Scoffone, E. (1969), *J. Amer. Chem. Soc. 91*, 3297.
Scatturin, A., Tamburro, A. M., Rocchi, R., and Scoffone, E. (1967), *Chem. Commun.*, 1203.
Scoffone, E., Marchiori, F., Rocchi, R., Scatturin, A., and Tamburro, A. M. (1971), *in* Peptides, Scoffone, E., Ed., Amsterdam, North-Holland Publishing Co., p 233.
Scoffone, E., Marchiori, F., Rocchi, R., Vidali, G., Tamburro, A. M., Scatturin, A., and Marzotto, A. (1966), *Tetrahedron Lett.*, 943.
Sela, M., and Anfinsen, C. B. (1957), *Biochim. Biophys. Acta 24*, 229.
Sela, M., Anfinsen, C. B., and Harrington, W. F. (1957), *Biochim. Biophys. Acta 26*, 502.
Sherwood, L. M., and Potts, J. T., Jr. (1965a), *J. Biol. Chem. 240*, 3799.
Sherwood, L. M., and Potts, J. T., Jr. (1965b), *J. Biol. Chem. 240*, 3806.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Takahashi, T., Irie, M., and Ukita, T. (1969), *J. Biochem. (Tokyo) 65*, 55.
Tamburro, A. M., Scatturin, A., Rocchi, R., Marchiori, F., Borin, G., and Scoffone, E. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 298.
Tsong, T. Y., Hearn, R. P., Wrathall, D. P., and Sturtevant, J. M. (1970), *Biochemistry 9*, 2666.
Wellner, D., Silman, H. J., and Sela, M. (1963), *J. Biol. Chem. 238*, 1324.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 304.
Winchester, B. G., Mathias, A. P., and Robin, B. R. (1970), *Biochem. J. 117*, 299.
Woodfin, B. M., and Massey, V. (1968), *J. Biol. Chem. 243*, 889.
Wyckoff, H. W., Hardmann, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., and Richards, F. M. (1967), *J. Biol. Chem. 242*, 3984.

Human Serum Lipoproteins. Evidence for Three Classes of Lipoproteins in S_f 0–2*

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ABSTRACT: The lipoprotein composition of the S_f 0–2 lipoproteins from the serum of individuals and from pooled serum was studied. S_f 0–2 was found to contain three classes of lipoproteins: high density lipoproteins (HDL_1), low density lipoproteins (LDL_3), and a lipoprotein which shares antigenic determinants with LDL, LDL-a-1. HDL_1 has a sedimentation coefficient at d 1.002 g/cm^3 ($s_{1.002}$) of 4.6 S, and a molecular weight by Agarose gel chromatography of 0.5×10^6. The electrophoretic and immunological properties of HDL_1 are similar to those of HDL_2. Delipidated HDL_1 and HDL_2 were found by polyacrylamide gel electrophoresis to contain the same polypeptides. LDL_3 has electrophoretic and immunological properties similar to those of LDL isolated from the density range 1.030–1.040 g/cm^3, and $s_{1.002}$ = 8.4 S. Its molecular weight is 1.8×10^6. LDL-a-1 has $s_{1.002}$ = 12.7 S, and a molecular weight of 5.4×10^6. LDL-a-1 is also present in the density range 1.060–1.075 g/cm^3; when isolated from that density range, LDL-a-1 was found to have $s_{1.002}$ = 13.4 S. Both LDL-a-1 fractions have a pre-β mobility by Agarose gel immunoelectrophoresis. Anti-LDL-a-1 antisera were absorbed with LDL (d 1.030–1.040 g/cm^3). The absorbed antisera precipitated with LDL-a-1 but not with LDL.

Serum lipoproteins S_f 0–2 are normally present in low concentrations, approximately 15–35 mg/100 ml. Elevated levels have been reported to be associated with high levels of very low density lipoproteins (Nichols, 1967).

There is some uncertainty in the literature about the types of lipoproteins which occur in S_f 0–2 (d 1.050–1.060 g/cm^3) and in the density range 1.060–1.075 g/cm^3. Both low and high density lipoproteins[1] have been found by immunochemical methods in the density range 1.050–1.063 g/cm^3 (Alaupovic, 1968; Lee and Alaupovic, 1970) and in the density range 1.063–1.110 g/cm^3 (Ayrault-Jarrier *et al.*, 1963; Alaupovic, 1968). The major questions raised by these observations are as follows. (1) Are HDL and LDL of different hydrated density but occur in these density ranges due to incomplete separation during centrifugation? (2) Are HDL and LDL which occur in these density ranges of similar hydrated density but nonetheless distinct molecular species? (3) Are the lipoproteins which occur in these density ranges hybrid molecules containing both HDL and LDL antigens? Answers to these questions are essential before we can proceed to study the details of polypeptide composition of the lipoproteins of these hydrated density ranges.

We have determined the types of lipoproteins which occur in S_f 0–2 (d 1.050–1.060 g/cm^3) and in the density range 1.060–1.075 g/cm^3. Lipoproteins were characterized by sedimentation and flotation analyses in the analytical ultracentrifuge, isopycnic density gradient centrifugation, Agarose gel chromatography, polyacrylamide gel electrophoresis, and immuno-

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[1] Abbreviations used are: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; the polypeptides are designated by their carboxyl-terminal amino acids as R-Thr, R-Gln, R-Glu, and R-Ala.

electrophoreses and immunodiffusion. Evidence is presented that S_f 0–2 is composed of at least three distinct classes of lipoproteins.

