The plasma of rhesus monkeys which were fed a normal chow diet was shown to contain several low density lipoproteins of which three major species, namely, LDL-I, LDL-II, and LDL-III were isolated by a combination of isopycnic and rate zonal density gradient ultracentrifugation. Of the 30 animals tested, 28 contained LDL-I and LDL-II, and 15 had, in addition to the first two components, LDL-III. Several exhibited additional minor LDL components. LDL-III with a mean buoyant density of 1.030 g/ml, had an unexpectedly large molecular weight \(3.47 \times 10^6\) when compared to LDL-I \(3.32 \times 10^6\) and LDL-II \(2.75 \times 10^6\) which had buoyant densities of 1.027 and 1.036 g/ml, respectively. The unusual physical properties of LDL-III were found to be due to a higher content and nature of its protein moiety, represented only by apo B according to electrophoretic criteria and radioimmunoassay. The protein of LDL-III (apo LDL-III) also differed from the other two in having a higher galactose and sialic acid content. Moreover, relative to LDL-I and LDL-II, LDL-III exhibited differences in behavior when using techniques of circular dichroism, radioimmunoassay, agarose- and sodium dodecyl sulfate-gel electrophoresis, which could be attributed to its protein moiety. In contrast, the molar lipid content of LDL-III was almost identical to that of LDL-I, and differential scanning calorimetry showed that the neutral lipids of the three LDLs undergo almost identical transitions below body temperature. When all of the data were analyzed according to the method of Shen et al. (Shen, B. W., Scanu, A. M., and Kezdy, F. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 837-841), the major structural difference between LDL-III and the other two LDLs appeared to relate to the degree of occupancy and conformation of the apoprotein in the surface of the particles.

In order to elucidate their role in lipid metabolism and atherosclerosis, plasma low density lipoproteins (LDL) of both normal and dietary-induced hyperlipidemic rhesus monkeys have been studied by various physicochemical techniques. During the course of these investigations, studies from this and other laboratories showed that the LDL class of normolipemic rhesus monkeys is heterogeneous in size and density (1–5). The heterogeneity of rhesus LDL was assessed by agarose-gel filtration and flotation velocity in the analytical ultracentrifuge. Both of these methods indicated the presence of two LDLs—one of relatively low and the other of higher density. Nelson and Morris (4) reported the presence of a dense LDL in 10 out of 30 monkeys with a molecular weight ranging between 3.5 to \(3.7 \times 10^6\) and a protein content higher than in normal rhesus LDL. Rudel et al. (6) isolated three different fractions of LDL from monkeys made slightly hyperlipidemic by a low cholesterol, high fat diet, and also reported the presence of a dense LDL, with properties similar to that described by Nelson and Morris (4).

The knowledge of the existence of multiple species of LDL in normolipidemic rhesus monkeys prompted us to investigate the structural basis for this heterogeneity and also to devise suitable methodology for the isolation of the various LDL species. To this purpose, we utilized a combination of physical, chemical, and immunological methods which led to results interpretable in the context of recent views on lipoprotein structure. The presentation of these results is the objective of this report.

**EXPERIMENTAL PROCEDURES**

Adult male rhesus monkeys were obtained from the Food Research Institute, University of Wisconsin, Madison. The animals weighed between 6 to 8 kg, and were singly caged and maintained on a regular Purina primate chow diet. Each monkey was fasted 16 h before 30 ml of plasma was collected biweekly by plasmapheresis, as previously described (1). Their serum cholesterol level was between 90 and 140 mg/dl and their serum triglyceride values were between 20 and 40 mg/dl.

