Physicochemical Characterization of Rhesus Low Density Lipoproteins†

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ABSTRACT: The serum low density lipoprotein (LDL; ρ 1.019–1.050 g/ml) of the normal Macaca mulatta monkey (rhesus), kept on a low-fat Purina primate chow diet, was isolated by ultracentrifugal flotation, and its physicochemical properties were compared with those previously reported for human LDL. Rhesus LDL was found to be chemically similar to human LDL. The values for the sedimentation ($s_{25,w}^0$) and diffusion ($D_{25,w}^0$) coefficients were 7.09 S and 2.50×10^{-7} cm^2 sec^{-1}, respectively. The intrinsic viscosity was 3.40 ml g^{-1}. The partial specific volume of rhesus LDL, determined in an Anton Paar precision density meter, was 0.960 ml g^{-1}. Molecular weights, calculated from a combination of s^0 and D^0 and of s^0 and $[\eta]$, were in agreement with the weight-average molecular weight, $\bar{M}_w$, of 2.29×10^6 obtained by high-speed sedimentation equilibrium. In addition, a Z-average molecular weight, $\bar{M}_z$, of 2.73×10^6 was calculated because curvature in the graphs of log c vs. r^2 indicated that rhesus LDL was heterogeneous. From the frictional ratio of 1.02, a maximum hydration of 0.1 g of H_2O/g of lipoprotein was obtained. On electron micrographs, rhesus LDL appeared spherical with a mean diameter of 196 Å, which was substantiated by hydrodynamic analysis.

Human low density lipoprotein (LDL)[1] has been extensively characterized (Oncley et al., 1957; Pollard et al., 1969; Lindgren et al., 1969; Fisher et al., 1971; Schumaker, 1973; Lee and Alaupovic, 1974), but little information is available on the physicochemical properties of this lipoprotein class in the Macaca mulatta monkey (rhesus). Since this monkey is one of the experimental animals used in the study of atherosclerosis, a systematic investigation of the characteristics of its LDL may prove valuable for a better understanding of the pathogenesis of this disease. In this report, we will describe the physicochemical properties of rhesus LDL and compare them with those of their human counterpart.[2] While this work was in progress, Lee and Morris (1974) published a report on rhesus LDL; their data are analyzed in the Discussion.

Materials and Methods

Isolation of Lipoproteins. Two male rhesus monkeys, 1–2 years of age, were obtained from the Food Research Institute, University of Wisconsin, Madison. They were singly caged and maintained on a modified Purina primate chow diet, very low in fat and low in cholesterol. Their serum cholesterol was less than 150 mg/100 ml, and total lipids were less than 600 mg/100 ml.

Each animal was fasted 20 hr before being subjected to plasmapheresis. About 20 ml of blood was drawn and mixed with a standard citrate–dextrose solution to prevent coagulation. The plasma was separated from the blood cells by low-speed centrifugation, and Na_2EDTA, adjusted to pH 7.0, was immediately added to a final concentration of 0.05%. The blood cells were washed with physiological salt solution and returned into the animal's circulation. The pooled plasma was processed for lipoprotein isolation within 24 hr from blood withdrawal.

All centrifugations were carried out in a Spinco L2-65B preparative ultracentrifuge at 10°, with a Spinco 30.2 rotor. Plasma was centrifuged for 20 hr at 30,000 rpm. The floating VLDL was removed by aspiration and the plasma density was increased to ρ 1.050 g/ml with solid NaCl.[3] The LDL was removed after a spin at 30,000 rpm for 24 hr, diluted with deionized, distilled water to a medium density of 1.019 g/ml, and then recentrifuged for 24 hr at 30,000 rpm. The bottom milliliter was saved and adjusted to ρ 1.050 g/ml with solid NaCl and overlayered with ρ 1.050 salt solution containing 0.05% Na_2EDTA. After a final centrifugation for 48 hr at 30,000 rpm, the floating LDL was removed and stored under nitrogen at 4°. Human LDL (ρ 1.019–1.063 g/ml) was prepared as previously described (Scanu et al., 1968).

Agarose Gel Column Chromatography. LDL was fractionated by gel filtration on a column (1.27 × 90 cm) packed with Sepharose 4-B (Pharmacia) and equilibrated at 4° with 0.15 *M* NaCl (pH 7.0) containing 0.02% NaN_3 and 0.025% Na_2EDTA. The column was monitored at 280 nm with an ISCO Model UA-4 column monitor. The flow rate was 4 ml/hr. Fractions of approximately 2.0 ml were collected in pretared tubes and weighed so that the elution weight of the lipoprotein could be calculated. The distribution coefficient of the eluted lipoproteins was evaluated from the expression $K_d = (V_e - V_0)/(V_i - V_0)$, where V_e is

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[1] Abbreviations used are: LDL, low density lipoprotein; Na_2EDTA, disodium ethylenediaminetetraacetate.

[2] A preliminary report of our results has already appeared (Scanu et al., 1974).

[3] We found that the complete LDL fraction was present in the density interval 1.019–1.050 and that by using this narrower range we were able to eliminate an as yet uncharacterized component that was present in our preparations isolated between ρ 1.019 and 1.063.

the elution weight of solvent corresponding to the peak concentration of the eluting protein, V_0 is the weight of solvent that is external to the gel matrix, and V_i is the total weight of solvent in the column. The void volume of the column was determined with *Escherichia coli* bacteriophage T4 (mol wt $\sim 300 \times 10^6$); V_i was determined with 2-mercaptoethanol.