Materials and Methods

Preparation of Lipoproteins. Lipoproteins were isolated from pooled human sera and from the sera of individual donors by sequential preparative ultracentrifugation (DeLalla and Gofman, 1954; Havel *et al.*, 1955) at 16°. Specifically, the following procedure was used. The nonprotein solvent density of the sera was adjusted to 1.050 g/cm^3 with solid NaCl. Centrifugation was then carried out in a 30.2 rotor at 27,000 rpm for 29 hr. The top 3.5 ml of each tube was removed with a tube slicer. After diluting the bottom fraction to 6 ml with *d* 1.050-g/cm^3 NaCl solution, the density was readjusted to 1.060 g/cm^3 with solid NaCl and recentrifuged in a 40.3 rotor at 32,000 rpm for 26 hr. The top 0.7 ml was carefully pipetted off from each tube and constituted the S_f 0–2 lipoproteins. The infranatant obtained after removal of the S_f 0–2 lipoproteins was brought to a density of 1.075 g/ml with solid NaCl and centrifuged in a 40.3 rotor at 32,000 rpm for 28 hr. The top 0.7 ml of each tube was carefully pipetted off. This fraction contained the lipoproteins of *d* 1.060–1.075 g/cm^3. Both the S_f 0–2 lipoproteins and the lipoproteins of *d* 1.060–1.075 g/cm^3 were washed once by recentrifugation in a *d* 1.120-g/cm^3 NaCl solution at 35,000 rpm for 26 hr. Alternatively, these fractions were washed once at their respective upper density limits at 34,000 rpm for 26 hr. LDL of *d* 1.030–1.040 g/cm^3 and *d* 1.040–1.050 g/cm^3, and HDL of *d* 1.080–1.120 g/cm^3 were similarly isolated. They were centrifuged once again at each separation density. After isolation, the preparations were dialyzed against 0.01 M Tris-HCl (pH 8.2) overnight and then concentrated with either Carbowax 20M (Union Carbide) or Aquacide II (Calbiochem). Lipoproteins were subsequently dialyzed against appropriate salt solutions prior to ultracentrifugal analyses. All sera, dialyzing solutions, and salt solutions used contained 0.001 M EDTA, adjusted to pH 7.4 with solid NaOH. The density of the salt solutions was checked by refractometry.

Ultracentrifugation Studies. Flotation analysis was performed at *d* 1.0653 g/cm^3 (NaCl, 20°) in a Spinco Model E ultracentrifuge equipped with RTIC temperature control unit and schlieren optical system, using an AnH rotor and a 12-mm charcoal-filled Epon double-sector centerpiece. Sedimentation analysis was carried out under similar conditions but in a 0.1 M NaCl solution of *d* 1.002 g/cm^3. Boundary positions were located by the maximum ordinate technique and the flotation and sedimentation coefficients evaluated in the usual manner (DeLalla and Gofman, 1954; Schachman, 1959). Most of the determinations were made at protein concentrations of 0.25% or less, except in two cases of the *d* 1.060–1.075-g/cm^3 preparation, when the protein concentrations were about 0.35%. Because of the multicomponent nature of the sample, no corrections were made for concentration dependence. Unless otherwise indicated, speed of centrifugation was 52,000 rpm.

Isopycnic Density Gradient Analysis. Density gradient centrifugation was performed in a 7–21% sucrose gradient containing 0.001 M EDTA. Three-tenths of 1 ml of the lipoprotein fraction which had been dialyzed against 0.1 M NaCl solution overnight was layered onto the gradient (4.7 ml) and centrifuged in a SW 50.1 rotor at 40,000 rpm for 24 hr in a Spinco Model L2-65B ultracentrifuge at 18°. After centrifugation, the bottom of each tube was punctured, and three-drop fractions collected, and then analyzed for protein content. Gradients formed as described above, but with dialysate in place of sample were centrifuged concurrently. Three-drop fractions were similarly collected and refractive index was measured in a Bausch and Lomb precision refractometer at 20°. Fraction densities were then calculated from refractive indices.

Agarose Gel Chromatography. The S_f 0–2 lipoproteins and lipoproteins of *d* 1.060–1.075 g/cm^3 were subfractionated by gel chromatography on 6% beaded Agarose gel (Bio-Gel A-5m, 200–400 mesh; Bio-Rad Laboratories, Calif.) at 6–8° using a 1.5 × 90 cm column. This gel was selected to obtain maximum resolution. Before use, the column was coated with a 1% solution of dimethyldichlorosilane in benzene (Bio-Rad Laboratories, Calif.). To prevent clogging at the bottom by the fine particles, a small amount of Bio-Gel A-5m (100–200 mesh), about 2 cm in height, was first put on top of the nylon support. The eluting buffer was 0.1 M Tris-HCl–0.15 M NaCl–0.001 M EDTA (pH 8.2). A pressure head of 20–30 cm was applied from a mariotte flask to keep the flow rate at 3–4 ml/hr, and fractions were collected at 1.5 ml/tube. The sample volumes were below 1.5% of the total volume of the column. Blue Dextran 2000 and ^{125}I were used to measure precisely the void volume (V_0) and the total solvent volume ($V_0 + V_i$), respectively, of the column. The values for partition coefficient (K_d) of the solute were obtained from the relation $K_d = (V_e - V_0)/V_i$ (Gelotte, 1960), where V_e is the elution peak volume of the solute, V_i is the inner volume of the gel, and V_0 is the void volume.

Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis of the whole lipoproteins was performed essentially according to Narayan *et al.* (1966). The lipoproteins were prestained with Sudan Black B as described by McDonald and Ribeiro (1959). For the delipidation of lipoproteins the procedure of Albers *et al.* (1971) was used. Polyacrylamide gel electrophoresis of the delipidated lipoproteins was carried out at pH 8.9 in 8 M urea by the method of Davis (1964). The protein was fixed with 10% trichloroacetic acid and stained with Amido-Schwarz in 7% acetic acid.