For the separation of the total lipoprotein, the plasma was adjusted to \(1.21 \text{ g/ml with solid NaBr and centrifuged for 23 h at 10°C in a Ti-60 rotor at 59,000 rpm using a Beckman preparative ultracentrifuge.} \) To remove the NaBr, the lipoproteins were dialyzed exhaustively against 0.01% Na2EDTA and 0.01% NaN3, pH 7.0, and then fractionated on a 0 to 30% NaBr gradient at 20°C in the SW-40 rotor at 39,000 rpm. Isopycnic equilibrium was reached after 66 h. In order to remove HDL from one of the LDL subspecies (LDL-III), rate zonal ultracentrifugation was used (SW-40 rotor, 20,000 rpm, 16 h, 20°C). To prepare the sample, one milliliter of LDL-III which had been dialyzed against 1.4 g/ml NaBr solution was carefully layered under 11.6 ml of a linear 0 to 30% NaBr gradient using a syringe fitted with a long needle. Then 0.6 ml of 1.48 g/ml NaBr was introduced into the bottom of the centrifuge tube until the sample had been raised to a thin cylindrical starting zone. After separation, the lipoproteins were pumped out at a rate of 0.8 ml/min through an ISCO UA-5 monitor set at 280 nm and collected as 0.4 ml fractions. The following lipoproteins were identified: VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; SLDL, small dense lipoprotein; LDL, low density lipoprotein.
Heterogeneity of Rhesus LDL

Chemical Analysis—The protein content was determined by the Lowry method using bovine serum albumin as a standard (15) and corrected for the different chromophoricity of apo LDL, relative to bovine serum albumin. By comparing the Lowry protein content of a solution of r-LDL in 0.001 N HCl (obtained from fractionated LDL-I and LDL-II) with the dry weight equivalent, we found that the color yield of apo LDL was 9% higher than that of bovine serum albumin. This value was obtained after subtracting from the dry weight of apo LDL the contribution of the carbohydrate moiety (6%) and the residual lipid content (0.2% phospholipid, no detectable neutral lipids and free fatty acids). Lipid phosphorus was measured essentially according to the method of Bartlett (16) and total cholesterol according to Zak et al. (17). Total lipids were extracted from LDL according to a slight modification of the procedure of Polch et al. (18), and were separated by thin layer chromatography on freshly activated precoated Silica Gel G plates (Analtech, Inc.). The developing systems were: hexane/diethyl ether/glacial acetic acid (90:20:0.5, v/v/v) for neutral lipids, and chloroform/methanol/glacial acetic acid/water (25:42:3, v/v/v/v) for phospholipids. The determination of the triglycerides was conducted by the method of Kritchevsky et al. (19). Free cholesterol and triglyceride values were obtained from their respective ratios to total cholesterol (sum of free and esterified cholesterol) after separation of the lipoproteins by thin layer chromatography and were then normalized to the total cholesterol value of the lipoprotein solution. Cholesterol ester was obtained by difference from total and free cholesterol and then multiplying by 1.67 to correct for the fatty acid portion. Fatty acids of cholesterol esters were characterized as previously described (3). Amino acid analyses of the apo LDL preparations were carried out in a Beckman model 121 amino acid analyzer using manual procedure described (4). For carbohydrate analysis, internal standard mannitol was first added to the soluble apo LDL in 0.001 N HCl before its neutralization with NaOH and subsequent lyophilization. The carbohydrates were released from the freeze-dried apo LDL in methanolic 1 N HCl by heating at 85°C for 16 h. The samples were then neutralized with silver carbonate, and hexosamines were converted to their N-acetyl derivatives with acetic anhydride. The methylglycosides were analyzed as their trimethylsilyl ether derivatives by gas-liquid chromatography according to the method of Dawson and Clamp (20).