Density Gradient Ultracentrifugation. The experiments were conducted at 25° in a Beckman L2-65B ultracentrifuge at 38,000 rpm for 66 hr. Linear sucrose or NaBr gradients were preformed in tubes for the Spinco SW-40 rotor. Gradients of 12.2 ml were constructed from 0% solute to either 15% sucrose or 10% NaBr. All solutions contained 0.025% Na_2EDTA and 0.02% NaN_3 and were adjusted to pH 7.0. Rhesus LDL dialyzed against 0.025% Na_2EDTA and 0.02% NaN_3 (pH 7.0) was added to the top of the gradient. A gradient tube containing dialysate instead of LDL was used as control. After centrifugation, the tubes were punctured at the bottom and 0.6-ml fractions were collected and monitored at 280 nm for absorbancy. The densities of the fractions from the control tube were determined by analysis with a Zeiss Abbe refractometer, Model A, which was thermoregulated at 20°.

Analytical Ultracentrifugation. The sedimentation, flotation, and diffusion coefficients of monkey LDL were determined at 25.0° in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. The lipoprotein preparations were dialyzed against a salt solution of pH 7.0 containing 0.2 *M* KCl, 0.0125 *M* sodium phosphate, and 0.025% Na_2EDTA (ρ 1.008 g/ml). For flotation analysis, the lipoproteins were dialyzed against ρ 1.063 NaCl, 0.025% Na_2EDTA. The dialysate was used to dilute the lipoproteins to the desired concentration for analysis. Both sedimentation and flotation measurements were conducted at 44,000 rpm with an An-D rotor and a double sector cell. Apparent sedimentation and flotation coefficients were calculated from a series of concentrations and plotted against protein concentration. The coefficients at zero concentration were obtained from the intercept of the least-squares line of these plots.

The diffusion experiments were carried out at 3,600 rpm at a temperature of 25°. Solvent was layered over the solution by means of a synthetic boundary centerpiece. The apparent values of the translational diffusion constant (D_{app}), at various concentrations, were evaluated from the area and height of the boundary of the schlieren peaks by means of standard procedures (Chervenka, 1970).

The schlieren patterns were transcribed onto tracing paper with a 10× Nikon Model 6C microcomparator. The areas were then determined with a Keuffel and Esser compensating polar planimeter and represented as averages of five readings. The diffusion coefficient, $D_{25,w}{}^0$, was evaluated from the intercept of the least-squares plot of D_{app} vs. concentration.

Partial Specific Volume. The densities of the dialysate and the lipoprotein solutions were measured with a Precision Density Meter DMA-02 (Anton Paar, Graz, Austria). All measurements were conducted with the cell compartment maintained at 25 ± 0.02°. No solutions were measured until a stable reading for consecutive aliquots of deionized distilled water had been obtained. For each individual sample, the precision was $\pm 1 \times 10^{-5}$ sec at a present count of 1×10^4. After each measurement, the cell was washed with copious amounts of saline, deionized distilled water, and finally with absolute ethanol. The cell was then dried with air until a constant reading on the instrument was obtained. Measurements were continued only when the readings for air before and after each sample were the same. Upon completion of the experiment, the instrument constant was rechecked with deionized distilled water used as a standard. Densities were measured with an error of about ±0.00002 g/ml. The apparent partial specific volume, $\bar{v}_{app}$, was calculated from the equation

$$\bar{v}_{app} = \frac{1}{\rho_0} + \frac{1}{c}\frac{\rho - \rho_0}{\rho_0}$$

where ρ_0 is the density of the solvent (0.2 *M* KCl–0.0125 *M* sodium phosphate–0.025% EDTA, pH 7.0), ρ the density of the solution, and c the lipoprotein concentration in grams per milliliter (Schachman, 1957). The calculated values of $\bar{v}_{app}$ were then plotted as a function of protein concentration, and the extrapolated value at infinite dilution was taken as the partial specific volume, $\bar{v}_2$. No concentration dependence was observed.

Determination of Molecular Weight. High-speed sedimentation equilibrium experiments, using a 12-mm thick charcoal filled Epon centerpiece, were carried out essentially as described by Yphantis (1964). The lipoprotein sample was dialyzed exhaustively against 0.2 *M* KCl–0.0125 *M* phosphate buffer (pH 7.0) containing 0.025% Na_2EDTA and 0.02% NaN_3. Lipoprotein concentrations between 0.2 and 0.85 mg/ml were prepared by dilution with the dialysate. Column heights of 3 mm were used routinely. No fluorocarbon or silicone oil base fluid was used because of the likelihood of lipoprotein denaturation at the solution–fluid interface. Attainment of equilibrium was usually checked after 24 hr by establishing that no further fringe displacement with time had occurred at a given radial distance, r, from the center of rotation. The temperature was maintained at 25° with the R.T.I.C. unit.

Following each experiment, a photograph of the base-line pattern was taken according to the method outlined by Teller (1973), to correct for window distortion. Both blank and sample fringe displacements were measured at 200-μ intervals on the x coordinate by following one light fringe. Every point was an average of five measurements, in which the mean value never differed from the middle point by more than 5 μ. The base-line data were smoothed graphically before being applied to the equilibrium data. Plots of log c vs. r^2 exhibited substantial curvature and were therefore computer fitted to a least-squares polynomial of second order; and values of d log c/dr^2 were then obtained by taking the first derivative of the polynomial at each measured radial distance. This allowed the calculation of the weight-average molecular weight, M_w, at each point, from the equation

$$M_w = [(2RT/(1 - \bar{v}_2\rho)\omega^2)](\text{d log } c/dr^2)$$

where R is the gas constant, T the absolute temperature, $\bar{v}_2$ the partial specific volume of the solute, ρ the density of the solution, ω the angular velocity, c the concentration, and r is the distance from the axis of rotation. The Z-average effective molecular weight of the entire solution, as shown by Yphantis (1964), is simply the weight average at the base of the cell, i.e., $\bar{M}_z = M_w(b)$. The weight-average effective molecular weight of the complete solution is the number average obtained at the base of the cell, i.e., $\bar{M}_w = M_n(b)$ (Yphantis, 1964). In order to determine these quantities it was necessary to do a short extrapolation to the bottom of the cell. The number-average molecular weight at the base of the cell was calculated from the equation

$$M_n(b) = \frac{c(r)}{\int_{r_m}^{r_b} c(r)d(r^2/2)} \frac{RT}{(1 - \bar{v}_2\rho)\omega^2} \quad (1)$$

where $c(r)$ is the concentration in fringes at a distance r from the center of rotation, and r_m and r_b are the radial distances to the meniscus and the bottom, respectively. The integral in the denominator was obtained by graphical integration.

