Physicochemical Properties of Low-Density Lipoproteins of Normal Human Plasma

EVIDENCE FOR THE OCCURRENCE OF LIPOPROTEIN B IN ASSOCIATED AND FREE FORMS

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1. Low-density (d 1.006–1.063 g/ml) lipoproteins from normal human plasma were separated by differential preparative ultracentrifugation into six subfractions. Each low-density (LD) lipoprotein subfraction contained lipoprotein B as the major and lipoproteins A and C as the minor lipoprotein families. 2. Three lipoprotein B subfractions (LP-B), LP-B-III (d 1.019–1.030 g/ml), LP-B-IV (d 1.030–1.040 g/ml) and LP-B-V (d 1.040–1.053 g/ml) were prepared from the corresponding LD lipoprotein subfractions by immunoprecipitating small amounts of lipoproteins A and C. 3. Determination of hydrodynamic properties indicated that LD lipoproteins consisted of three molecular segments characterized by a stepwise change in the molecular weight: LDL-I and LDL-II subfractions (d 1.006–1.019 g/ml) with an average mol.wt. of 4.75×10^6 , LDL-III (d 1.019–1.030 g/ml) with a mol.wt. of 3.99×10^6 , and LDL-IV, LDL-V and LDL-VI (d 1.030–1.053 g/ml) with a mol.wt. of 2.85×10^6 . 4. All three lipoprotein B subfractions had an average mol.wt. of 3.16×10^6 . 5. The LDL-I and LDL-II subfractions consisted of lipoprotein B and lipoprotein C families which were present in the form of an association complex. This was isolated from serum by immunoprecipitation with antibodies to lipoprotein B. The complex had a mol.wt. of 4.35×10^6 . 6. The results indicate a fundamental difference between the LD lipoprotein subfractions with d 1.006–1.019 g/ml and those subfractions with d 1.030–1.063 g/ml. In the former, lipoprotein B occurs as a part of an association complex, whereas in the latter it occurs as a separate entity.

Human plasma low-density lipoproteins consist of two macromolecular distributions characterized by S_r 0–12 and S_r 12–20 (Oncley, 1963; Ewing *et al.*, 1965; Nichols, 1967). The heterogeneity of both subclasses in regard to particle size, hydrated density and molecular weight has been attributed to discrete changes in the lipid and protein content of the lipoprotein molecules (Oncley *et al.*, 1957; Shore & Shore, 1962; Nichols, 1967; Dearborn & Wetlaufer, 1969). To acquire further insight into the polydisperse character and structural features of low-density lipoproteins, we separated the normal human plasma LD lipoproteins* into six subfractions by differential centrifugation and studied their immunological and chemical properties (Lee & Alaupovic, 1970). Our results showed that LD lipoproteins consist of a mixture of immunologically distinct lipoprotein species characterized by lipoprotein B as the major, and lipoproteins A and C as the minor, lipoprotein families.

* Abbreviations: VLD lipoproteins, very-low density lipoproteins ($S_r > 20$); LD lipoproteins, low-density lipoproteins (S_r 0–20).

Thus the polydispersity of LD lipoproteins stems not only from subtle changes in the chemical composition within the major lipoprotein B family, but also from the presence of various amounts of lipoprotein A and C families in each segment of the low-density range.

To define further the relationship and interactions between the three lipoprotein families and to acquire additional information for a structural model of the lipoprotein B family, we determined the hydrodynamic properties of six LD lipoprotein and three lipoprotein B subfractions. The complex nature of LD lipoprotein subfractions within the range d 1.006–1.019 g/ml was analysed with antibodies to major plasma apolipoproteins.

Experimental

Materials

Blood was drawn from healthy young men and women who had fasted overnight. The blood was collected in plastic bags (Abbott Laboratories, North Chicago, Ill., U.S.A.) containing 2115 U.S.P. units of

sodium heparin in saline, with 0.075M-sodium citrate as the buffer. It was allowed to cool for 2h at 5°C, and the plasma was recovered by low-speed centrifugation. Only plasma samples with normal lipid contents (total cholesterol <200mg/100ml, triglyceride <120mg/100ml and phospholipid <250mg/100ml) were used for the isolation of LD lipoproteins. To obtain serum, solid CaCl₂ (0.25g/100ml of plasma) was added to plasma specimens. These were then allowed to clot overnight at 4°C. The sera were recovered by low-speed centrifugation.

Methods

Isolation of LD lipoprotein subfractions. Six LD lipoprotein subfractions were isolated from plasma by differential ultracentrifugation at 5°C as described by Lee & Alaupovic (1970). The six subfractions were designated according to the solvent density (density excluding that contributed by plasma proteins and lipoproteins) ranges, in the following order of increased densities: LDL-I (*d* 1.006–1.009g/ml), LDL-II (*d* 1.009–1.019g/ml), LDL-III (*d* 1.019–1.030g/ml), LDL-IV (*d* 1.030–1.040g/ml), LDL-V (*d* 1.040–1.053g/ml) and LDL-VI (*d* 1.053–1.063g/ml). After removal of the VLD lipoproteins (*d* <1.006g/ml) from plasma samples, a combined LDL-I+II (*d* 1.006–1.019g/ml) subfraction was isolated from the infranate by adjusting the solvent density to 1.019g/ml with solid NaBr and ultracentrifuging in a Ti 50 rotor (Spinco model L or L-2 ultracentrifuge) at 105000g for 22h.

Preparation of lipoprotein B subfractions by immunoprecipitation. The LP-B-III (*d* 1.019–1.030g/ml), LP-B-IV (*d* 1.030–1.040g/ml) and LP-B-V (*d* 1.040–1.053g/ml) subfractions were prepared by treating the corresponding LD lipoprotein subfractions with goat antibodies to lipoprotein A and rabbit antibodies to lipoprotein C as described previously (Lee & Alaupovic, 1970). The lipoprotein A and C were precipitated by adding to each subfraction equivalent amounts of the lipoprotein-free antisera, as determined by a serial microdilution technique. The reaction mixtures were allowed to stand for 24h at 5°C, and the antigen–antibody precipitates were removed by low-speed centrifugation. Supernatants adjusted to the upper solvent density of each corresponding subfraction were centrifuged at 150000g for 24h to remove the rabbit or goat serum proteins. Purified lipoprotein B subfractions removed from the top layer showed no reaction with goat antibodies to rabbit whole serum or with rabbit antibodies to goat whole serum.