Immunological Methods. ANTIGEN PREPARATION. LDL (*d* 1.030–1.040 g/cm^3) was prepared from pooled sera as described above. The polypeptides R-Thr and R-Gln were prepared as described previously (Borut and Aladjem, 1971). The polypeptides R-Ala and R-Glu were isolated from VLDL as described (Albers and Scanu, 1971). LDL-a-1 was prepared as follows. Approximately 300 μg of the S_f 0–2 protein was placed on each of 10–12 polyacrylamide gel columns. The bands corresponding to LDL-a-1 (see Figure 6) were cut out of the gels, pooled, and crushed in a mortar with a pestle.

ANTISERA PREPARATION. Anti-LDL antisera were prepared as previously described (Aladjem *et al.*, 1957). These antisera did not react with HDL, nor with human serum albumin, nor with the polypeptides R-Ala or R-Glu isolated from VLDL. Anti-R-Thr and anti-R-Gln were those of previous studies (Borut and Aladjem, 1971; Albers and Aladjem, 1971). Anti-LDL-a-1 antisera were prepared as follows. The purified LDL-a-1 obtained from polyacrylamide gel electrophoresis was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were immunized as described previously (Albers and Dray, 1968). Antisera specific for the a-1 specificity were prepared as follows. Anti-LDL-a-1 was absorbed with LDL (*d* 1.030–1.040 g/cm^3) until it no longer reacted with LDL; these absorbed antisera were designated anti-a-1.

GEL DIFFUSION. Immunodiffusion and immunoelectrophoresis were carried out in 1% Agarose in 0.05 M Tris-HCl (pH

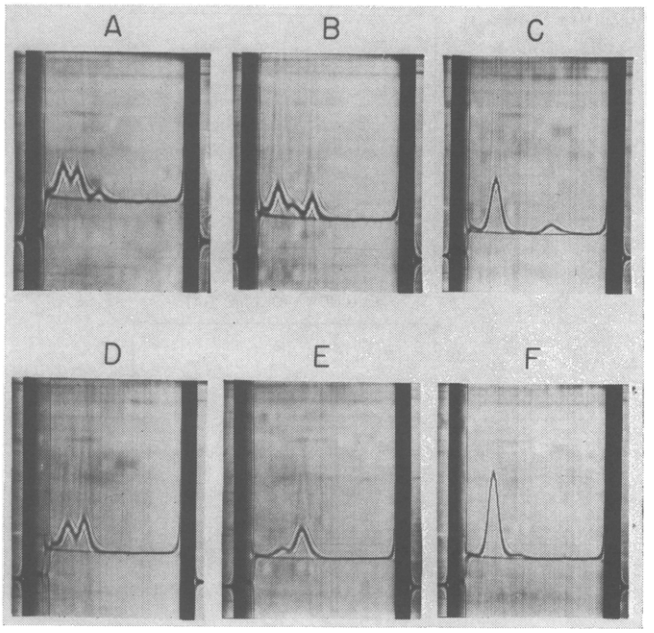

FIGURE 1: Analytical ultracentrifuge analysis of lipoprotein fractions in 0.1 M NaCl–1 × 10^{-3} M EDTA (pH 7.2). (A) S_f 0–2, from serum 70; (B) S_f 0–2, from serum 87; (C) lipoprotein of d 1.060–1.075 g/cm³, from pool 1; (D) S_f 0–2, from serum 19; (E) S_f 0–2, from serum 35; (F) lipoprotein of d 1.060–1.075 g/cm³, from serum 35. The rotor speed was 52,000 rpm except in part D which was 48,000 rpm. Photographs were taken at 32 min (A and B), 40 min (D), and 48 min (C, E, and F) after reaching speed. The phase-plate angle was 45° in A and B; 50° in D; 55° in E and F; 60° in C. Direction of sedimentation is from left to right.

8.4) and in 0.05 M barbital buffer (pH 8.6), respectively (Albers and Aladjem, 1971).

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951); human serum albumin (Pentex) was used as the standard.

Results

Analytical Ultracentrifugation. Representative schlieren patterns of the S_f 0–2 lipoproteins obtained by analytical ultracentrifugation in an NaCl solution of d 1.002 g/cm³ are shown in Figure 1 (A,B,D,E). The S_f 0–2 lipoproteins gave at least two peaks with sedimentation coefficients of 4.6 ± 0.1 and 8.4 ± 0.3 S, respectively. In addition, preparations isolated from serum pools showed a third peak with a sedimentation coefficient of 12.7 ± 0.3 S as shown in Figure 1A,B. This 12.7S component was also found in 4 of 13 S_f 0–2 preparations isolated from the sera of individuals. By analytical ultracentrifugation of S_f 0–2 at d 1.0653 g/cm³, only one peak was obtained with S_f = 1.6 ± 0.2 S.

Representative schlieren patterns of the lipoproteins of d 1.060–1.075-g/cm³ run at d 1.002 g/cm³ are shown in Figure 1C,F. All preparations isolated from serum pools contained at least two sedimenting components, a major component of 4.7 ± 0.1 S and a minor component of 13.4 ± 0.2 S. An 8.4S component was occasionally seen, which occurred at very low concentrations (Figure 1F). All preparations isolated from the serum of individuals contained the 4.7S component. The 13.4S component was found in four of seven sera. The four sera which contained the 13.4S component also contained the 12.7S component in S_f 0–2.