The molecular weight of rhesus LDL was also determined from the sedimentation and diffusion coefficients, s^0 and D^0, by the Svedberg equation

$$M = RTs^0/D^0(1 - \bar{v}_2\rho) \quad (2)$$

For an additional check, the molecular weight was calculated from the sedimentation coefficient and intrinsic viscosity, $[\eta]$, by means of the Scheraga–Mandelkern equation:

$$M^{2/3} = Ns^0[\eta]^{1/3}\eta_0/\beta(1 - \bar{v}_2\rho) \quad (3)$$

where N is Avogadro's number, η_0 is the viscosity of the solvent, and β is a function of the shape of the particle.

Viscosity measurements were made in Cannon-Manning No. 50 semi-micro viscometers (Cannon Instrument Co., State College, Pa.). These viscometers had a flow time of over 200 sec for water. The kinetic energy correction for such flow times is small and was ignored (Van Holde and Sun, 1962). The viscometers required a charge of 0.5 ml and were mounted vertically in a constant-temperature bath which was maintained at 25 ± 0.02°. Before measurement, solutions were cleared of lint and dust by a low-speed centrifugation. When warranted, solutions were filtered through 0.45-μ Millipore filter membranes. Readings of flow times were taken until they were reproducible to ±0.1 sec. Solution densities were determined with the Anton Paar density meter as described above.

Experimental data were expressed in terms of the relative viscosity according to the equation $\eta_{rel} = \eta/\eta_0 = \rho t/\rho_0 t_0$, where ρ_0 and t_0 are the density and the flow time for the reference liquid, d and t being the corresponding values of the lipoprotein solutions.

The value for the intrinsic viscosity was obtained by extrapolation of the reduced viscosity, $\eta_{red} = (\eta - \eta_0)/\eta_0 C$, to zero lipoprotein concentration, i.e., $\lim_{c\to 0}(\eta_{red}) = [\eta]$, where $[\eta]$ is the intrinsic viscosity (Tanford, 1961).

Immunology. Double diffusion studies and production of antisera to human LDL were carried out as previously reported (Scanu et al., 1973). Immunoelectrophoresis of rhesus LDL was done on precast agarose films (Pfizer Diagnostics Division) with pH 8.6 barbital serving as the electrode buffer. Electrophoresis was conducted for 40 min at 90 V.

Electron Microscopy. Studies were carried out in a Phillips EM 200 unit, on specimens that were negatively stained with 1% phosphotungstic acid (pH 7.2) and stored at 4°. The conditions were essentially those described previously (Pollard et al., 1969).

Polyacrylamide–Agarose Disc Gel Electrophoresis. The electrophoretic procedure was that of Davis (1964). We used a 3.75% running gel, pH 8.9, to which 1% agarose was added to improve the handling of the gel, and a 2.5% spacing gel with a pH of 6.7. The electrode buffer was pH 8.3 Tris-glycine buffer. Coomassie Blue was used for staining according to the method of Chrambach et al. (1967).

Amino Acid Analysis. LDL was subjected to density gradient ultracentrifugation on a linear gradient constructed from 15% sucrose, as described above. The samples examined were taken from the narrow central portion of the band. Lipid was removed from the lipoprotein with 2:1 chloroform–methanol after dialysis against 0.05 M NaCl (pH 7.0)–0.01% Na_2EDTA (Folch et al., 1957). The extracted protein was dried over P_2O_5 and hydrolyzed, after careful deaeration, in 6 N HCl at 110°. Losses due to hydrolysis were determined by extrapolation from 24-, 48-, and 72-hr hydrolysates. Analyses were carried out at least in duplicate, in a Beckman Model 121 amino acid analyzer equipped with an automatic sample injector, scale expander, and integrator. The cysteine and cystine content of apo-LDL was estimated by determination of cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan was determined spectrophotometrically according to the method of Edelhoch (1967).

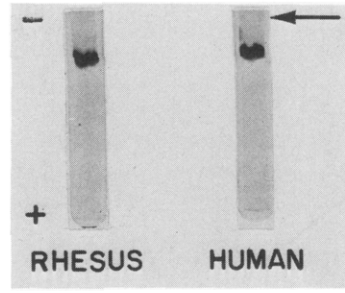

FIGURE 1: Electrophoresis of rhesus LDL and human LDL in 3.75% polyacrylamide–1% agarose gels after staining with Coomassie Blue.

Chemical Analysis. Protein content was determined by Lowry's method, with bovine serum albumin as standard (Lowry et al., 1951). Lipid phosphorus was measured essentially according to the method of Bartlett (1959). The phospholipid concentration (as lecithin) was obtained by multiplying the phosphorus content by a factor of 25.4, which is based on an estimated average molecular weight of 787 for egg lecithin. Free cholesterol was determined by a slight modification of the Abell–Kendall method (Abell et al., 1952). Total cholesterol and triglyceride were determined with an Autoanalyzer II (Technicon Instruments Corp) (Rush et al., 1971). The cholesterol channel of the autoanalyzer was calibrated with samples of known Abell–Kendall values that were supplied by the Center for Disease Control Laboratory (Atlanta, Ga.).