To isolate totally the lipoprotein B family, the human whole serum was precipitated with goat antibodies (the γ -globulin fraction) to lipoprotein B. The equivalent amounts of serum and immunoglobulin were determined by a serial microdilution technique.

The reaction mixture was left overnight at 5°C. The precipitated antigen–antibody complex was collected by low-speed centrifugation and washed with cold 0.15M-NaCl, pH7.0, until it was free of albumin, as demonstrated by an immunodiffusion test. The antigen–antibody precipitate was dissociated in 0.2M-glycine–HCl buffer, pH3.2, as described by Kostner & Holasek (1969). To ensure complete dissociation of antigen–antibody complex, the pH of the final solution was adjusted to 3.2 with 0.2M-HCl. The separation of lipoprotein B from γ -globulins was accomplished by ultracentrifugation of the dissociated antigen–antibody complex in a sucrose density gradient. The gradient was formed in a centrifuge tube by layering 5ml of a sucrose solution in saline (*d* 1.125g/ml, pH7.0) under 3ml of a sucrose solution in 0.2M-glycine–HCl buffer (*d* 1.083g/ml). The solution (3–4ml) of dissociated antigen–antibody complex in 0.2M-glycine–HCl buffer, pH3.2, was layered on the top of the sucrose gradient and centrifuged in the Ti 50 rotor at 105000g for 40h. The yellow-coloured lipoprotein layer was removed by a tube-slicing technique and dialysed exhaustively against a NaCl solution of *d* 1.0075g/ml containing 0.05% EDTA, pH7.0. The dialysed lipoproteins were centrifuged in a Ti 60 rotor at 150000g for 24h at 10°C. The VLD lipoproteins were removed by cutting the tubes through the middle of the clear zone, approx. 2.5–3.0cm from the top. The infranant was dialysed against a NaCl solution (*d* 1.020g/ml) containing 0.05% EDTA, pH7.0. After centrifugation at 150000g for 24h, the combined LDL-I and LDL-II subfractions (*d* 1.006–1.019g/ml) floating in the top layer (2.5–3.0cm) were removed by a tube-slicing device and labelled as IP-I+II.

Isolation of goat antibodies (the γ -globulin fraction) to human lipoprotein B. To eliminate serum lipoproteins and proteins other than immunoglobulins (the γ -globulin fraction), goat antiserum to human lipoprotein B and human whole serum were mixed in proportions determined by a serial microdilution technique and left overnight at 5°C. The precipitated antigen–antibody complex was collected by low-speed centrifugation, washed and dissociated as described by Kostner & Holasek (1969). The liberated goat γ -globulins were separated from lipoprotein B by ultracentrifugation in a sucrose gradient, as described in the previous paragraph. The sedimented γ -globulin fraction (2cm at the bottom of the tube) was collected with a syringe and dialysed successively against glass-distilled water and 0.15M-NaCl, pH7.0. The γ -globulin fraction was immunochemically homogeneous and gave a single immunoprecipitin line with human lipoprotein B.

Immunological methods. The immunological characteristics of LD lipoprotein and lipoprotein B subfractions were studied by double diffusion and immunoelectrophoresis as described previously (Lee

& Alaupovic, 1970). Separate antisera to lipoprotein families A, B and C were prepared and characterized as described previously (Alaupovic *et al.*, 1972; Kostner & Alaupovic, 1972). The antiserum to lipoprotein A contained antibodies to A-I and A-II polypeptides of apolipoprotein A; the antiserum to lipoprotein B contained only antibodies to lipoprotein B; the antiserum to lipoprotein C consisted of antibodies to C-I, C-II and C-III polypeptides of apolipoprotein C.

Specific gravity. The specific gravity of salt solutions was measured at 25°C by a precision hydrometer (Fisher Scientific Co., Pittsburgh, Pa., U.S.A.) calibrated with water at 15°C by the manufacturer. The density of salt solutions at 25°C was calculated by multiplying the values for specific gravity by 0.9991, the density of water at 15°C.

Ultracentrifugal redistribution of salts. Several solutions of known densities of NaCl, NaBr and KBr were centrifuged in a Ti 50 rotor at 105 000g for 22h at 5°C. After centrifugation, the salt solutions were carefully removed from the centrifuge tubes with a Pasteur pipette at 1 cm intervals. The refractive index, n , of these fractions was determined with a precision refractometer (Bausch and Lomb Inc., Rochester, N.Y., U.S.A.) at 25°C ± 0.05°C. The temperature was maintained by circulating water from a constant-temperature water bath (Neslab Instruments, Portsmouth, N.H., U.S.A.). Standard graphs for density ρ versus n were constructed from four or five different concentrations of each salt solution. From the standard graphs, a measured n was converted into ρ for each solution.

Ultracentrifugal analyses. The analytical ultracentrifugation of LD lipoprotein and lipoprotein B subfractions was performed in a Spinco model E ultracentrifuge equipped with schlieren and Rayleigh interference optical systems and an RTIC temperature-control unit. All experiments were performed at 25°C. Plate measurements were made with a Nikon microcomparator (Nikon Co., Tokyo, Japan).

Sedimentation- and flotation-velocity experiments were performed in a single- or double-sector cell with a Kel-F centrepiece at various rotor speeds (4200–52640 rev./min). The observed sedimentation coefficients were reduced to values corresponding to water at 20°C ($s_{20,w}$), as described by Schachman (1957). The flotation coefficients, S_r , were calculated as outlined by DeLalla & Gofman (1954). The sedimentation coefficients ($s_{20,w}^0$) and flotation coefficients (S_r^0) at infinite dilutions were calculated from values obtained at three or four different lipoprotein concentrations (2–8 mg/ml).