RESULTS

Density Gradient Ultracentrifugation Studies—From each of 30 monkeys maintained on a normal Purina monkey chow diet, the whole of the plasma lipoproteins was isolated by a single ultracentrifugation at density 1.21 g/ml NaBr and then fractionated to isopycnic equilibrium in the ultracentrifuge on a 0 to 10% NaBr or NaCl gradient. Representative profiles of rhesus LDL from four different monkeys are given in Fig. 1 that illustrate the heterogeneity within a single individual and also among different monkeys. Based on these results we could divide the monkeys into two groups: one (13 animals) containing two major LDLs floating at density 1.027 and 1.036 g/ml and the other one (15 animals) containing an additional major LDL, the whole of the plasma lipoproteins was isolated by a single ultracentrifugation at density 1.21 g/ml NaBr and then fractionated to isopycnic equilibrium in the ultracentrifuge on a 0 to 10% NaBr or NaCl gradient. Representative profiles of rhesus LDL from four different monkeys are given in Fig. 1 that illustrate the heterogeneity within a single individual and also among different monkeys. Based on these results we could divide the monkeys into two groups: one (13 animals) containing two major LDLs floating at density 1.027 and 1.036 g/ml, and the other one (15 animals) containing an additional major component, LDL-III, with a buoyant density of 1.050 g/ml (Fig. 1a). Some of the monkeys exhibited LDL profiles (Fig. 1, c and d) which differed appreciably from the patterns shown in Fig. 1, a and b, but no attempt was made to analyze them. The ratio of the major LDLs to each other was representative for each monkey, but varied considerably among the animals studied. In the six monkeys that were examined in detail, the profiles remained relatively unchanged over a 2-year period. LDL-I and LDL-II could not be completely resolved by density-gradient ultracentrifugation because of their overlapping buoyant densities, but LDL-III was easily separated from the less dense LDLs although small amounts of HLs were present, as shown by agarose-gel electrophoresis. This HDL contaminant could be removed by gel filtration on Sepharose 4B or 6B; however, neither column could resolve the three LDLs. Since the recoveries of LDL-III after gel filtration were often poor, we utilized instead rate zonal ultracentrifugation. This technique is usually utilized to separate...
Heterogeneity of Rhesus LDL

Heterogeneity of Rhesus LDL

FIG. 1. Density gradient ultracentrifugation of rhesus low density lipoproteins. Sodium chloride gradient, 0 to 10% in 0.01% Na₂EDTA and NaN₃ (pH 7.0). Total rhesus lipoproteins were spun at 39,000 rpm for 66 h in the SW-40 rotor at 20°C. After separation, the gradients were pumped out with saturated NaCl and their absorbance profile at 280 nm was recorded with an ISCO UA-5 monitor. a, typical low density lipoprotein profile of a rhesus monkey exhibiting three components: fractions I, II, and III have mean hydrated densities of 1.027, 1.036, and 1.050 g/ml and are called LDL-I, LDL-II, and LDL-III, respectively. The arrows define the fractions that were pooled for analysis. b, typical LDL profile of a rhesus monkey having only two components: fractions I and II have hydrated densities of 1.027 and 1.036 g/ml and are called LDL-I and LDL-II, respectively. c, a typical LDL profile of one rhesus monkey which has an additional component in the same region occupied by LDL-I and LDL-II. d, a typical LDL profile of another rhesus monkey which appears to have two LDL-III components. The densities of the fractions were obtained from refractometric measurements of a control gradient at 20°C.

proteins on the basis of their sedimentation rates in density gradients, but works equally well by flotation. We reasoned that since LDL-III was an order of magnitude larger than HDL, it also had a proportionately higher flotation rate in high salt solutions where differences in the buoyancy factor are minimized. Indeed, only an overnight spin in an SW-40 rotor at a relatively low speed (20,000 rpm) in a 0 to 30% NaBr gradient afforded the separation of LDL-III from HDL (Fig. 2).

Electrophoresis of Lipoprotein Particles—Electrophoresis on 1% agarose films resolved LDL-III reasonably well from LDL-I and LDL-II, because the former had pre-β mobility

FIG. 2. Rate zonal separation of LDL-III from HDL contaminant of similar buoyant density. The sample in d 1.4 g/ml NaBr was layered under a linear 0 to 30% NaBr gradient and centrifuged in the SW-40 rotor at 20,000 rpm and 20°C for 16 h. The gradient was pumped out with d 1.48 g/ml of NaBr at a rate of 0.8 ml/min and the profile was recorded at 280 nm with an ISCO UA-5 monitor.