Sucrose Density Gradient Centrifugation. A single, somewhat skewed distribution was obtained when S_f 0–2 was centrifuged for 2.2 × 10^8 g min in a 7–21% sucrose density gradient. The result of one experiment is shown in Figure 2. More than 85% of the lipoprotein was distributed within the density range 1.042–1.060 g/cm³ with a peak at d 1.051 g/cm³. Very similar results were obtained with each of three other S_f 0–2 preparations. The value of 1.051 g/cm³ for the hydrated density of S_f 0–2 coincides with the estimated value 1.05 g/cm³ obtained by Lindgren and Nichols (1960).

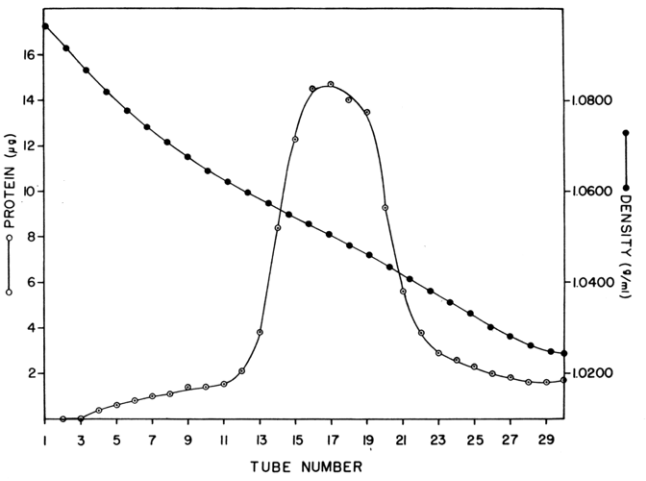

FIGURE 2: Density gradient elution from a 7–21% sucrose gradient of S_f 0–2 isolated from serum 87.

Agarose Gel Chromatography. For calibration, LDL (d 1.030–1.040 g/cm³) and HDL_2 (d 1.080–1.120 g/cm³) were each isolated from two serum pools. The results of a representative run of a mixture of LDL and HDL are shown in Figure 3. The run described in Figure 3 yielded two major and two minor fractions. The first minor fraction appeared at the void volume. The second minor fraction appeared at an elution volume (V_e) of 128 ml, slightly before the total solvent volume ($V_0 + V_i$) of 130 ml. This second minor fraction was more prominent with preparations which were concentrated with Carbowax. Preparations which were concentrated with Aquacide II contained negligible absorbing materials at this elution volume (see Figures 3 and 4B). The first major fraction occurred at V_e of 75 ml and by immunochemical methods was found to be LDL. The second major fraction occurred at V_e of 93 ml and, by similar methods, was found to be HDL. There was little overlap between the LDL and HDL lipoprotein fractions. S_f 0–2 was then isolated from the serum of four individuals and from three serum pools. The results of two representative runs are shown in Figure 4. The run de-

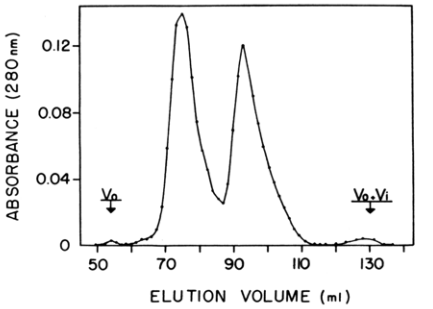

FIGURE 3: Absorbance of eluates from Bio-Gel A-5m of a mixture of LDL (d 1.030–1.040 g/cm³) and HDL_2 (d 1.080–1.120 g/cm³) equilibrated with Tris–NaCl (pH 8.2) buffer.

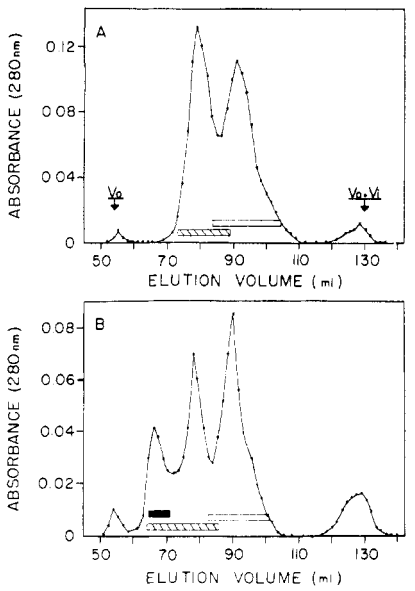

FIGURE 4: Absorbance of eluates from Bio-Gel A-5m of S_f 0–2 equilibrated with Tris–NaCl (pH 8.2) buffer. (A) S_f 0–2 from serum 19; (B) S_f 0–2 from pool 1. Precipitation with anti-LDL, ▨; precipitation with anti-a-1, ■; precipitation with anti-R-Thr, ▭. The void volume and total solvent volume of the column are indicated by vertical arrows labeled V_0 and (V_0 + V_i), respectively.

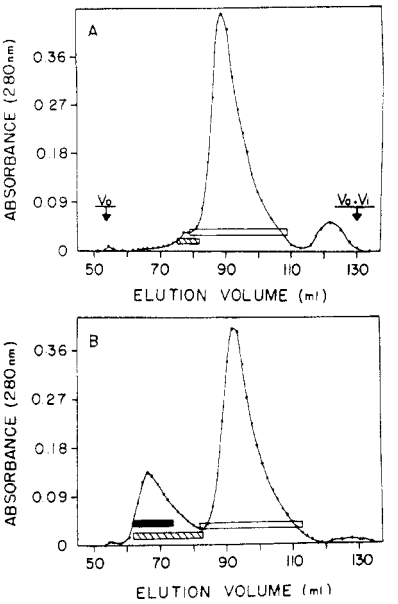

FIGURE 5: Absorbance of eluates from Bio-Gel A-5m of the lipoproteins of d 1.060–1.075 g/cm³. Conditions were the same as those in Figures 3 and 4. (A) Lipoprotein from serum 16; (B) lipoprotein from serum 45. Immunochemical reactivity as indicated in Figure 4.

scribed in Figure 4A contained two major and two minor fractions. The two minor fractions had elution volumes similar to those of the minor fractions in Figure 3. The first major fraction occurred at V_e of 78 ml, the second major fraction at V_e of 91 ml. These two fractions were found in every S_f 0–2 preparation. In the experiment described in Figure 4B, two minor and three major fractions were obtained. The first major fraction had V_e of 66 ml. The other two major fractions had elution volumes similar to those of the major fractions in Figure 4A.