Hydrated density, $\bar{d}$, was determined by measuring the sedimentation and/or flotation coefficients of lipoprotein subfractions at four or five different solvent densities, which were attained by dialysing the lipoproteins in appropriate NaCl solutions containing

0.02% EDTA, pH 6.8–7.0. Values for the hydrated density were obtained by interpolating the $s\eta$ versus ρ data (η = solvent viscosity; ρ = solvent density) to zero sedimentation. The values for the partial specific volume, $\bar{v}$, were obtained from the relation $\bar{v} = 1/\bar{d}$. The temperature coefficient of $\bar{v}$ for lipoproteins was assumed to be the same as that for the common proteins, i.e., $\Delta\bar{v} = -0.0004$ ml/g per °C for the conversion of $\bar{v}$ from values at 25°C into 20°C values.

Diffusion coefficients of lipoprotein subfractions were measured in NaCl solutions of d 1.003–1.005 g/ml containing 0.02% EDTA, pH 6.8–7.0, in a synthetic boundary cell at rotor speed of 12 590 rev./min. The apparent diffusion coefficients, D_{app} , were calculated by applying the height-area method. The zero-time corrections were made from a plot of D_{app} versus the reciprocal of time. Diffusion coefficients at infinite dilutions, D^0 , were extrapolated from measured values obtained at three different lipoprotein concentrations (2–8 mg/ml). The diffusion coefficients at infinite dilution were reduced to diffusion coefficients, $D_{20,w}^0$ and $D_{25,w}^0$, by the standard procedure (Schachman, 1957).

Determination of diameter. The diameters of lipoprotein subfractions were estimated, assuming spherical particles, according to the equation (Oncley & Gurd, 1953):

$$S_r^0 = (1.063 - \bar{d})(\text{diam.})^2/1.88$$

where S_r^0 is flotation coefficient at infinite dilution (in Svedberg units), $\bar{d}$ is hydrated density (in g/ml) and diam. is lipoprotein diameter (in nm).

Determination of molecular weight. The molecular weights of lipoprotein subfractions were determined by three different methods. (1) Sedimentation equilibrium. High-speed sedimentation-equilibrium experiments were done at 25°C in a double-sector interference cell with sapphire windows by the methods of Yphantis (1964) and Van Holde (1967). The concentration of lipoprotein subfractions was 0.5 mg/ml. Bjorklund & Katz (1956) observed that molecular-weight determination of LD lipoproteins by light-scattering was not dependent on the concentration. Since light-scattering is a technique equivalent to sedimentation equilibrium, no concentration dependence of molecular weight was considered in the latter method. The rotor speed was 13 000 rev./min. Photographs were taken every hour after the initial 20h until the consecutive patterns resulted in an identical slope for $\log j$ versus r^2 , where j is the fringe displacement and r is the distance in cm from the centre of rotation. Water blanks were run by the method of Yphantis (1964). The blank corrections were negligible. The weight-average molecular weight was calculated from the slope of linear plots of $\log j$ versus r^2 according to the equation:

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{2.303 d(\log j)}{dr^2}$$

where M is the molecular weight, R the gas constant (8.314×10^7 ergs/°K per mol), T the absolute temperature, $\bar{v}$ the partial specific volume of the solute (lipoprotein), ρ the solvent density and ω the angular velocity (2π rev./min).

(2) s/D method. The molecular weight of lipoprotein subfractions was calculated from sedimentation velocity and diffusion coefficient by the Svedberg equation [$M = RTs/D(1 - \bar{v}\rho)$]. The s_{25}^0 and D_{25}^0 measured at the same solvent density ρ were used. When values for s_{25}^0 were unavailable, corresponding $S_0^0\eta$ values at solvent density 1.063 g/ml were used, according to the equation:

$$M = \frac{-RTS_0^0\eta}{D_{25,w}^0(1 - 1.063\bar{v})}$$

(3) Stokes spheres. When diffusion coefficients were unavailable, the molecular weight of lipoprotein subfractions was estimated from a relationship based on the assumption that Stokes frictional factor for spheres applies to lipoprotein molecules. The molecular diameter, diam., was calculated from a relationship presented in the previous paragraph. The minimum molecular weight was calculated from hydrated molecular volume, which had been estimated from S_0^0 , Avogadro's number and hydrated density according to the equation (Oncley, 1963):

$$M = 317 \cdot \text{diam.}^3 \cdot \bar{d}$$

Determination of viscosity. Data for viscosity of solvents were taken from the International Critical Table or measured with a Cannon-Ubbelohde semi-micro viscometer at $25^\circ\text{C} \pm 0.01^\circ\text{C}$ in a constant-

temperature water bath (Neslab Instruments, Portsmouth, N.H., U.S.A.).

Results

Ultracentrifugal redistribution of salts

To evaluate the salt redistribution occurring during ultracentrifugation, the NaCl solutions (d 1.0060 and d 1.0649 g/ml), NaBr solutions (d 1.0300, d 1.0500 and 1.0620 g/ml) and KBr solutions (d 1.1984 g/ml) were centrifuged in a Ti 50 rotor at 40000 rev./min for 22 h at 5°C . The resulting salt redistributions are depicted in Fig. 1. Under identical conditions, the NaCl solutions formed narrower density gradients than the corresponding NaBr solutions. Results also showed that the extent of salt redistribution increased with rising solution densities of the same salt. Although not shown in the figure, the extent of salt redistribution depended on the speed of rotation: an increase in the gravitational force produced a wider gradient of salt solutions of the same densities. The density range of lipoproteins floating in the top layer of centrifuge tubes was frequently designated in the literature according to the density of the initial salt solution. However, owing to the salt redistribution during ultracentrifugal runs, the actual density of floating lipoproteins is substantially lower than that of the initial solution. The density differences between the floating lipoproteins and the original solvents became greater with each successive increase in the density of background solutions. In this study, the differences were established by actual determination of hydrated densities (average densities) of the isolated lipoproteins in the analytical ultracentrifuge (Table 1).