Carbohydrate Analysis. Rhesus and human LDL in 0.001 N NaOH was delipidated by extraction with mixtures of ether and ethanol (Fless, 1971). The organic solvent and any noncovalently bound sugars were removed by dialysis against 0.001 M $NaHCO_3$ (pH 9.5). Internal standard arabitol was added to the soluble apo-LDL before neutralization with HCl and subsequent lyophilization. Carbohydrate was released from the freeze-dried apo-LDL by refluxing with dry 1 N HCl in anhydrous methanol. The sugars were analyzed as their trimethylsilyl ether derivatives by gas–liquid chromatography, according to the method of Dawson and Clamp (1970).

Results

Assessment of Purity. Rhesus LDL exhibited a single band with a mobility equal to that of human LDL on acrylamide–agarose electrophoresis (Figure 1). By agarose column chromatography, only one component could be demonstrated with a K_d of 0.43. Human LDL behaved similarly, but eluted with a K_d of 0.40. Rhesus LDL, like human LDL, banded as a single component by sucrose or NaBr

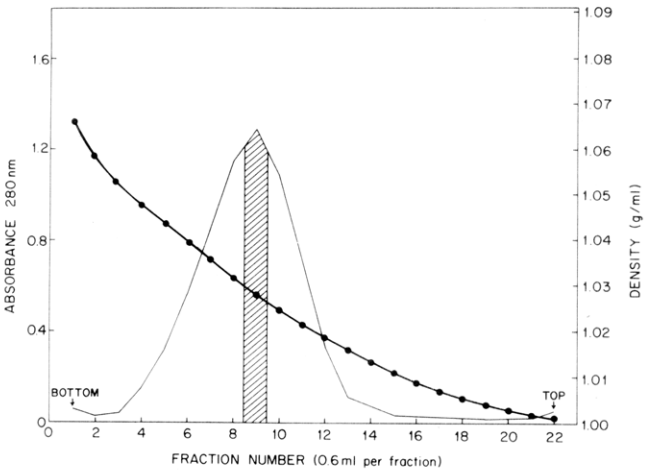

FIGURE 2: Sucrose gradient 0–15% in 0.025% Na_2EDTA and 0.02% NaN_3 (pH 7.0). Rhesus LDL was spun at 38,000 rpm for 66 hr in the SW-40 rotor of a Beckman Model L2-65B centrifuge at 25°. Fractions of 0.6 ml were collected from the bottom and monitored for absorbance at 280 nm. The shaded area represents the "narrow cut" LDL which was taken for molecular weight analysis by sedimentation equilibrium.

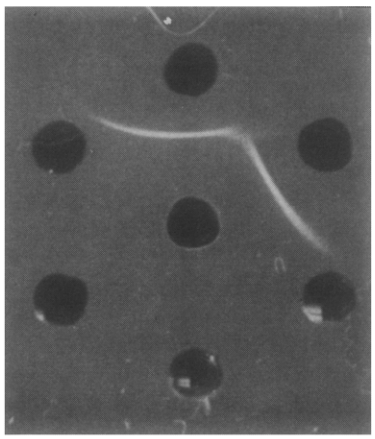

FIGURE 3: Double immunodiffusion photograph of the reaction of human LDL and rhesus LDL against anti-human LDL which is located in the center well. Human LDL was present in the top well; rhesus LDL was in the two o'clock position.

density gradient ultracentrifugation (Figure 2).

Immunological Properties. Rhesus LDL, when reacted with antisera raised in the rabbit against human LDL, produced a single line of precipitation which exhibited partial identity with human LDL (Figure 3). By immunoelectrophoresis, rhesus LDL gave a single line of precipitation when reacted with antisera raised in the rabbit against whole human serum.

Electron Microscopic Studies. Electron micrographs of LDL revealed free-standing spherical particles with an average diameter of 196 ± 10 Å (Figure 4). The morphology was similar to that reported for human LDL (Forte et al., 1968).

Composition of Rhesus LDL. The lipid–protein composition of rhesus LDL indicated a close similarity with the results reported for normal man (Table I). The triglyceride content was lower, and that of free cholesterol higher, in rhesus than in human LDL. The amino acid composition of rhesus LDL is shown in Table II. No major difference was found between human and monkey apo-LDL within experimental error. The carbohydrate composition of apo-LDL for the two species is given in Table III. Like human apo-LDL, rhesus apo-LDL contains mannose, galactose, glucosamine, and sialic acid. The content of neutral sugars, i.e., mannose and galactose, is slightly less in rhesus LDL whereas that of glucosamine and sialic acid is almost identical with that in human LDL.

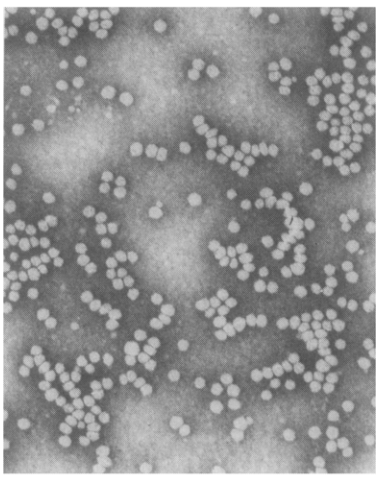

FIGURE 4: Electron micrograph of rhesus LDL × 150,000.

Table I: Percentage Composition of Rhesus and Human LDL.

	Rhesus	Human[a]
Triglyceride	2.5 ± 0.2	11, 12
Phospholipid	23.6 ± 1.0	22, 20
Free cholesterol	14.0 ± 1.2	8, 10
Cholesterol ester	36.0 ± 0.5	37, 36
Protein	24.0 ± 2.0	21, 22

[a] The first number is an average value from Oncley et al. (1957); the second number is taken from Hatch and Lees (1968).

Table II: Amino Acid Analysis of Rhesus and Human LDL Protein.