Every eluate from every run was tested with anti-LDL, anti-a-1, and anti-R-Thr antisera. Material from any of the first fractions did not react with any of the antisera. The fractions with V_e = 66 ml reacted with anti-LDL and anti-a-1 but not with anti-R-Thr. The fractions with V_e = 78 ml reacted with anti-LDL but did not react with anti-a-1 or anti-R-Thr. The fractions with V_e = 91 ml reacted with anti-R-Thr but not with anti-a-1 or anti-LDL. Based upon these results, we shall use the nomenclature LDL-a-1, LDL_3, and HDL_1 to describe the three types of lipoproteins found in S_f 0–2. Lipoprotein which reacts with both anti-LDL and anti-a-1 was designated LDL-a-1, whereas lipoprotein which reacts with anti-LDL but not with anti-a-1 was designated LDL_3. Lipoprotein which reacts with anti-R-Thr was designated HDL_1.[2] All S_f 0–2 preparations, whether isolated from individuals or from serum pools, contained LDL_3 and HDL_1. All S_f 0–2 preparations isolated from pooled serum and those isolated from two of four individuals contained, in addition, LDL-a-1. By our sensitive immunochemical procedures LDL-a-1 was not detected in the preparations which lacked the 12.7S component.

[2] HDL_1 has been considered synonymous with S_f 0–2 (Lindgren and Nichols, 1960) and S_f 0–3 (Shore and Shore, 1962; Nichols, 1967). We shall consider HDL_1 as those lipoproteins in S_f 0–2 which have immunochemical and chemical properties similar to those of HDL_2.

We also studied the lipoproteins which occur in the density range 1.060–1.075 g/cm³. Sera from three individuals and from three serum pools were studied. The results of two representative runs are shown in Figure 5. A major fraction with V_e of approximately 91 ml, corresponding to HDL (d 1.060–1.075 g/cm³), was found in every run. Pooled serum and serum from one individual, contained in addition a fraction with a V_e = 66 ml as shown in Figure 5B. Preparations which contained this fraction contained both the 4.7S and 13.4S components. Preparations from the sera of two individuals which lacked this fraction, contained only the 4.7S component. When the LDL-a-1 fractions from several runs were pooled and rechromatographed, only one fraction was obtained which reacted with anti-LDL and anti-a-1; its V_e = 66 ml.

The molecular weights of LDL (d 1.030–1.040 g/cm³) and of HDL_2 have been reported to be 2.3×10^6 (Adams and Schumaker, 1969) and 0.4×10^6 (Hazelwood, 1958), respectively. Using these values and our experimental values of K_d, we calculated the molecular weights of the fractions by the method of Andrews (1964). These results, as well as the sedimentation coefficients and immunochemical properties are summarized in Table I.

Polyacrylamide Gel Electrophoresis. S_f 0–2 and lipoproteins of d 1.060–1.075 g/cm³ were run on polyacrylamide gel electrophoresis. Results obtained with S_f 0–2 are shown in Figure 6A,B. Three major bands were obtained. Each of 25 different preparations examined contained bands 2 and 3. Band 2 has electrophoretic mobility similar to LDL of d 1.030–1.040 g/cm³; it appeared as a single broad band or as two closely spaced bands. Band 3 has electrophoretic mobility similar to and indistinguishable from HDL_2. LDL-a-1 obtained from Agarose gel chromatography gave a single band corresponding to band 1 (Figure 6C). All preparations which lacked LDL-a-1 also lacked the 12.7S component. We conclude that when S_f 0–2 is applied onto the gel, band 1 is LDL-a-1, band 2 is LDL_3, and band 3 is HDL_1.

Every lipoprotein preparation of d 1.060–1.075 g/cm³ contained band 3, corresponding to HDL_2. Band 2, corresponding

TABLE I: Some Characteristics of Human Serum Lipoproteins Occurring in the Density Range 1.030–1.120 g/cm³.

Density Range (g/cm³)	Lipoprotein	$S_{20,1.002}$	Pptn with Antisera: Anti-a-1	Anti-LDL	Anti-R-Thr	Elution Vol (ml)[a]	Partition Coef	Mol Wt × 10⁻⁶[f]
1.030–1.040	LDL		−	+	−	75	0.28 (2)[c]	2.3[d]
1.080–1.120	HDL_2		−	−	+	93	0.51 (2)	0.4[e]
1.050–1.060	LDL-a-1	12.7 ± 0.3[b] (4)[c]	+	+	−	66	0.16 (2)	5.4
	LDL_3	8.4 ± 0.3 (3)	−	+	−	77–79	0.32 ● 0.01[b] (7)[c]	1.8
	HDL_1	4.6 ± 0.1 (3)	−	−	+	90–91	0.48 ± 0.01 (5)	0.5
1.060–1.075	LDL-a-1	13.4 ± 0.2 (4)	+	+	−	65.5–66.5	0.16 ± 0.01 (4)	5.4
	HDL	4.7 ● 0.1 (4)	−	−	+	89.5–92.5	0.49 ± 0.01 (6)	0.5

[a] From Bio-Gel A-5m. [b] Mean plus and minus standard deviation. [c] Number of preparations, each obtained from different individuals or serum pools. [d] Adams and Schumaker (1969). [e] Hazelwood (1958). [f] Molecular weight calculated on the basis of elution volume, except as otherwise noted.

to LDL, was occasionally seen as a faint band. Seven of sixteen preparations contained band 1. All preparations which contained band 1 in S_f 0–2 also contained band 1 in *d* 1.060–1.075 g/cm³.