Fig. 1. Salt redistribution after preparative ultracentrifugation: NaCl (●), NaBr (■) and KBr (○)

Initial densities of salt solutions are indicated by broken lines. The continuous lines represent density gradients after ultracentrifugation. Runs were carried out in a Ti 50 rotor at 40000 rev./min for 22 h at 5°C .

Table 1. *Physicochemical properties of LD lipoprotein subfractions*

Isolation procedure and methods for determination of physicochemical parameters were as described in the Experimental section. Each of the identical subfractions listed was isolated from a different donor.

Low-density lipoprotein preparation	Density range (g/ml)	Hydrated density d (g/ml)	Flotation coefficient S_0^0	Sedimentation coefficient $s_{20,w}^0$	$10^{-7} \times$ Diffusion coefficient (cm^2/s)		Diameter (nm)	$10^{-6} \times$ Mol.wt. estimated from			Frictional ratio f/f_0
					$D_{25,w}^0$	$D_{20,w}^0$		s^0/D^0	S_0^0/D^0	Stokes sphere	
LDL-I	1.006-1.009	1.0016	19.2	—	—	—	24.3	—	—	4.57	—
LDL-I+II	1.006-1.019	1.0030	19.1	2.96	—	—	24.4	—	—	4.65	—
LDL-I+II	1.006-1.019	1.0065	18.7	—	—	—	24.9	—	—	4.94	—
LDL-II	1.009-1.019	1.0120	16.7	—	—	—	24.8	—	—	4.92	—
LDL-II	1.009-1.019	1.0130	15.8	—	1.93	1.68	24.4	—	4.87	4.67	1.03
LDL-II	1.009-1.019	—	—	—	1.98	1.73	—	—	—	—	—
LDL-III	1.019-1.030	1.0180	12.4	—	2.05	1.79	22.7	—	3.99	3.91	1.03
LDL-III	1.019-1.030	—	—	5.05	1.93	1.73	—	—	—	—	—
LDL-IV*	1.030-1.040	1.0315	6.42	—	—	—	19.6	—	—	2.45	—
LDL-V	1.040-1.053	1.0335	5.90	7.44	2.11	1.84	19.4	2.73	2.86	2.38	1.12
LDL-V*	1.040-1.053	1.0340	5.92	—	—	—	19.6	—	—	2.46	—
LDL-VI	1.053-1.063	1.0357	5.45	8.6	2.12	1.85	19.4	2.96	2.85	2.39	1.12
LDL-VI	1.053-1.063	1.0420	4.40	—	2.14	1.87	19.8	—	2.98	2.58	1.09

* Isolation at 14°C.

Immunochemical characterization of LD lipoprotein subfractions

Confirming our previous results (Lee & Alaupovic, 1970; Alaupovic *et al.*, 1972), immunological testing showed that each of the six LD lipoprotein subfractions reacted positively not only with antibodies to lipoprotein B, but also with antibodies to lipoprotein C (and lipoprotein A). However, the shape of immunodiffusion lines of subfractions LDL-I and LDL-II differed markedly from those of subfractions LDL-III, LDL-IV, LDL-V and LDL-VI. Whereas the immunoprecipitin lines of the former subfractions fused with antibodies to both lipoprotein B and C, the crossed precipitin lines of the latter subfractions exhibited a non-identity reaction (Plate 1). These patterns suggest that the lipoprotein B and C families exist in LDL-I and LDL-II subfractions as association complexes and in LDL-III–LDL-VI subfractions as separate physicochemical entities. Although lipoprotein A was detected in some LDL-I and LDL-II subfractions, this lipoprotein family occurs mainly as a separate entity in LDL subfractions of higher densities.

Physicochemical characterization of LD lipoprotein subfractions

A more pronounced curvature of the baseline in the schlieren patterns of a NaBr solution (d 1.20 g/ml) compared with that of a NaCl solution (d 1.063 g/ml), clearly indicated that the former underwent more extensive salt redistribution than the latter (Fig. 2). To minimize the error in establishing the density and viscosity of solvents caused by the ultracentrifugal salt redistribution, all physicochemical parameters were determined in NaCl solutions of low ionic strength.

The results of physicochemical characterization of LD lipoprotein subfractions are shown in Table 1. As was expected, the hydrated density, $\bar{d}$, and the

sedimentation coefficient, $s_{20,w}^0$, increased, and the flotation coefficient, S_f^0 , decreased with increasing density of lipoprotein subfractions. The representative flotation and sedimentation patterns of LD lipoprotein subfractions are shown in Figs. 3 and 4. LDL-I and LDL-II subfractions were characterized by asymmetrical flotation peaks with S_f^0 values of 19.2 and 15.8–16.7 respectively. The schlieren patterns of the higher-density subfractions (LDL-III–LDL-VI) exhibited single, symmetrical peaks with the flotation coefficients ranging from 12.4 to 4.4 and sedimentation coefficients ranging from 5.0 to 8.6. However, at low lipoprotein concentrations the sedimenting or floating peaks had an asymmetrical shape. This suggested the presence of more than one macromolecular species. The sedimentation and flotation coefficients of all LD lipoprotein subfractions were concentration-dependent. The slopes of s or S_f versus lipoprotein concentrations for different LD lipoprotein subfractions were all negative (Fig. 5). The results of duplicate determinations of s or S_f were within ± 0.05 .

