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Study of Abnormal Plasma Low-Density Lipoprotein in Rhesus Monkeys with Diet-Induced Hyperlipidemia†

Gunther M. Fless, Robert W. Wissler, and Angelo M. Scanu*

ABSTRACT: Male rhesus monkeys were divided into three groups: five were fed a regular primate chow diet and were used as controls; four received an "average" American diet; and five a special low-fat primate chow diet supplemented with 25% coconut oil and 2% cholesterol. In all of these animals, the plasma low-density lipoproteins (LDL) were isolated by ultracentrifugal flotation between densities of 1.019 and 1.050 g/ml. The LDL of the five control monkeys had variable molecular weights, with a mean value of $3.12 \pm 0.21 \times 10^6$ (range: 2.92×10^6 to 3.45×10^6), and an average partial specific volume of 0.969 ± 0.003 ml/g; both were assessed by flotation equilibrium analysis in the analytical ultracentrifuge. In the individual animals, however, the physical properties of LDL were invariant with time. The administration of either an "average" American diet or a coconut oil–cholesterol diet was accompanied by hypercholesterolemia associated with changes in LDL which were characterized by increases in molecular weight to $3.52 \pm 0.21 \times 10^6$ (average of nine monkeys) and in partial specific volume to 0.973 ± 0.002 ml/g. These changes were particularly evident when the molecular weight of LDL from monkeys in the normolipidemic state was compared with that obtained from the same monkeys during the hyperlipidemic state. Chemical analyses revealed that the particles from the hyperlipidemic animals had a relatively higher cholesteryl ester content, a slight increase in phospholipids, and a marked decrease to nearly complete absence of triglycerides. The other lipoprotein components, protein, carbohydrate, free cholesterol, and fatty acids, did not vary significantly from those of control LDL. It is concluded that the administration of atherogenic diets causes structural changes in LDL which appear to be accounted for, at least in part, by changes in the composition of the lipid moiety. The changes in physical and chemical properties noted in the LDL of rhesus monkeys with experimentally induced hypercholesterolemia contrast with the apparent structurally normal LDL from rhesus monkeys with spontaneous hypercholesterolemia reported previously.

The rhesus monkey is a good model for the study of experimental atherosclerosis since advanced atheromatous lesions closely resembling those observed in human subjects accompany the hyperlipidemias induced by diets rich in fat and cholesterol (Taylor et al., 1962, 1963; Wissler, 1968). It was recently observed that hyperlipidemic sera, particularly the low-density lipoproteins (LDL)[1] from rhesus monkeys, stimulate proliferation and cholesteryl ester accumulation in stationary cultures of aortic smooth muscle cells, whereas sera or LDL from normolipidemic animals do not exhibit such an effect (Fischer-Dzoga et al., 1971, 1974; Bates and Wissler, 1976). This important difference in cellular response prompted us to examine the structural relationship between the LDL of control monkeys and that of animals fed atherogenic diets. In this report, we describe some of the physicochemical properties of rhesus plasma LDL (ρ 1.019–1.050 g/ml) during diet-induced hyperlipidemia. A description of the properties of LDL from normolipidemic monkeys has already appeared (Fless and Scanu, 1975).

Materials and Methods

Animals and Diets. Male rhesus monkeys were obtained from the Food Research Institute, University of Wisconsin, Madison. Five animals serving as controls were fed a regular Purina primate chow diet. Four monkeys were placed on a human diet which represents an "average" American diet as described by Wissler and Vesselinovitch (1975). Five other animals were fed a diet that consisted of 70.5% of a modified, low-fat Purina primate chow supplemented with 25% coconut oil, 2% cholesterol, 1% vitamin mix, and 1.5% gelatin. This diet was modified slightly from that published previously by Wissler et al. (1962). Each animal was fasted 16–18 h before 30

† From the Departments of Medicine, Biochemistry, and Pathology, The University of Chicago, Pritzker School of Medicine, and the Franklin McLean Memorial Research Institute, Chicago, Illinois, 60637, operated by The University of Chicago under Contract [E (11-1)-69] for the US-ERDA. *Received June 17, 1976.* This work was supported by Grants HL 08727 and HL 15062 from the United States Public Health Service and funds from the United States Energy, Research, and Development Administration. G.M.F. was supported by the United States Public Health Service Postdoctoral Fellowships 1-F22 HL 00517 and CA-05250.

[1] Abbreviations: LDL, low density lipoprotein; n-LDL, LDL from normolipidemic monkeys; hyp-LDL, LDL from monkeys fed an "average" American diet or a coconut-oil-supplemented diet; apo-B, apolipoprotein B from apo-LDL; Na_2EDTA, disodium ethylenediaminetetraacetate.

ml of plasma was collected biweekly by means of plasmapheresis, as previously described (Fless and Scanu, 1975). The rhesus monkeys that were fed normal monkey chow had serum cholesterol levels varying between 100 and 150 mg/dl; those on the "average" American diet had a level between 250 and 450 mg/dl; and the animals on the diet high in coconut oil and cholesterol had a level between 500 and 1000 mg/dl. Serum triglyceride values for all monkeys were less than 100 mg/dl.