	Moles of Amino Acid/100,000 g of Amino Acid Residues Recovered	
	Rhesus	Human
Lysine	64.0 ± 1.8	67.8 ± 0.9
Histidine	21.1 ± 1.0	21.2 ± 0.9
Arginine	28.5 ± 1.1	28.6 ± 0.9
Aspartic acid and asparagine	93.3 ± 0.8	94.2 ± 1.1
Threonine[a]	62.1 ± 0.5	62.5 ± 0.6
Serine[a]	76.5 ± 1.3	74.6 ± 0.9
Glutamic acid and glutamine	109.7 ± 1.7	106.2 ± 2.1
Proline	39.5 ± 1.9	39.0 ± 1.7
Glycine	41.2 ± 0.9	40.5 ± 2.0
Alanine	51.2 ± 1.1	53.4 ± 1.1
Half-cystine[b]	4.7 ± 0.1	4.9 ± 0.2
Valine	50.3 ± 0.8	47.9 ± 1.0
Methionine	14.4 ± 0.5	14.0 ± 0.9
Isoleucine	50.8 ± 0.7	49.5 ± 0.8
Leucine	105.5 ± 1.0	106.2 ± 1.4
Tyrosine	27.2 ± 0.8	29.0 ± 1.6
Phenylalanine	41.6 ± 1.3	42.9 ± 0.8
Tryptophan[c]	9.0 ± 0.3	

[a] Corrected for decomposition from 24-hr, 48-hr, and 72-hr hydrolysates. [b] Determined as cysteic acid after performic acid oxidation by the method of Moore (1963). [c] Determined by the spectrophotometric method of Edelhoch (1967).

Table III: Carbohydrate Composition of Rhesus and Human Apo-LDL.

	% by Weight	
	Rhesus	Human
Mannose	1.92	2.48
Galactose	0.82	1.06
N-Acetylglucosamine	1.70	1.78
Sialic acid	2.13	2.14

Table IV: Physical Properties of Rhesus LDL Compared to Human Values.

Physical Property	Rhesus	Human
1. $s_{25,w}^0$ (S)	7.09 ± 0.15	7.85[a]
2. $s_{25,1.063}^0$ (S)	−5.75 ± 0.28	−6.38[b]
3. $D_{25,w}^0 \times 10^7$ (cm^2 sec^{-1})	2.50 ± 0.26	2.17[c]
4. $[\eta]$ (ml/g)	3.4 ± 0.2	3.4[d]
5. $\bar{v}_2$ (ml/g)	0.960 ± 0.005	0.967[c]
6. f/f_0	1.02	1.10, 1.11[c,g]
7. δ_1 (g of H_2O /g of LDL)	0.1	0.34[c]
8. $\bar{M}_w \times 10^{-6}$		
(sedimentation equilibrium)	2.29 ± 0.12	2.2, 3.2[e]
(light scattering)		2.77[f]
9. $\bar{M}_z \times 10^{-6}$		
(sedimentation equilibrium)	2.73 ± 0.26	
10. $M \times 10^{-6}$ (from s^0 and D^0)	2.18	2.73[c]
11. $M \times 10^{-6}$ (from s^0 and $[\eta]$)	2.37	
12. Diameter (Stokes) (Å)	198	202, 229[c,h]
(EM) (Å)	196 ± 10	216[i]

[a] Value taken from Fisher et al. (1971) and corrected for concentration from data of Lee and Alaupovic (9174). [b] Value taken from Fisher et al. (1971) and converted from $s_{25,1.20}^0$ to $s_{25,1.063}^0$ as outlined by Schumaker (1973). [c] Value taken from Fisher et al. (1971). [d] Value taken from Toro-Goyco (1958). [e] First value from Scanu et al. (1968); second value from Lee and Alaupovic (1974). [f] Value from Bjorklund and Katz (1956). [g] Value taken from Schumaker (1973). [h] Value taken from DeBlois et al. (1973). [i] Value taken from Forte et al. (1968).

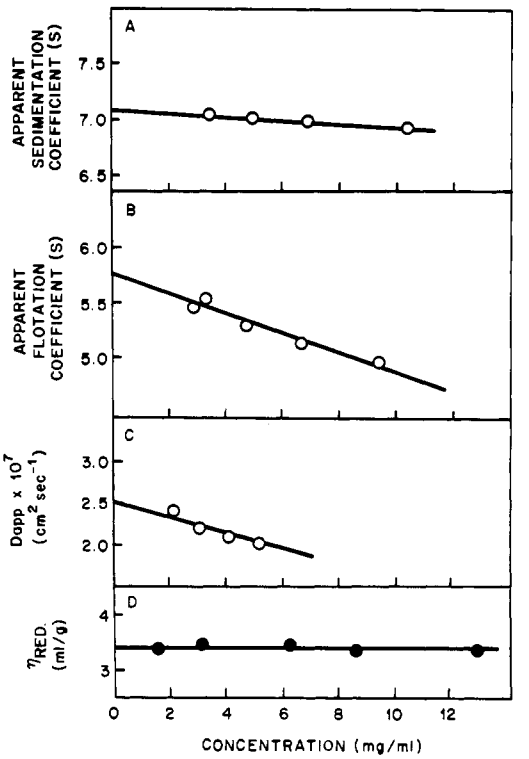

FIGURE 5: (A) Apparent sedimentation coefficient of rhesus LDL plotted as a function of lipoprotein concentration (○). The solvent was 0.2 *M* KCl–0.0125 *M* phosphate (pH 7.0) containing 0.025% Na_2EDTA. The rotor speed was 44,000 rpm at 25°. (B) Concentration dependence of the flotation coefficient in ρ 1.063 g/ml of NaCl containing 0.025% Na_2EDTA (pH 7.0). Same rotor speed and temperature as in A. (C) Concentration dependence of the apparent diffusion coefficient of rhesus LDL. Measurements were made in 0.2 *M* KCl–0.0125 *M* phosphate (pH 7.0) containing 0.025% Na_2EDTA. Rotor speed was 3600 rpm at 25°. (D) Measurement of intrinsic viscosity in 0.2 *M* KCl–0.0125 *M* phosphate (pH 7.0) containing 0.025% Na_2EDTA and 0.02% NaN_3, at 25°. The concentration is expressed as lipoprotein protein (●).