The hydrated density, $\bar{d}$, of lipoprotein subfractions was estimated from a plot of $s\eta$ versus ρ at zero sedimentation. Theoretically, the intercept values of density, ρ , at $s = 0$ for the straight lines of $s\eta$ (and $-S_f\eta$) versus ρ are concentration-independent as long as the measurements are performed at the same lipoprotein concentration. Selected graphs of $s\eta$ versus ρ for LD lipoprotein subfractions are presented in Figs. 6 and 7. Straight lines were obtained with all LD lipoprotein subfractions when determinations were carried out in NaCl solutions. Values for duplicate determinations of $\bar{d}$ were within ± 0.0005 g/ml. To investigate the effect of high salt concentration on S_f values, some lipoprotein subfractions were also analysed in NaBr solutions of d 1.0800 and d 1.1995 g/ml. The results were compared with S_f values extrapolated from the straight line of $s\eta$ versus ρ determined at three different low concentrations of NaCl

Fig. 2. Salt redistribution during analytical ultracentrifugation

(a) Schlieren patterns of LDL-IV in a NaBr solution, d 1.20 g/ml; pictures were taken 4 and 16 min after a speed of 44770 rev./min was reached, at a bar angle of 60° . (b) Schlieren patterns of LDL-III in a NaCl solution, d 1.063 g/ml; pictures were taken 4 and 36 min after a speed of 52000 rev./min was reached, at a bar angle of 70° .

EXPLANATION OF PLATE I

Immunodiffusion of LDL-II (A) and LDL-V (D) subfractions in 1% agarose gel

Well B contains antibodies to lipoprotein C and well C contains antibodies to lipoprotein B.

Fig. 3. Flotation patterns of LD lipoprotein subfractions

(a) LDL-I and (b) LDL-II at solvent density 1.063 g/ml; pictures were taken 8 min after a speed of 42040 rev./min was reached. (c) LDL-III at solvent density 1.063 g/ml. (d) LDL-IV at solvent density 1.080 g/ml. (e) LDL-V at solvent density 1.063 g/ml. (c)–(e) Pictures were taken 16 min after a speed of 52640 rev./min was reached.

Fig. 4. Sedimentation patterns of LD lipoprotein subfractions

(a) LDL-II (upper pattern) and LDL-III (lower pattern) at solvent density 1.0030 g/ml; the picture was taken 16 min after a speed of 52640 rev./min was reached. (b) LDL-IV and (c) LDL-V subfractions at solvent density 1.0041 g/ml; pictures were taken 36 min after a speed of 52640 rev./min was reached.

Fig. 5. Concentration dependence of flotation coefficients of LDL-II (●) and LDL-III (○) and of sedimentation coefficients of LDL-V (■) and LDL-VI (□) subfractions

See the Experimental section for the experimental conditions.

solutions, the highest density being 1.063 g/ml. As was expected from the results of the salt redistribution experiments, in which the lipoproteins floated against a solvent of decreasing density, the S_r values estimated in the NaBr solution of d 1.080 g/ml were 8–11% lower, and those in the NaBr solution of 1.1995 g/ml 12–13% lower, than the extrapolated S_r values.

Because of salt redistribution during preparative ultracentrifugation and the difference in temperature coefficients of densities of salt solution and lipoprotein, the hydrated density of LD lipoprotein subfractions isolated by flotation at a particular solvent density was usually lower than the solvent density employed (Table 1). The difference between the actual hydrated densities of the isolated lipoproteins and the density of the solvents used depends on the density and nature of the salt solutions and the distribution of lipoproteins in any individual case.

Diffusion coefficients were determined for all LD lipoprotein subfractions, except for LDL-I and LDL-IV. The apparent diffusion coefficients, $D_{app.}$, were all linear functions of reciprocal time for different concentrations of lipoproteins, with LDL-V subfraction shown as an example (Fig. 8). There was a tendency for diffusion coefficients of LD lipoprotein subfractions to be inversely concentration-dependent. The results of duplicate determinations of

Fig. 6. Determination of hydrated densities of LDL-I (●), LDL-II (○), LDL-II (■) and LDL-III (□) subfractions

The LDL-II subfractions were two different preparations. See the Experimental section for the experimental conditions.

Fig. 7. Determination of hydrated densities of LDL-V (●), LDL-VI (○) and LDL-VI (■) subfractions

The LDL-VI subfractions were two different preparations. See the Experimental section for the experimental conditions.

D were within $\pm 0.02 \times 10^{-7} \text{ cm}^2/\text{s}$. The values for diffusion coefficients at infinite dilution reduced to $D_{20,w}^0$ and $D_{25,w}^0$ are listed in Table 1. Based on the similarity in the values of diffusion coefficients, the LD lipoprotein subfractions can be divided into two groups; one represented by LDL-II and LDL-III subfractions with $D_{25,w}^0$ of 1.93×10^{-7} – $2.05 \times 10^{-7} \text{ cm}^2/\text{s}$, and the other by LDL-V and LDL-VI with $D_{25,w}^0$ of 2.11×10^{-7} – $2.14 \times 10^{-7} \text{ cm}^2/\text{s}$. The latter results agreed closely with that reported in the literature for the whole low-density range. Fisher *et al.* (1971) determined the $D_{25,w}^0$ of LD lipoproteins to be $2.17 \times 10^{-7} \text{ cm}^2/\text{s}$.

Results of the determination of Stokes diameter and molecular weight (Table 1) clearly indicated that LD lipoprotein subfractions can be divided into three groups based on these two parameters. Whereas LDL-I and LDL-II subfractions represent one major group, and LDL-IV, LDL-V and LDL-VI subfractions the other, the LDL-III subfraction seems to be an inter-

Fig. 8. Apparent diffusion coefficients, $D_{app.}$, of various concentrations of LDL-V subfraction as functions of reciprocal time

The initial lipoprotein concentration is represented by ○, the one-half by ■ and the one-third by ●. See the Experimental section for the experimental conditions.

mediate, overlapping lipoprotein preparation. The average Stokes diameter of single or combined LDL-I and LDL-II preparations was 24.5 nm. The Stokes diameter of LDL-V and LDL-VI subfractions was 19.6 nm. In contrast with these two extreme values, the diameter of LDL-III subfraction was 22.7 nm.