FIG. 3. Electrophoresis of rhesus LDLs on (A) 1% agarose films and (B) 4% agarose. The agarose films in A labeled I, II, and III refer to LDL-I, LDL-II, and LDL-III, respectively, and were stained with Coomassie blue; the gel labeled P refers to plasma from a rhesus monkey having three LDLs and was stained with Fat Red 7B. The 4% agarose gels in B were stained with Coomassie blue.
Heterogeneity of Rhesus LDL

**TABLE I**

*Per cent chemical composition of rhesus LDL*

<table>
<thead>
<tr>
<th>LDL species</th>
<th>Protein</th>
<th>Protein-bound carbohydrate</th>
<th>Phospholipid</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-I</td>
<td>18.5 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>26.1 ± 0.4</td>
<td>8.1 ± 0.1</td>
<td>37.5 ± 0.6</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td>LDL-II</td>
<td>20.8 ± 0.5</td>
<td>1.2 ± 0.1</td>
<td>25.3 ± 0.2</td>
<td>7.8 ± 0.2</td>
<td>37.3 ± 0.2</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>LDL-III</td>
<td>24.0 ± 0.5</td>
<td>2.0 ± 0.2</td>
<td>25.3 ± 0.8</td>
<td>7.4 ± 0.2</td>
<td>34.0 ± 0.4</td>
<td>6.1 ± 0.0</td>
</tr>
</tbody>
</table>

**TABLE II**

*Number of components per lipoprotein particle*

The following molecular weights were used for the calculations: triglyceride 880; phospholipid, 775; cholesteryl ester, 650; cholesterol, 387. For comparison with human lipoproteins as reported by Shen et al. (29), it was more suitable to calculate the number of amino acid residues per particle by assuming an average residue weight of 100.

<table>
<thead>
<tr>
<th>LDL species</th>
<th>Amino acid</th>
<th>Phospholipid</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-I</td>
<td>6142</td>
<td>1118</td>
<td>695</td>
<td>1915</td>
<td>336</td>
</tr>
<tr>
<td>LDL-II</td>
<td>5729</td>
<td>888</td>
<td>554</td>
<td>1578</td>
<td>246</td>
</tr>
<tr>
<td>LDL-III</td>
<td>8328</td>
<td>1133</td>
<td>664</td>
<td>1847</td>
<td>249</td>
</tr>
</tbody>
</table>

(Fig. 3A). Since the fasted normal rhesus monkeys had little VLDL, as shown by density-gradient ultracentrifugation, this pre-β band was essentially due to LDL-III.

A better resolution with regard to LDL-III was achieved by electrophoresis in tubes containing 4% agarose (Fig. 3B). In this case, however, the mobilities of the LDL fractions were reversed; LDL-III now migrating slower than both LDL-I and LDL-II, which were not resolved from each other.

**Chemical Composition**—The percentage chemical composition of the LDLs from the six monkeys studied in detail is given in Table I. The values represent means of LDL samples taken from four separate bleedings. The cumulative error in determining the chemical composition of a lipoprotein was about 6%. The most striking difference between the three lipoproteins is the increased percentage of the protein in going from LDL-I to LDL-II and LDL-III. There were no changes in the percentage of phospholipids, whereas free cholesteryl and triglyceride decreased in parallel. The percentage cholesteryl ester did not differ between LDL-I and LDL-II and was higher than in LDL-III. In addition, the ratio of triglycerides to cholesteryl ester plus triglyceride decreased from LDL-I to LDL-II and LDL-III.

Knowing the molecular weight of each LDL, the chemical composition of the three LDL fractions could be expressed by calculating the number of individual components per particle. As seen in Table II, each component of LDL-II was smaller in number relative to LDL-I or LDL-III, which had remarkably similar contents of phospholipid, free cholesteryl, and cholesteryl ester. However, in keeping with its greatly different buoyant density, the protein content of LDL-III was much greater than that of LDL-I or LDL-II. The triglyceride contents of LDL-II and LDL-III were similar but less than that of LDL-I.

The cholesteryl ester fatty acid compositions of the three LDLs shown in Table III was almost the same. Only three major fatty acids were present: palmitic, oleic, and linoleic acid in the approximate ratio of 1:2:4, respectively.

The three LDL fractions had the same phospholipid composition within experimental error (Table IV). The major phospholipid was phosphatidylcholine followed by a much smaller percentage of sphingomyelin. Phosphatidylethanolamine, phosphatidylinosine, phosphatidylglycolinositol, and lysophosphatidylcholine were only minor components.