The results of polyacrylamide gel electrophoresis of delipidated HDL_1 and HDL_2 are shown in Figure 7. Delipidated HDL_1 preparations gave two major and approximately nine minor bands. Similar results were obtained with delipidated HDL_2. The HDL_1 preparations appear to contain all the bands present in HDL_2 when HDL_1 and HDL_2 were run at equivalent concentrations. The similarity in the intensity of staining of the bands suggests that the bands obtained with delipidated HDL_1 could not be due simply to contamination of HDL_1 with HDL_2. We conclude, therefore, that HDL_1 contains all or nearly all the polypeptides of HDL_2.

Immunochemical Studies. The immunochemical relationship of LDL-a-1 to LDL and LDL_3 was studied by immunodiffusion in Agarose. LDL (*d* 1.030–1.040 g/cm³), LDL_3, and LDL-a-1 were placed in adjacent wells and tested against anti-LDL. Anti-LDL precipitated each of the above lipoproteins, and the three precipitin bands fused. We conclude that both LDL-a-1 and LDL_3 contain the major antigenic determinants of LDL. Anti-LDL-a-1 precipitated both LDL and LDL-a-1. When anti-LDL-a-1 was absorbed with LDL (*d* 1.030–1.040 g/cm³), the absorbed antisera (anti-a-1) still precipitated LDL-a-1 but did not precipitate LDL or LDL_3. This result indicates that LDL-a-1 contains antigenic determinants which are not present in LDL (*d* 1.030–1.040 g/cm³) or LDL_3.

As shown in Figure 8, LDL-a-1 reacted with both anti-LDL and anti-a-1. LDL-a-1 has pre-β mobility by Agarose gel immunoelectrophoresis. The reaction of S_f 0–2 with anti-R-

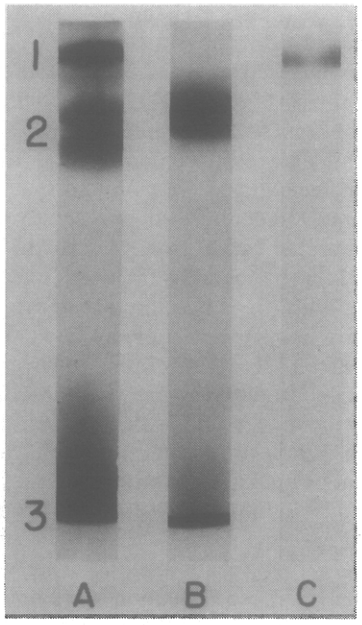

FIGURE 6: Polyacrylamide gel electrophoresis of lipoprotein fractions prestained with Sudan Black B performed in 3.5% acrylamide (pH 8.9). (A) S_f 0–2 containing LDL-a-1; (B) S_f 0–2 lacking LDL-a-1; (C) LDL-a-1 obtained after rechromatography on Bio-Gel A-5m column. The origin is at the top, the anode is at the bottom.

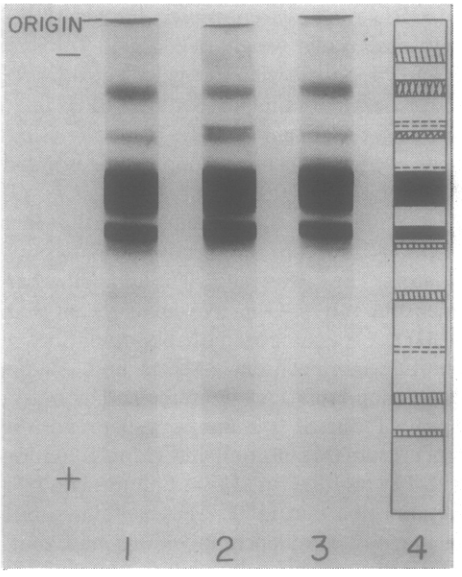

FIGURE 7: Polyacrylamide gel electrophoresis of delipidated lipoproteins performed in 7.5% acrylamide (pH 8.9) in 8 M urea. (1) Delipidated HDL_2 of *d* 1.080–1.120 g/cm³; (2) delipidated HDL_1 from serum 34; (3) delipidated HDL_1 from serum 35; (4) schematic representation of polyacrylamide gel results. Bands represented by dotted lines were not seen in all preparations.

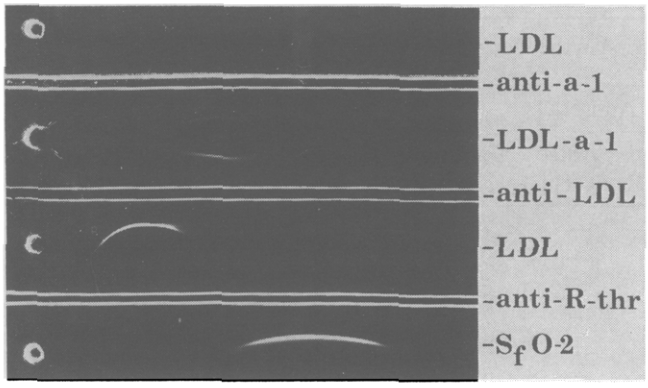

FIGURE 8: Immunoelectrophoresis of LDL (d 1.030–1.040 g/cm^3), LDL-a-1, and S_f 0–2 with anti-LDL, anti-a-1, and anti-R-Thr.