The molecular weights of LD lipoprotein subfractions were estimated by Svedberg's equation and by assuming Stokes sphere. Owing to a negligible salt redistribution in dilute NaCl solutions, the quantity s^0/D^0 was preferred to S_r^0/D^0 for more accurate estimation of the molecular weights of subfractions of higher hydrated densities (LDL-V and LDL-VI) by Svedberg's equation. On the other hand, one should avoid using the sedimentation coefficient to calculate the molecular weight of subfractions with hydrated density near that of water (subfractions LDL-I and LDL-II). This is because the buoyancy factor, $(1 - \bar{v}\rho)$, being close to zero, introduces an uncertainty into such calculations. The results of molecular-weight determinations further substantiated the division of LD lipoprotein subfractions into three groups. The average molecular weight of the LDL-I and LDL-II subfractions was 4.75×10^6 assuming Stokes spheres. There was little difference in the molecular weight (4.57×10^6 – 4.94×10^6) of various LDL-I and LDL-II preparations. A similar value (4.87×10^6) was obtained by Svedberg's equation (s_r^0/D^0). The average molecular weights of the LDL-V and LDL-VI subfractions were 2.45×10^6 assuming Stokes spheres, or 2.85×10^6 by Svedberg's equation (s^0/D^0). The corresponding molecular weights of the LDL-III subfraction were 3.91×10^6 or 3.99×10^6 respectively.

LD lipoprotein subfractions isolated from the upper part of the density spectrum (LDL-IV–LDL-VI) had frictional ratios considerably higher ($f/f_0 = 1.09$ – 1.12) than LDL-I, LDL-II and LDL-III

subfractions; these were characterized by a frictional ratio near unity ($f/f_0 = 1.03$).

The estimation of molecular weights of LDL-II and LDL-III subfractions by the Svedberg equation and by assuming Stokes spheres gave similar results (Table 1). This finding, predictable from the frictional ratios of near unity for these two subfractions, indicated that lipoproteins of the lower density range come closer to a spherical shape and/or contain less water of hydration than those of the higher-density range. For these latter lipoproteins (LDL-V and LDL-VI subfractions), the molecular weights based on the assumption of spherical shape were 13–19% lower than those calculated by Svedberg's equation. This discrepancy is obviously caused by some deviation of lipoproteins with higher hydrated densities from an assumed model of unhydrated spheres. Since the diffusion coefficients of lipoprotein subfractions were determined experimentally, the molecular weights calculated by Svedberg's equation should be considered more reliable than those based on the assumed spherical shape of anhydrous lipoprotein molecules. However, to verify these results by a different method, the molecular weights of two higher-density LD lipoprotein subfractions were determined

by sedimentation equilibrium (Table 2). Linear relationships of the plots of $\log j$ versus r^2 were maintained for all three lipoprotein preparations for at least 24 h after equilibrium had been reached. Despite some differences in the hydrated density and the flotation coefficient, the values for the molecular weight of LDL-IV and LDL-V subfractions were very similar. The average molecular weight of higher-density lipoprotein subfractions (LDL-IV and LDL-V) determined by sedimentation equilibrium (3.16×10^6) was slightly higher than those obtained by Svedberg's equation (2.73 – 2.98×10^6). Thus molecular weights of LD lipoprotein subfractions estimated by absolute methods were higher than those based on the assumption of Stokes spheres.

Physicochemical characterization of LDL-I+II (d 1.006–1.019 g/ml) subfraction isolated by immunoprecipitation

Immunological studies (Plate 1) had indicated that both LDL-I and LDL-II subfractions consisted mainly, if not exclusively, of lipoprotein B and C families present in the form of an association complex. The association of these two lipoprotein families into

Table 2. *Determination of molecular weight of some LD lipoprotein subfractions by sedimentation equilibrium*

Isolation procedure and methods for determination of physicochemical parameters were as described in the Experimental section. Slopes for $\log j$ versus r^2 for 26 and 50 h runs overlapped, indicating that the sedimentation equilibrium had been reached after 26 h.

Low-density lipoprotein preparation	Density range (g/ml)	Hydrated density d (g/ml)	Flotation coefficient S_f^0	$10^{-6} \times$ Mol.wt. estimated from	
				Sedimentation equilibrium	Stokes sphere
LDL-IV*	1.030–1.040	1.0315	6.42	3.21	2.45
LDL-V*	1.040–1.053	1.0340	5.92	3.17	2.46
LDL-V†	1.040–1.053	1.0325	—	3.10	—

* Isolated at 14°C.
† Isolated at 5°C.

Table 3. *Some physicochemical characteristics of combined LDL-I and LDL-II subfractions isolated by ultracentrifugation or immunoprecipitation*

Isolation procedures and methods for determination of physicochemical parameters were as described in the Experimental section.

Low-density lipoprotein preparation	Isolation procedure	Hydrated density d (g/ml)	Flotation coefficient S_f^0	Diameter (nm)	$10^{-6} \times$ Mol.wt. estimated from Stokes sphere
LDL-I+II	Isolated by ultracentrifugation at d 1.006–1.019 g/ml	1.0060	17.9	24.3	4.58
IP-I+II	Isolated from whole serum by precipitation with antibodies to lipoprotein B and ultracentrifugation at d 1.006–1.019 g/ml	1.0050	18.4	23.9	4.35

a physicochemical entity was confirmed by results of an experiment in which treatment of LDL-I or LDL-II subfractions with antibodies to lipoprotein C resulted in a complete co-precipitation of lipoprotein B. This fact confirmed the interpretation of prior immunological studies, strongly suggesting that the entire lipoprotein B family in this density range existed in a complex with lipoprotein C.

To verify the existence of the lipoprotein B-lipoprotein C complex within the range d 1.006–1.019 g/ml, preparation IP-I+II was isolated as described in the Experimental section. The immunoprecipitin lines of the IP-I+II preparation with antibodies to lipoprotein B and lipoprotein C fused with each other. Thus the existence of the association complex was confirmed by a different isolation method. The physicochemical properties of this lipoprotein B-lipoprotein C complex (preparation IP-I+II) are presented in Table 3. The values for the hydrated density, flotation coefficient, diameter and molecular weight of the IP-I+II preparation closely resembled those of the ultracentrifugally isolated LDL-I+II subfraction.