The amino acid compositions of apo LDL-I, apo LDL-II, and apo LDL-III are identical and were similar to that reported previously (1). The carbohydrate compositions of apo LDL-I and apo LDL-II were identical. However, apo LDL-III had a galactose and especially a sialic acid content much greater than that found in the apo LDL from LDL-I or LDL-II (Table V). In turn, there were no significant differences in the mannosyl and glucosamine content. The carbohydrate content of apo LDL-I and apo LDL-II was 6.0%, whereas that of apo LDL-III was 10.7%.

**Molecular Weight of the LDL Fractions**—Sedimentation and flotation equilibrium of LDL-I, LDL-II, and LDL-III were carried out in solutions of NaBr, pH 7.0 of different densities (1.5% NaBr, d = 1.012 g/ml; 12% NaBr, d = 1.094 g/ml; 20% NaBr, d = 1.151 g/ml). For all lipoproteins and at all densities employed, the plots of log c versus the square of the distance from the center of rotation were linear. The mean molecular weights and the mean apparent partial specific volumes of the individual lipoproteins from the six monkeys were obtained simultaneously from plots of solvent density p versus (2·d log ρ)/(d tracer) (Fig. 4) and are listed in Table VI. This analysis is possible by assuming that rhesus LDL like human LDL is not preferentially hydrated in high salt solutions (21). Surprisingly, LDL-III, which had a mean molecular weight of 4.47 × 10⁶, was larger than either LDL-II (2.75 × 10⁶) or LDL-I (3.32 × 10⁶). The apparent partial specific volumes of LDL-I, LDL-II, and LDL-III were 0.974, 0.965, and 0.952 ml/g, respectively. The reciprocal of the apparent partial specific volumes gave buoyant densities of 1.027, 1.036, and 1.050 g/ml for LDL-I, LDL-II, and LDL-III, respectively.

**Hydrodynamic Analysis**—The hydrodynamic analysis was carried out on the three LDLs of a single monkey. The flotation coefficients of the three LDLs were determined in a solution of d = 1.20 g/ml NaBr, pH 7.0 containing 0.01% Na₂EDTA. At infinite dilution, this symbol refers to the diffusion coefficient is not corrected for solvent viscosity and apparent partial specific volume. The diffusion coefficient, D₄₀, at infinite dilution in 0.15 M NaBr (pH 7.0) containing 0.01% Na₂EDTA was 1.78 ± 0.07 × 10⁻⁷ cm² s⁻¹ for LDL-I, 2.00 ± 0.10 × 10⁻⁷ cm² s⁻¹ for LDL-II, and 1.59 ± 0.07 × 10⁻⁷ cm² s⁻¹ for LDL-III. From the diffusion coefficient, molecular weight, and apparent partial specific volume we obtained the fractional ratio f/f₀, which contains effects due only to hydra-
The difference between the frictional ratios of LDL-I and LDL-II is not significant; however, the value ratios of LDL-I, LDL-II, and LDL-III were 1.07, 1.04, and 0.69 g of H₂O/g of LDL-III. From the diffusion measurements we calculated the anhydrous diameters of LDL-I, LDL-II, and LDL-III, by assuming spherical lipoprotein particles, to be 217 Å, 205 Å, and 222 Å, respectively. Mean diameters of approximately 150 free-standing LDL-I, LDL-II, and LDL-III particles were 234 ± 3, 212 ± 2, and 240 ± 2 Å (Fig. 6). Thus the sizes of LDL-I and LDL-III as determined for LDL-II and confirm the same observation obtained with the ultracentrifugal method.

**Electron Microscopy**—Typical micrographs of the three negatively stained species of LDL are shown in Fig. 5. The free-standing particles appeared spherical and showed no apparent substructure although they had a granular appearance similar to the pattern found on the background. The mean diameters of approximately 150 free-standing LDL-I, LDL-II, and LDL-III particles were 234 ± 3, 212 ± 2, and 240 ± 2 Å (Fig. 6). Thus the sizes of LDL-I and LDL-III as measured by electron microscopy are similar but larger than that of LDL-II and confirm the same observation obtained with the ultracentrifugal method.