Hydrodynamic Analysis of Rhesus LDL. The sedimentation coefficient determined in 0.2 *M* KCl–0.0125 *M* phosphate buffer (pH 7.0) containing 0.025% Na_2EDTA was found to be 7.09 S when corrected to 25° and water (Figure 5A). The sedimentation coefficient exhibited only a small concentration dependence whereas this dependence was found to be comparatively more pronounced in the flotation experiments (Figure 5B). The value of the flotation coefficient was 5.75 S in ρ 1.063 g/ml of NaCl (pH 7.0) containing 0.025% Na_2EDTA. The diffusion coefficient was measured in 0.2 *M* KCl–0.0125 *M* phosphate buffer (pH 7.0) containing 0.025% Na_2EDTA. The translational diffusion coefficient, corrected to 25° and water, was 2.50×10^{-7} cm^2 sec^{-1} and was obtained from plots of D_{app} vs. concentration (Figure 5C). Using the values of s^0 and D^0, we calculated the molecular weight of rhesus LDL from the Svedberg equation (eq 2). The value of 2.18×10^6 was close to the weight-average molecular weight of rhesus LDL obtained directly from the sedimentation equilibrium (see the following section and Table IV). A similar molecular weight, 2.37×10^6, was estimated from the Scheraga–Mandelkern equation (eq 3). We chose the value $\beta = 2.16 \times 10^6$, because the intrinsic viscosity suggested that native rhesus LDL has a very compact globular structure. The intrinsic viscosity of rhesus LDL determined in this study is 3.4 ml/g, which is identical with the value obtained for human LDL by Toro-Goyco (1958) (Figure 5D). The results of the hydrodynamic analysis of rhesus LDL, together with values for human LDL from the literature, are summarized in Table IV.

Molecular Weight of Rhesus LDL. The molecular weight of rhesus LDL was also determined by meniscus-depletion sedimentation equilibrium (Yphantis, 1964). In Figure 6, the logarithm of the solute concentration c is plotted in terms of Rayleigh fringe displacements, μ (in microns), against the square of the distance, r (in centimeters), from the center of rotation. The curvature of the graph indicates heterogeneity. The concentration dependence of the weight-average molecular weight determined for each point in the cell, as shown in Figure 7, was found by evaluation of $d \log c/dr^2$ from the data given in Figure 6. The partial specific volume of rhesus LDL used in these calculations was 0.960 ± 0.005 ml/g, the value obtained with the Anton Paar precision density meter. The weight-average molecular weight ranges from a value of approximately 1.5×10^6 at the meniscus to about 2.7×10^6 at the bottom of the cell. These limiting values hold true only for the conditions listed in Figure 7 because, for a heterogeneous system that is not in chemical equilibrium, a different molecular weight distribution will be observed for each different initial loading concentration (Harris et al., 1969). However, as stated by Yphantis (1964), the quantities of interest are not the point values of the molecular weight aver-

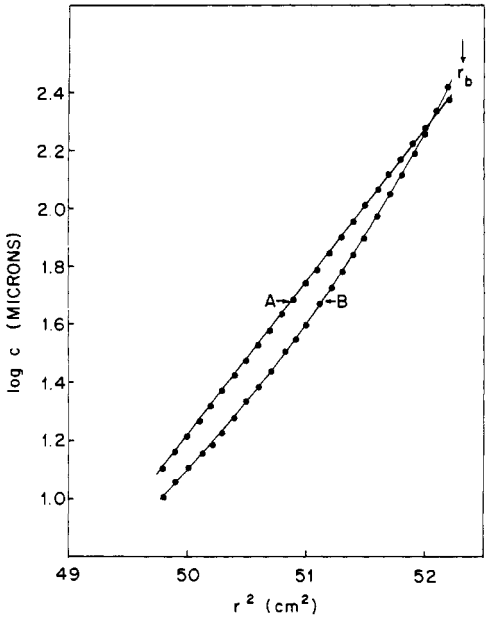

FIGURE 6: Sedimentation equilibrium of rhesus LDL: (A) "narrow cut" LDL taken from the central portion of the band of the sucrose gradient; (B) whole LDL isolated between ρ 1.019 and 1.050.

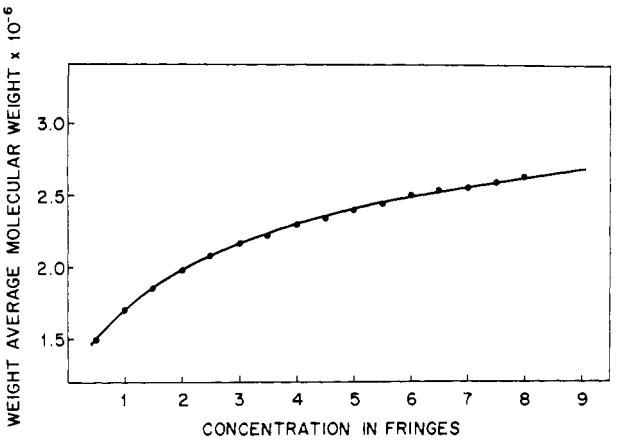

FIGURE 7: Dependence of the molecular weight of rhesus LDL (ρ 1.019–1.050) on concentration. Evaluated from the curve in Figure 6 as described in the text.

ages, but rather the averages of these quantities over all contents of the cell so that the initial solution is characterized as a whole. Accordingly, the $\bar{M}_{w,app}$ for rhesus LDL (ρ 1.019–1.050) was determined from eq 1, and $\bar{M}_{z,app}$ from the weight-averaged molecular weight at the bottom of the cell. The least-squares plot of the reciprocals of the molecular weight averages vs. concentration (Figure 8) indicates only slight concentration dependence for the weight-average molecular weight and a somewhat larger dependence for the Z-average molecular weight. We obtained a $\bar{M}_z$ of 2.73×10^6 and a $\bar{M}_w$ of 2.29×10^6 for rhesus LDL at infinite dilution, where the superscript bar refers to the average quantities over all contents of the cell. Rhesus LDL, like human LDL, was thus shown to be heterogeneous with respect to molecular weight.