Physicochemical characterization of lipoprotein B subfractions

In contrast with lipoproteins within the range d 1.006–1.019 g/ml (LDL-I+II), treatment of LD lipoprotein subfractions of higher densities (d 1.030–1.053 g/ml) with antibodies to either lipoprotein A or lipoprotein C failed to produce simultaneous coprecipitation of lipoprotein B. This indicated that, above d 1.030 g/ml, lipoprotein A and C families were not associated with lipoprotein B, but occurred as separate physicochemical entities. Immunoprecipitation of small amounts of lipoproteins A and C from subfractions LDL-III, LDL-IV and LDL-V yielded corresponding immunologically homogeneous subfractions LP-B-III, LP-B-IV and LP-B-V. In contrast with LD lipoprotein subfractions, the lipoprotein B subfractions displayed single symmetrical schlieren boundaries even at low lipoprotein concentrations. The lipoprotein B subfractions of increasing hydrated densities (Fig. 9) were characterized by similar diffusion coefficients, diameters and molecular weights (Table 4). A decrease in the flotation coefficients and an increase in the sedimentation coefficients with increasing density of lipoprotein B subfractions is mainly due to a difference in their respective buoyancy factors. In comparison with the corresponding LD lipoprotein subfraction, the LP-B-III had a lower flotation coefficient, diameter and molecular weight. On the other hand, the LP-B-IV and LP-B-V subfractions had higher flotation coefficients, diameters and molecular weights than the corresponding LDL-IV and LDL-V subfractions. The former result may reflect the absence of a residual lipoprotein B-lipo-

Table 4. *Physicochemical properties of lipoprotein B subfractions*

Isolation procedure and methods for determination of physicochemical parameters were as described in the Experimental section.											
Low-density lipoprotein preparation	Density range (g/ml)	Hydrated density d (g/ml)	Flotation coefficient S_f^0	Sedimentation coefficient $s_{20,w}^0$	$10^{-7} \times$ Diffusion coefficient (cm ² /s)		Diameter (nm)	$10^{-6} \times$ Mol.wt. estimated from			Frictional ratio f/f_0
					$D_{25,w}^0$	$D_{20,w}^0$		s^0/D^0	S_f^0/D^0	Stokes sphere	
LP-B-III	1.019–1.030	1.0190	11.0	5.30*	2.24	1.95	21.7	—	3.32	3.27	1.00
LP-B-IV	1.030–1.040	1.0266	8.31	7.49	2.10	1.83	20.9	3.23	3.25	2.97	1.08
LP-B-V	1.040–1.053	1.0329	6.80	8.69	2.10	1.83	20.8	3.17	3.29	2.93	1.07

* This value was obtained from a single lipoprotein concentration.

Fig. 9. Determination of hydrated densities of LP-B-III (●), LP-B-IV (■ and ○) and LP-B-V (□) subfractions

See the Experimental section for the experimental conditions.

protein C complex, in the LP-B-III subfraction, the presence of which contributed to a higher flotation coefficient and a higher molecular weight of the LDL-III subfraction. The increased values of the hydrodynamic parameters of LP-B-IV and LP-B-V subfractions presumably result from the removal of separate lipoprotein A and C families which are responsible for the lower flotation coefficients and molecular weights of LDL-IV and LDL-V subfractions. The frictional ratios of lipoprotein B subfractions suggest that, similarly to their corresponding LD lipoprotein subfractions, the lipoprotein molecules of higher hydrated densities either deviated from a spherical shape and/or were hydrated. The molecular weights of LP-B-IV and LP-B-V subfractions as determined by Svedberg's equation were, indeed, higher than those estimated by assuming Stokes spheres.

Discussion

In this study, the molecular weights of LD lipoprotein subfractions were estimated by Svedberg's equation and sedimentation equilibrium whenever possible and compared with values based on the assumed Stokes spheres. In most instances, a comparison of LD lipoprotein preparations of similar hydrated densities indicated excellent agreement between results of this study and those reported in the literature. For example, the value of 2.73×10^6 for the molecular weight of LDL-V was the same as that estimated by Fisher *et al.* (1971) for the major peak of human LD lipoproteins (d 1.020–1.063 g/ml) by Svedberg's equation. By using light-scattering technique, Bjorklund & Katz (1956) showed that comparable LD subfractions with S_r 5.9 and S_r 6.4 had anhydrous molecular weights of 2.77×10^6 and 2.80×10^6 respectively. On the other hand, a comparison of the molecular weights estimated by sedimentation equilibrium showed that values for LDL-IV and LDL-V subfractions (3.21×10^6 and 3.14×10^6)

differed considerably from those of a LD preparation (d 1.019–1.063 g/ml), which was found to be 2.1×10^6 – 2.3×10^6 (Scanu *et al.*, 1968). The relatively low molecular weight of the latter lipoprotein preparation was probably due to a partial specific volume of 0.964, a value corresponding to a hydrated density of 1.0373. This value for the hydrated density far exceeds values reported in the literature for comparable LD lipoprotein preparations or for LDL-IV and LDL-V subfractions in this study. Fisher *et al.* (1971) found a value of 1.034 g/ml for the hydrated density of the major peak of LD lipoproteins. Bjorklund & Katz (1956) established that the hydrated densities for the two LD subfractions with S_r 8.1 and S_r 6.4 were 1.0274 and 1.0328 g/ml respectively. Lindgren *et al.* (1969) reported hydrated densities of S_r 0–12 preparations to be 1.0304 ± 0.0035 g/ml for men and 1.0284 ± 0.0031 g/ml for women. Molecular weights based on the hydrated density values of 1.034 or 1.030 g/ml would be 10–30% higher than that based on a hydrated density of 1.037 g/ml.