**Differential Scanning Calorimetry Studies**—The circular dichroic spectra of the three LDLs from two representative animals were analyzed between 215 and 230 nm and their structural composition was determined according to Chen et al. (24) (Fig. 8). Both LDL-I and LDL-II contained 50% α-helix and 30% β-structure, while LDL-III contained 40% α-helix and 25% β-structure. In the calculations we did not include data below 215 nm because of possible significant contributions to the total ellipticity of the lipoproteins by the lipid moieties, as pointed out by Chen and Kane (25).

**Circular Dichroism Studies**—The circular dichroic spectra of the isolated cholesteryl esters of rhesus LDL was obtained by differential scanning calorimetry of the isolated cholesteryl esters of rhesus LDL. The data are plotted according to the equation $\rho = \frac{-4.606 RT d \log c/d r^2}{M\bar{v}_{-\alpha} w^2 + 1/\bar{v}_{+\beta}}$. The rotor speed was varied as a function of solvent density: at d 1.151 g/ml, the speed was 4,360 rpm; at d 1.094 g/ml, the speed was 7,120 rpm; and at d 1.012 g/ml, the speed was 9,920 rpm. The temperature was maintained at 20°C.

**Equilibrium Centrifugation of LDL-I (C), LDL-II (○), and LDL-III (□) obtained from a single monkey.** The data are plotted according to the equation $\rho = \frac{-4.606 RT d \log c/d r^2}{M\bar{v}_{-\alpha} w^2 + 1/\bar{v}_{+\beta}}$. The rotor speed was varied as a function of solvent density: at d 1.151 g/ml, the speed was 4,360 rpm; at d 1.094 g/ml, the speed was 7,120 rpm; and at d 1.012 g/ml, the speed was 9,920 rpm. The temperature was maintained at 20°C.

**TABLE IV**

<table>
<thead>
<tr>
<th>LDL species</th>
<th>Phosphatidyl choline</th>
<th>Sphingomyelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-I</td>
<td>72.6 ± 1.7</td>
<td>13.3 ± 0.6</td>
</tr>
<tr>
<td>LDL-II</td>
<td>76.5 ± 2.7</td>
<td>14.0 ± 2.4</td>
</tr>
<tr>
<td>LDL-III</td>
<td>74.0 ± 0.8</td>
<td>13.1 ± 1.1</td>
</tr>
</tbody>
</table>

**TABLE V**

<table>
<thead>
<tr>
<th>Carbohydrate composition of rhesus apo LDL</th>
<th>LDL species</th>
<th>Apo LDL-I and II</th>
<th>Apo LDL-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>0.094 ± 0.008</td>
<td>0.094 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>0.047 ± 0.005</td>
<td>0.097 ± 0.015</td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.065 ± 0.008</td>
<td>0.070 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.073 ± 0.008</td>
<td>0.185 ± 0.015</td>
<td></td>
</tr>
</tbody>
</table>
Heterogeneity of Rhesus LDL

the LDL-II standard, indicating that the antibodies used recognized identical antigenic determinants in all of the three lipoproteins. However, LDL-III proved to be only 46% as reactive as either LDL-I or LDL-II suggesting that in LDL-III, a considerable number of antigenic determinants were either masked or altered.

SDS-gel electrophoresis (10% gels) indicated that the major contaminant of the LDL-III fraction unpurified by rate zonal ultracentrifugation was A-I. Therefore, the purified LDL-III, together with LDL-I and LDL-II, were examined by a double radioimmunoassay for rhesus A-I. It was found that less than 1% of the LDL protein reacted with the antibody, indicating

Radioimmunoassay—To assess their immunochemical reactivity, the three LDLs were tested by a double antibody radioimmunoassay for apo B. The displacement curves of LDL-I, LDL-II, and LDL-III in each monkey were parallel to

![Graph](http://www.jbc.org/)

**Fig. 6.** Particle size distribution of rhesus LDL obtained by electron microscopy. Approximately 150 free-standing particles from each lipoprotein preparation were measured on multiple photographs taken from different areas of the grid.