Thr is also shown in Figure 8. A single precipitin zone with electrophoretic mobility similar to that of HDL_2 was observed. Gel diffusion and immunoelectrophoretic zones formed multiple bands of the kind previously observed (Aladjem, 1966), provided high concentrations of reactants were used. These multiple zones are interpreted to indicate heterogeneity of each of the three classes of lipoproteins.

All HDL_1 and delipidated HDL_1 preparations reacted with anti-R-Thr and anti-R-Gln.

Discussion

The results of the present study show that S_f 0–2 (d 1.050–1.060 g/cm^3) contains at least three classes of lipoproteins; HDL_1, LDL_3, and a lipoprotein which shares antigenic determinants with the latter, LDL-a-1. LDL-a-1 was also found in the density range of 1.060–1.075 g/cm^3; LDL-a-1 was detected in the sera of some individuals but not in the sera of others.

HDL_1 was isolated from the density range of 1.050–1.060 g/cm^3 and shown by sucrose density gradient centrifugation to have a hydrated density of approximately 1.051 g/cm^3. It has electrophoretic and immunochemical properties similar to those of HDL_2; its apparent molecular weight is 0.5×10^6, compared to a molecular weight of 0.4×10^6 for HDL_2 (Hazelwood, 1958). By polyacrylamide gel electrophoresis, HDL_1 was found to contain all the polypeptides of HDL_2. Shore and Shore (1970) have reported that R-Thr and R-Gln, the major polypeptides of HDL, occur in lipoproteins isolated from the density range of 1.050–1.063 g/cm^3. Havel *et al.* (1970) have fractionated delipidated lipoprotein from the density range of 1.040–1.070 g/cm^3 from the serum of a patient with hyperlipoproteinemia. They detected R-Thr and a number of VLDL polypeptides but not R-Gln. We have recently shown that HDL_2 and HDL_3 are composed of subpopulations with differing polypeptide compositions (Albers and Aladjem, 1971). One of these subpopulations contains R-Thr but not R-Gln. However, all of our S_f 0–2 lipoprotein preparations which came from presumably normal individuals contained HDL_1 and both R-Thr and R-Gln. These findings can be reconciled with the assumption that HDL_1 also contains subpopulations with differing polypeptide compositions and that some individuals lack one of these subpopulations.

LDL_3 is the second class of lipoprotein which is present in S_f 0–2. LDL_3 has electrophoretic and immunochemical properties similar to those of LDL of d 1.030–1.040 g/cm^3. Its apparent molecular weight is 1.8×10^6. It is, thus, somewhat smaller than LDL of d 1.030–1.040 g/cm^3, which has a molecular weight of 2.3×10^6 (Adams and Schumaker, 1969). LDL_3 was found in all S_f 0–2 preparations.

The third class of S_f 0–2 lipoprotein was designated LDL-a-1. It was found in pooled sera and in the sera of some but not all individuals. This lipoprotein is different from HDL_1 and LDL_3 in its immunochemical and physicochemical properties, as shown in Table I. LDL-a-1 has all the major antigenic determinants of LDL; in addition, it has other determinant(s) which are not present in either LDL_3 or HDL_1.

The question arises whether there is any relation between LDL-a-1 and antigenic "variants" of LDL described in the literature. Numerous variants have been reported to occur in man (Blumberg *et al.*, 1962; Allison and Blumberg, 1965; Berg, 1963), and in the rabbit (Albers and Dray, 1969a,b). Most of these were discovered with the use of isoimmune antisera. Unlike these, LDL-a-1 and Lp(a) (Berg, 1963) were detected using absorbed rabbit immune sera. LDL-a-1 and Lp(a) have certain properties in common: pre-β mobility by Agarose electrophoresis, as shown in Figure 8, and the results of Simons *et al.* (1970), respectively, and very slow mobility on 3.5% polyacrylamide gel as shown in Figure 6 and the results of Weigandt *et al.* (1969), respectively. There are also certain differences between our findings with LDL-a-1 and those which have been reported for Lp(a). We found LDL-a-1 in the density range of 1.050–1.075 g/cm^3. Lp(a) has been reported to occur in several density ranges: 1.019–1.063 g/cm^3 (Berg, 1964), 1.031–1.035 g/cm^3 (Rittner and Goenechea, 1969), 1.050–1.125 g/cm^3 (Weigandt *et al.*, 1968), and 1.064–1.120 g/cm^3 (Schultz *et al.*, 1968). Harvie and Schultz (1970) reported that the Lp(a) antigen became detectable when sera, in which Lp(a) was not detected initially, were concentrated about 120-fold. They suggested that all individuals have some Lp(a). We did not detect LDL-a-1 in the sera of certain individuals even though, in some experiments, lipoproteins of d 1.050–1.075 g/cm^3 were concentrated approximately 110-fold. The concentrated lipoprotein fractions in which we could not detect LDL-a-1 also lacked three other properties characteristic of LDL-a-1: the 13S component determined by analytical ultracentrifugation, the 0.16 K_d peak determined by Agarose gel chromatography, and the slow migrating band on polyacrylamide gel electrophoresis.

On the basis of electron microscopic studies, it has been reported that the S_f 0–3 (d 1.040–1.060 g/cm^3) lipoproteins are "remarkably uniform" (Puppione *et al.*, 1970). Our gel chromatography data clearly indicate that there are three types of lipoproteins in this fraction and that these lipoproteins are distinctly different with respect to size. We have recently corroborated by electron microscopy the differences in size of the gel chromatography fractions (to be published).