Most of the reported studies (Lindgren *et al.*, 1951, 1969; Adams & Schumaker, 1969) on the molecular-weight determination of LD lipoprotein preparations were based on the assumption that these molecules are anhydrous spheres; the measured parameters were flotation coefficients and hydrated densities. Lindgren *et al.* (1969) found a molecular weight of 2.12×10^6 for LD lipoprotein from men and 2.36×10^6 for women, whereas Adams & Schumaker (1969) found values of 2.1×10^6 – 2.6×10^6 . When based on the same assumption and measured parameters, the molecular weights (2.38×10^6 – 2.58×10^6) of LD lipoprotein subfractions (LDL-IV, LDL-V and LDL-VI) of comparable hydrated densities were within the range reported in the literature. However, the inverse relationship between the hydrated density and molecular weight (Lindgren *et al.*, 1969) within these three density ranges was not observed. Frictional ratios of LDL-V and LDL-VI subfractions clearly indicate that these lipoproteins were hydrated and/or deviated from a spherical shape. This conclusion supports a similar view expressed by Fisher *et al.* (1971). Therefore the molecular-weight determinations based on Stokes frictional factor may be applied only to lipoprotein subfractions of low hydrated densities. Determination of molecular weight of LD lipoprotein or lipoprotein B subfractions (IV–VI) of high hydrated density should be performed by one of the absolute methods.

Determination of hydrodynamic properties of the six LD lipoprotein subfractions clearly showed that the low-density spectrum consists of three molecular segments characterized by a stepwise change in the molecular weight: LD lipoprotein subfractions LDL-I and LDL-II within the range d 1.006–1.019 g/ml with an average molecular weight of 4.6×10^6 – 4.9×10^6 , subfraction LDL-III of d 1.019–1.030 g/ml

with a molecular weight of 4.0×10^6 , and subfractions LDL-IV, LDL-V and LDL-VI within the range d 1.030–1.063 g/ml with a molecular weight of 2.9×10^6 . Several investigators (Oncley *et al.*, 1957; Shore & Shore, 1962; Nichols, 1967; Dearborn & Wetlauffer, 1969; Lindgren *et al.*, 1972) have suggested that the heterogeneity of LD lipoprotein particles in regard to particle size, hydrated density and molecular weight may be attributed to discrete changes in the lipid and protein content of lipoprotein molecules. Our previous studies (Lee & Alaupovic, 1970) on the chemical composition of the same six LD lipoprotein subfractions confirmed these reports and suggestions. Our results indicated, however, that, as with the molecular weights, the six subfractions exhibited a stepwise rather than a gradual change in the total lipid/protein or phospholipid/protein ratios. The average total lipid/protein ratio of LDL-I and LDL-II subfractions was 1.5, whereas that of LDL-IV, LDL-V and LDL-VI subfractions was 1.1; the LDL-III subfraction had an intermediate value of 1.2.

Recent studies on the quantitative determination of apolipoproteins in LD lipoproteins (Lee & Alaupovic, 1972) showed that apolipoprotein C accounted for 34–38% of the total protein content of subfractions LDL-I+II (d 1.006–1.019 g/ml), but only 3–5% of that of subfractions LDL-III–V (d 1.019–1.053 g/ml). These results indicate not only a remarkable parallel between the molecular weights, total lipid/protein ratios and ApoB/ApoC/ApoA proportions of LD lipoprotein subfractions, but also a sudden change in the numerical values of these parameters around d 1.019 g/ml. In an attempt to explain this abrupt change in numerical values, we used monospecific antibodies to lipoprotein B to precipitate lipoproteins from LDL-I+II. Immunological testing showed that precipitated lipoproteins contained both the lipoproteins B and C. In contrast, free lipoprotein B was isolated from each LD lipoprotein subfraction within the range d 1.019–1.063 g/ml. We suggest that these findings can best be explained by a concept invoking the existence of distinct lipoprotein families as the basic constituents of the lipid-transport system (Alaupovic, 1968, 1972; Alaupovic *et al.*, 1972). According to the lipoprotein family concept, the lipoprotein families occurring in the low-density segment of the lipoprotein spectrum exist in two different physicochemical forms. In the range above d 1.019 g/ml, lipoprotein families are present predominantly as separate physicochemical entities that can be isolated by the use of monospecific immunoabsorbents. However, in the range below d 1.019 g/ml, the lipoprotein families B and C and, if present, small quantities of A form a lipoprotein complex or complexes. The structural integrity of this complex(es) is preserved in both electric and gravitational fields. A stepwise change in the molecular weight, total lipid/protein ratios and ApoB/ApoC/ApoA proportions of LD lipoprotein

subfractions thus correlates with and depends upon the occurrence of lipoprotein B either as a part of an association complex or as a separate entity. In some individuals, a small amount of an association complex of lipoprotein B—lipoprotein C has also been observed in LD lipoprotein subfractions of higher densities.

The possibility that lipoproteins in LDL-I+II subfraction contain apolipoproteins B and C in a single molecule has not yet been ruled out. We prefer, however, the concept of association complexes for the following reasons. First, the large lipoprotein segment with densities higher than 1.019 g/ml consists of separate lipoprotein B and lipoprotein C molecules each of which is characterized by a full complement of neutral lipids and phospholipids. So far, lipoprotein species bearing smaller amounts of triglyceride and the same proportions of apolipoproteins B and C have not been isolated from this density range. Secondly, it has already been shown (Kook *et al.*, 1970) that VLD lipoproteins can be dissociated by heparin treatment into lipoprotein families A and B.

The similarity of phospholipid/protein ratios (Lee & Alaupovic, 1970) and molecular weights of lipoprotein B subfractions in the range d 1.019–1.063 g/ml indicates that the polydispersity of this lipoprotein family is caused mainly by a subtle change in the ratios of cholesterol esters and triglycerides rather than phospholipids and protein. It seems, then, that each segment of the polydisperse system of lipoprotein B family consists of the same phospholipid–protein structural unit, which is characterized by the capacity to maintain the structural integrity of triglyceride–cholesterol ester core of differing composition. The absolute amount of this unit seems to be practically constant throughout the spectrum of lipoprotein B.

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