It was of interest to determine the molecular weight of rhesus LDL isolated from a narrow density interval. Therefore, LDL (ρ 1.019–1.050) was placed on a linear gradient constructed from 15% sucrose and buffer and spun to isopycnic equilibrium at 25°. Only the LDL from a narrow

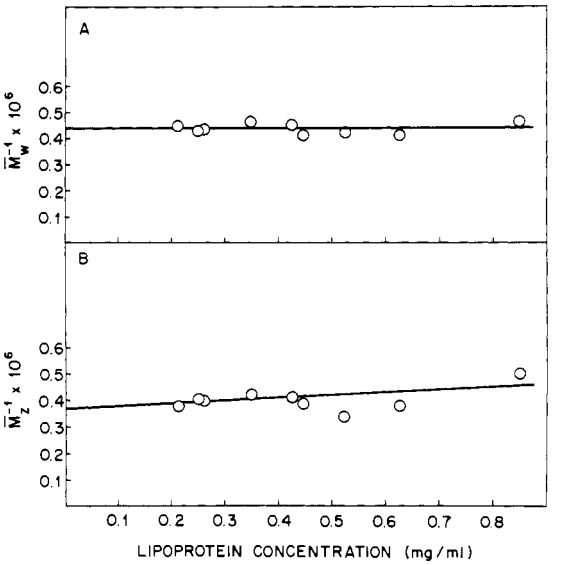

FIGURE 8: Concentration dependence of the molecular weight average of rhesus LDL. (A) Weight-average molecular weight of the complete solution. (B) Z-average molecular weight of the complete solution.

central portion of the gradient, representing an approximate density interval of 1.032–1.036 g/ml, was taken for molecular weight analysis (Figure 2). This material appeared to be homogeneous with respect to molecular weight, as evidenced by the linearity of the plot of log c vs. r^2 shown in Figure 6. The molecular weight of this sample was calculated for a partial specific volume of 0.967 ml/g, which is the inverse of the buoyant density in a NaBr gradient (Schumaker, 1973). The buoyant density in NaBr (1.034 g/ml) was used instead of the lower value obtained in sucrose (1.028 g/ml), because NaBr is more representative of the conditions used in sedimentation equilibrium, where 0.2 M KCl was employed as the solvent. Furthermore, this choice was based on the fact that the $\bar{v}_2$ measured for whole LDL (ρ 1.019–1.050 g/ml) is representative of a solution of particles heterogeneous in size and probably in partial specific volume (Schumaker, 1973). Under these conditions, the weight-average molecular weight of the narrow cut LDL at infinite dilution was 2.51×10^6.

Discussion

The results of the present studies have shown that rhesus LDL of ρ 1.019–1.050 is heterogeneous when determined by meniscus depletion sedimentation equilibrium, in spite of the apparent homogeneity indicated by electrophoretic, chromatographic, and density gradient centrifugal studies. This observed heterogeneity is in agreement with the current concept about human LDL, that it consists of a distribution of particles varying in size, hydrated density, and molecular weight, the variation probably being related to changes in the lipid and protein content of the particles (Oncley et al., 1957; Shore and Shore, 1962; Nichols, 1967; Dearborn and Wetlaufer, 1969; Lee and Alaupovic, 1974). It is of interest to note that, although the molecular weight of human LDL has recently been determined by sedimentation and flotation equilibrium the plot of log c vs. r^2 showed no obvious curvature (Scanu et al., 1968; Lee and Alaupovic, 1974; Nelson et al., 1974). These data may be taken to indicate homogeneity of human LDL, but they do not represent proof, because curvature due to heterogeneity could be cancelled out by nonideality (Chervenka, 1970). When

the data obtained from our sedimentation equilibrium studies were treated according to the method outlined by Yphantis and Teller, definite curvature in the plot of log c vs. r^2 was detected (Yphantis, 1964; Teller, 1973). From the analysis of these plots, and using a value of 0.960 ml/g for the partial specific volume, we obtained a weight-average molecular weight ($\bar{M}_w$) for rhesus LDL of 2.29 ± 0.12 × 10^6 and a Z-average molecular weight ($\bar{M}_z$) of 2.73 ± 0.26 × 10^6. The weight-average molecular weight determined by sedimentation equilibrium is somewhat higher than the molecular weight average of 2.18 × 10^6 obtained from s and D. This is in agreement with an important generalization which states that the molecular weight values derived from the transport method are somewhat lower than those obtained by sedimentation equilibrium (Creeth and Pain, 1967). The average molecular weight of human LDL is 2.7 ± 0.4 × 10^6 as determined by a number of investigators (Fisher et al., 1971; Bjorklund and Katz, 1956; Lee and Alaupovic, 1974; Nelson et al., 1974; Adams and Schumaker, 1969; Schumaker, 1973). The wide range in the reported values may indicate individual differences but may also reflect different experimental conditions and methods of analysis. The weight average molecular weight of rhesus LDL reported here therefore falls into the lower end of the range of values reported for human LDL. This observation is in accordance with the data from gel filtration on Sepharose 4B which indicated that the distribution coefficient for rhesus LDL is larger than that for human LDL.