![Graph](http://www.jbc.org/)

**Fig. 7.** Differential scanning calorimetry endotherms obtained for (a) LDL-I, (b) LDL-II, and (c) LDL-III. The fractions taken represent pools from all six animals and from several bleedings in order to have sufficient material for analysis.
The most novel aspect of our studies is the documentation of the existence in normolipemic rhesus monkeys of an LDL species, which we have referred to as LDL-III, with properties remarkably different from those of the other two major LDL species observed, LDL-I and LDL-II. LDL-III did not obey the usual negative size-density correlation described for human LDL (26) in that, despite having the highest density of the three LDL species examined, it also had the greatest molecular weight. Frictional ratios calculated from the corresponding diffusion coefficients and molecular weights showed that LDL-III had significantly higher values than those of LDL-I and LDL-II (Table VI). This fact is likely attributable to a greater degree of hydration of LDL-III over LDL-I and LDL-II rather than to particle asymmetry which was not supported by electron microscopy. Indirect evidence for the quasispherical nature of human or monkey LDL comes from small angle x-ray scattering (27, 28).

LDL-III also differed from LDL-I and LDL-II when examined through a compositional analysis as was done by Shen et al. (29) to formulate a general model of human plasma lipoproteins. The unifying concept in that model is that circulating lipoproteins have a hydrophobic core of cholesteryl esters and triglycerides surrounded by the tightly packed hydrophobic tails of phospholipids and free cholesterol, while the head group of the phospholipids, together with the protein, occupy the outer surface of the lipoprotein particles. In the case of our rhesus monkeys, the volume and the surface area of the hydrophobic core of each LDL obeyed the same relationship observed with the human model (Fig. 10, A and B). Chemical analysis indicated that the cholesteryl ester and phospholipid

that all three species were essentially free of A-I. Furthermore, no small molecular weight proteins were detected in any of the lipoprotein fractions on 10% SDS gels after the final purification. When the three LDLs were characterized by SDS-gel electrophoresis on either 4% acrylamide or 4% agarose, single bands were obtained (Fig. 9). The apoprotein of LDL-III moved considerably slower than that of LDL-I or LDL-II on 4% agarose in contrast to the 4% acrylamide gels because the latter were almost impenetrable to the apoproteins.

![Figure 8: Circular dichroic spectra of the three species of LDL from one rhesus monkey at 22°C; LDL-I (- - -); LDL-II (---); and LDL-III (----).](http://www.jbc.org/)

![Figure 9: Electrophoresis of rhesus LDL on gels containing SDS A, 4% acrylamide; B, 4% agarose.](http://www.jbc.org/)

![Figure 10: Comparison of the space and surface fitting of the hydrophobic core of the three different rhesus LDLs with human lipoproteins through compositional analysis according to Shen et al. (29). A, space-fitting of the hydrophobic core with cholesteryl esters and triglycerides; B, surface-fitting of the hydrophobic core with the hydrophobic tails of the phospholipids and free cholesterol. The arrow signifies the position of rhesus LDL.](http://www.jbc.org/)
Heterogeneity of Rhesus LDL

