo VLDL it became evident by all techniques employed, regardless of the method of solubilization (SDS or titration). These results suggest that lipids play an immunological role in serum lipoproteins, acting either as haptens or by their stabilizing effect on the apoprotein conformation, as indicated by the spectral studies. At the moment it is not possible to distinguish between these possibilities, which may well be interrelated. More information on the intrinsic antigenic properties of the protein-free lipid moieties of serum lipoproteins is required. Further, some caution must be observed when the lipid-free moieties of these lipoproteins are used as immunizing agents. These proteins are potentially capable of lipid binding (50). Thus, it cannot be ruled out that some degree of relipidation may occur when they are injected into the animal used for immunization. It is apparent that we are confronted with a complex problem for which no ready answer is yet available.

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