As suggested by the linearity of the log c vs. r^2 plot, the molecular weight heterogeneity was significantly reduced when the analyses were conducted on a narrow LDL cut taken from the central portion of the band after sucrose gradient ultracentrifugation. The weight-average molecular weight of this fraction was 2.51 × 10^6 when calculated with a $\bar{v}_{app}$ of 0.967 ml/g.

The diffusion coefficient of rhesus LDL, measured in 0.2 M KCl, gave a value $D_{25,w}{}^0 = 2.50 \times 10^{-7}$ cm^2 sec^{-1}. For human LDL, values of $D_{25,w}{}^0$ have been reported to vary between 2.01 and 2.25 × 10^{-7} cm^2 sec^{-1} (Fisher et al., 1971; Wong, 1972; DeBlois et al., 1973; Schumaker, 1973). Although these measurements were made in solutions containing relatively low and high salt concentrations, identical results were obtained in both solvents (Fisher et al., 1971). The somewhat greater value of the diffusion coefficient of rhesus LDL is therefore consistent with a particle size smaller than that of human LDL analyzed by Fisher et al. (1971).

The knowledge of the diffusion coefficient, D^0, the anhydrous molecular weight, M, and the partial specific volume, $\bar{v}_2$, of rhesus LDL permits one to draw certain conclusions about the shape and hydration of the particle from the equation

$$RT/ND^0 = (6\pi\eta f/f_0)[3M(\bar{v}_2 + \delta_1\bar{v}_1{}^0)/4\pi N]^{1/3}$$

which contains two unknown quantities: f/f_0, the deviation of the shape of the lipoprotein hydrodynamic particle from a sphere, and δ_1, the hydration of the particle (Tanford, 1961). Using the weight-average molecular weight obtained from sedimentation equilibrium and assuming no hydration, one can ascribe the frictional ratio, f/f_0, of 1.02 entirely to asymmetry and take it to indicate that rhesus LDL behaves hydrodynamically almost like a sphere. The electron-microscopic data showed that rhesus LDL is indeed spherical (Figure 4). If, in turn, we assume that $f/f_0 = 1$, the deviation of this ratio from unity may be ascribed to hydration. The maximum hydration obtained in this manner is 0.1 g of H_2O/g of LDL. The radius, R_0, of a hydrated sphere of equivalent molecular weight and partial specific volume can be calculated from the relation

$$R_0 = [(3M/4)(\bar{v}_2 + \delta_1\bar{v}_1{}^0)]^{1/3}$$

if δ_1 is taken to be 0.1 g of H_2O/g of LDL (Tanford, 1961). The hydrated radius of rhesus LDL thus obtained is 99 Å, giving a diameter of 198 Å. The diameter of 196 Å obtained from electron microscopy is in excellent agreement with the above value. The calculated parameters of rhesus LDL are listed in Table IV, together with corresponding values for human LDL. The overall information obtained thus far suggests that rhesus LDL may have a smaller particle size than human LDL and may be less hydrated, although similar in chemical composition, but analysis from a larger sample of monkeys are needed to make this conclusion valid.

Lee and Morris (1974) have also characterized rhesus LDL from normal animals. Their data, however, show some differences from our findings in terms of amino acid composition (content in glycine, valine, methionine, and isoleucine), as well as higher triglyceride and lower cholesterol values. Furthermore, by flotation equilibrium Lee and Morris found a weight-average molecular weight 3.3 × 10^6, which is significantly higher than that obtained in the current study. We cannot account for this difference except to ascribe it to differences in the animals used or in their diet. The use by Lee and Morris of fluorocarbon oil to improve the resolution of the fringes may be questioned since this oil is likely to interact with the lipoprotein particles, thus forming artifacts (Smith et al., 1972).

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Fusion of Fatty Acid Containing Lecithin Vesicles†

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ABSTRACT: The rate and temperature at which fusion of purified homogeneous lecithin vesicles containing several per cent fatty acid occurs have been determined by analysis of proton nuclear magnetic resonance (nmr) spectra. Dimyristoyllecithin with myristic acid as 2% of the lipid was found to fuse rapidly at temperatures between 17 and 20°, and dimyristoyllecithin with 4% lauric acid was found to fuse rapidly at temperatures between 11 and 15°, while dimyristoyllecithin with 4% palmitic acid did not fuse at an appreciable rate anywhere in the range 17–37°. These results, along with data on dipalmitoyl- and dilauroyllecithin, are discussed in terms of a possible dependence on separation of a fatty acid rich phase especially conducive to fusion.

Membrane fusion plays an important part in biological functions, such as storage, reproduction, stimulation, and response (Poste and Allison, 1973). It has been studied in a number of systems, most of which involve whole cells. Several agents have been found to stimulate fusion in these systems. Certain viruses, such as Sendai virus, have been shown to induce cell fusion (Okada, 1969; Poste, 1972). Lysolecithin causes erythrocytes to fuse (Poole *et al.,* 1970), though at the cost of cell viability. Fusion of mammalian cells has resulted from exposure to mixed phospholipid vesicles (Papahadjopoulos *et al.,* 1973), and a host of lipids have been found to induce fusion of hen erythrocytes (Ahkong *et al.,* 1973), among them unsaturated fatty acids, retinol, and α-tocopherol.

No uniform theory of membrane fusion has emerged from these studies, partly due to the inability to characterize at a molecular level the cell components primarily responsible for fusion. In an attempt to overcome this problem, recent investigations have turned to model systems involving phospholipid vesicles of well-defined composition. These structures have been thoroughly characterized (Huang, 1969; Chapman *et al.,* 1967; Johnson, 1973) and are known to undergo fusion processes. Fusion with cells which afterwards retain viability (Grant and McConnell, 1973), with multilamellar or vesicle structures (Papahadjopoulos *et al.,* 1974), and among vesicles (Taupin and McConnell, 1972; Prestegard and Fellmeth, 1974) has been

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