Composition of these lipoproteins was virtually the same. Furthermore, differential scanning calorimetry indicated that those cholesterol esters responsible for the transition have similar thermotropic behavior. Therefore, these observations, taken together, suggest that the three LDLs have a similar internal organization. On the other hand, the packing of the protein at the lipoprotein surface of LDL-I, LDL-II, and particularly LDL-III as shown by the plots of \( r^2/n_{mol} \) versus \( n_{mol} \) indicated a significant deviation from the one calculated for human lipoproteins (Fig. 11). Since the tail packing of the phospholipid molecules on the surface of the core is the same in the rhesus monkey and in man as shown in Fig. 10B, it appears valid to assume that the molecular area occupied by the phospholipid head group and, thereby the slope of the plots, should also be identical. Indeed the slope of the straight regression line drawn through the data points determined for LDL-I and LDL-II is similar within experimental error to the one determined for the human lipoproteins. With this information, we felt it valid to assume that both groups of LDL had identical slopes although only limited data were available for LDL-III. From the y-intercept of the two lines, we calculated that the molecular area per amino acid was 13 \( \bar{A}^2 \) for both LDL-I and LDL-II and 9 \( \bar{A}^2 \) for LDL-III. These results may be taken to indicate that either the protein moiety of the rhesus LDL is packed more tightly on the LDL surface than its human counterpart (molecular area per amino acid, 15.6 \( \bar{A}^2 \)), or that, particularly in the case of LDL-III, only a fraction of its protein moiety would be in direct contact with the surface, implying a difference in protein conformation. This latter conclusion is consistent with the circular dichroic studies, indicating that LDL-III contained significantly less \( \alpha \)-helix and \( \beta \)-structure than LDL-I and LDL-II, in spite of the same amino acid composition. In addition, apo LDL-III differed from the other two apo LDLs in containing more galactose and sialic acid, a fact which probably is a contributory factor to the change in conformation of the surface protein. It is known that both changes in conformation and carbohydrate content may in part be responsible for the increased hydration of LDL-III. Kuntz and Kaufmann (30) have pointed out that polar groups which are so prevalent in carbohydrates, especially when ionized, avidly bind water. Moreover, unfolded proteins, although to a smaller extent, bind more water than proteins in a more compact or ordered configuration, due to an increased surface exposure of the polar groups. Both the greater carbohydrate content of apo LDL-III and its different conformation may also explain the low reactivity of this lipoprotein to anti-LDL antibodies, as indicated by the results of the radioimmunoassay. Also, the large number of negatively charged sialic acid molecules of LDL-III was probably responsible for the greater mobility of this lipoprotein on 1% agarose gels.

The protein of LDL-III also differed from that of LDL-I and LDL-II by electrophoresis on 4% agarose gels containing SDS. Single bands were observed in all three cases, with apo LDL-III exhibiting a distinctly slower mobility. This may indicate that apo LDL-III has a larger size, but the abnormal mobility may also be due to its higher carbohydrate content which is known to impart anomalous electrophoretic behavior to proteins dissolved in SDS (31).

The observation that normolipemic rhesus monkeys have multiple LDL species with different apo LDLs is of interest from the metabolic standpoint. According to currently accepted concepts, VLDL of human subjects are converted to LDL in a mole/mole relationship (32). If this concept applies to rhesus monkeys as well, it follows that perhaps LDL-I and LDL-II, on the one hand, and LDL-III on the other may be derived from different VLDL species, possibly originating from different organs, e.g., the liver and/or the intestine. Heterogeneity of human VLDL has been documented (33) and has also been observed in rhesus monkeys,4 however, the relationship between VLDL and LDL heterogeneity observed in this work remains to be established. Studies in this direction are now in progress in this laboratory. On the other hand, it is also possible that part of the LDL heterogeneity may be caused by a direct derivation of some LDL from either the liver or intestine (34).

Finally, the structural and functional significance of LDL-III remains unexplained. If LDL-III is truly absent in some of the animals, this may signify a non-essential role of this particle. However, it may be possible that this lipoprotein is present in circulation at a level not detectable with our current methodology. Should this be the case, then LDL-III may be turning over at a higher rate in these animals. The possible atherogenic importance of LDL-III is currently being studied in this laboratory in rhesus monkeys fed high-fat diets supplemented with cholesterol in both phases of progression and regression of the induced hyperlipidemia.

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Fig. 11. The packing of phospholipids and protein at the lipoprotein-water interface. A, data for human lipoproteins taken from Shen et al. (29). B, data for LDL-I (○) and LDL-II (●) are the individually analyzed LDLs from six monkeys. C, data for LDL-III (●) are from four monkeys. The equation describing the packing of phospholipids and proteins at the lipoprotein-water interface is \( r^2/n_{mol} = n_{mol}/4\pi + (\rho_{mol}/4\pi)n_{mol} n_{ex} \) where \( r \) is equivalent radius of the particle, \( n_{mol} \) and \( n_{ex} \) are the number of amino acids and phospholipids, respectively, and \( n_{ex} \) are the molecular areas of the amino acid and phospholipid at the outer surface of the particle (29).

REFERENCES


4 G. M. Fless and A. M. Scanu, unpublished observation.
Isolation and characterization of the three major low density lipoproteins from normolipidemic rhesus monkeys (Macaca mulatta).
G M Fless and A M Scanu


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