Lipoproteins with pre-β mobility are generally considered very low density lipoproteins (Levy *et al.*, 1966; Schumaker and Adams, 1969). LDL-a-1 was found to have pre-β mobility on Agarose. A lipoprotein reacting with anti-LDL and having pre-β mobility on paper and a density between 1.040 and 1.063 g per cm^3 has been reported (Sodhi, 1969). An electrophoretic variant of human LDL with pre-β mobility on paper and reported to be in the 1.063–1.210-g/cm^3 density range has been described (Seegers *et al.*, 1965). Other studies, such as polyacrylamide gel electrophoresis, Agarose gel chromatography, and analytical centrifugation were not performed to characterize these materials. No further comparison, therefore, between these and LDL-a-1 can be made. Whether or not LDL-a-1 is, in fact, Lp(a) could not be definitely determined since anti-Lp(a) antisera were not available for the present study.

We conclude that each of the three isolated and partially characterized lipoprotein classes have similar hydrated densities but different chemical, immunochemical and physicochemical properties. LDL-a-1, LDL_3, and HDL_1 are all obviously separate entities and not hybrid molecules.

Addendum

After this manuscript was submitted, Ehnholm *et al.* (1971) reported the purification and characterization of the Lp(a) antigen. Their Lp(a) preparations were similar to our LDL-a-1 with respect to the following properties: pre-β mobility in Agarose gel, slow migration rate in polyacrylamide gel disc electrophoresis, sharing of common antigens with LDL, and variation of quantity among individuals. There appears to be some difference, however, in the mean hydrated density of the two materials.

References

Adams, G. H., and Schumaker, V. N. (1969), *Anal. Biochem. 29*, 117.

Aladjem, F. (1966), *Nature (London) 209*, 1003.

Aladjem, F., Lieberman, M., and Gofman, J. W. (1957), *J. Exp. Med. 105*, 49.

Alaupovic, P. (1968), *Progr. Biochem. Pharmacol. 4*, 91.

Albers, J. J., and Aladjem, F. (1971), *Biochemistry 10*, 3436.

Albers, J. J., Albers, L. V., and Aladjem, F. (1971), *Biochem. Med. 5*, 48.

Albers, J. J., and Dray, S. (1968), *Biochem. Genet. 2*, 25.

Albers, J. J., and Dray, S. (1969a), *J. Immunol. 103*, 155.

Albers, J. J., and Dray, S. (1969b), *J. Immunol. 103*, 163.

Albers, J. J., and Scanu, A. M. (1971), *Biochim. Biophys. Acta 236*, 29.

Allison, A. C., and Blumberg, B. S. (1965), *Progr. Med. Genet. 4*, 176.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Ayrault-Jarrier, M., Levy, G., Wald, R., and Polonovski, J. (1963), *Bull. Soc. Chim. Biol. 45*, 349.

Berg, K. (1963), *Acta Pathol. Microbiol. Scand. 59*, 369.

Berg, K. (1964), *Acta Pathol. Microbiol. Scand. 62*, 600.

Blumberg, B. S., Dray, S., and Robinson, J. C. (1962), *Nature (London) 194*, 566.

Borut, T. C., and Aladjem, F. (1971), *Immunochemistry 8*, 851.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

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

Ehnholm, C., Garoff, H., Simons, K., and Aro, H. (1971), *Biochim. Biophys. Acta 236*, 431.

Gelotte, B. (1960), *J. Chromatogr. 3*, 330.

Harvie, N. R., and Schultz, J. S. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 99.

Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955), *J. Clin. Invest. 34*, 1345.

Havel, R. J., Shore, V. G., Shore, B., and Bier, D. M. (1970), *Circ. Res. 27*, 595.

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

Lee, D. M., and Alaupovic, P. (1970), *Biochemistry 9*, 2244.

Levy, R. I., Lees, R. S., and Fredrickson, D. S. (1966), *J. Clin. Invest.45*, 63.

Lindgren, F. T., and Nichols, A. V. (1960), *in* The Plasma Proteins, Vol. 2, Putnam, F., Ed., New York, N. Y., p 11.

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

McDonald, H. J., and Ribeiro, L. P. (1959), *Clin. Chim. Acta 4*, 458.

Narayan, K. A., Creinin, H. L., and Kummerow, F. A. (1966), *J. Lipid Res. 7*, 150.

Nichols, A. V. (1967), *Advan. Biol. Med. Phys. 11*, 109.

Puppione, D. L., Forte, G. M., Nichols, A. V., and Strisower, E. H. (1970), *Biochim. Biophys. Acta 202*, 392.

Rittner, C., and Goenechea, S. (1969), *Z. Immunitaetsforsch. Allerg. Klin. Immunol. 137*, 390.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.

Schultz, J. S., Shreffler, D. C., and Harvie, N. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 963.

Schumaker, V. N., and Adams, G. H. (1969), *Annu. Rev. Biochem. 38*, 116.

Seegers, W., Hirschhorn, K., Burnett, L., Robson, E., and Harris, H. (1965), *Science 149*, 303.

Shore, B., and Shore, V. (1962), *J. Atheroscler. Res. 2*, 104.

Shore, V., and Shore, B. (1970), *J. Amer. Oil Chem. Soc. 47*, 91A.

Simons, K., Ehnholm, C., Renkonen, O., and Bloth, B. (1970), *Acta Pathol. Microbiol. Scand. 78*, 459.

Sodhi, H. S. (1969), *Metabolism 18*, 852.

Utermann, G., and Weigandt, H. (1969), *Hum. Genet. 8*, 39.

Weigandt, H., Lipp, K., and Wendt, G. G. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 489.