Low density lipoproteins of ρ 1.019–1.050 g/ml were prepared as previously described (Fless and Scanu, 1975). Two out of seven control animals had multiple components of S_f 5 and 6 in their LDL fraction that could be separated by density gradient centrifugation on a 0 to 10% NaCl gradient. The buoyant densities were 1.048 and 1.036 g/ml, respectively. Therefore, for purposes of comparison we used only LDL from the five monkeys exhibiting a single component by flotation analysis in the ultracentrifuge.

Physicochemical Methods. The sedimentation, flotation, and diffusion coefficients of LDL were determined at 25 °C as previously described (Fless and Scanu, 1975). In some of the diffusion experiments, interference optics were used because of the relatively low lipoprotein concentration.

High-speed sedimentation equilibrium experiments were carried out at 25 °C in a Beckman Model E ultracentrifuge, as described by Yphantis (1964). Flotation equilibrium of LDL was conducted in high-density solutions of NaBr at sufficiently high speed to permit flotation of all of the macromolecular solute, so that the bottom of the cell was cleared (Nelson et al., 1974). By this procedure, the measurement of $d \log c/dr^2$ as a function of solvent density permits the simultaneous determination of the molecular weight and the partial specific volume.

Before analysis, solutions of LDL were dialyzed exhaustively against 0.15 M NaBr (pH 7.0) containing 0.025% Na_2EDTA. For a typical equilibrium experiment, the LDL solutions were diluted to concentrations between 0.1 and 1.0 mg/ml with the dialysate. To obtain solutions of higher density, we added various amounts of a concentrated NaBr solution of $\rho^{25} = 1.35$ mg/ml to the lipoprotein. Reference solutions were made by the addition of dialysis medium to the concentrated NaBr solution in place of the lipoprotein solution. Solvent densities were measured with a Precision Density Meter DMA-02 (Anton Paar, Graz, Austria), as previously described (Fless and Scanu, 1975). The analytical cell used was equipped with sapphire windows and a six-channel, 12-mm-thick charcoal-filled Epon centerpiece. Column heights of 3 mm were used without fluorocarbon or silicon oil. Equilibrium was considered established when no further fringe displacement had occurred with time at a given radial distance, r, from the center of rotation. For greater accuracy, we checked speeds manually instead of relying on the electronic speed control setting. Photographs of sample and baseline patterns were analyzed as previously described (Fless and Scanu, 1975).

The frictional ratio of the unhydrated lipoprotein particle was calculated from the relation

$$f/f_0 = RT/N\,D^0\,6\pi\eta[3M\bar{\nu}_2/4\pi N]^{1/3}$$

where R is the gas constant, T is the absolute temperature, N is Avogadro's number, D^0 is the diffusion coefficient corrected for concentration, η is the viscosity of the solvent, M is the anhydrous molecular weight obtained from sedimentation equilibrium, and $\bar{\nu}_2$ is the partial specific volume of the lipoprotein, also obtained from sedimentation equilibrium (Tanford, 1961). The solvation of LDL was calculated from the relation

$$f/f_{min} = f/f_0\,[(\bar{\nu}_2 + \delta_1\nu_1{}^0)/\bar{\nu}_2]^{1/3}$$

where f/f_{min} is the minimum possible frictional ratio, δ_1 represents the solvation, and $\nu_1{}^0$ is the specific volume of pure solvent (Tanford, 1961). The radius, R_0, of a spherical hydrated particle was obtained from the equation

$$R_0 = [3M/4\pi N(\bar{\nu}_2 + \delta_1\nu_1{}^0)]^{1/3}$$

Density gradient ultracentrifugation, agarose gel column chromatography, and acrylamide–agarose gel electrophoresis were done as previously described (Fless and Scanu, 1975). The method of Weber and Osborne (1969) was used for sodium dodecyl sulfate gel electrophoresis with 10% acrylamide gels.

Chemical Analysis. Lipids were extracted from LDL according to the procedure of Schmid et al. (1973), by use of 25 volumes of toluene–ethanol (3:2, v/v) containing 0.2 ml of glacial acetic acid per 100 ml. The total lipid extract dissolved in chloroform was separated by thin-layer chromatography on freshly activated, precoated Silica Gel G plates (Brinkmann Instruments, DesPlaines, Ill). The developing system, hexane–diethyl ether–glacial acetic acid (90:20:2, v/v/v), was used for neutral lipids, and chloroform–methanol–glacial acetic acid–water (25:15:4:2, v/v/v/v) was used for phospholipids. The neutral lipid fractions were visualized with 2′,7′-dichlorofluorescein, and the phospholipid fractions with iodine before they were scraped off and extracted with chloroform–methanol (1:1, v/v) for analysis.

Lipid phosphorus was measured according to the method of Bartlett (1959). Phospholipid concentrations were obtained by multiplication of the lipid phosphorus value by 25.1 (lecithin), 17.5 (lysolecithin), 24.1 (sphingomyelin), 24.1 (phosphatidylethanolamine), and 26.5 (phosphatidylserine and phosphatidylinositol combined). Total and free cholesterol were determined as described by Zak (1965). Cholesteryl ester was calculated as the difference between total and free cholesterol by means of the factor 1.65 for the ester cholesterol. Triglyceride was determined by the method of Kritchevsky et al. (1973).

Fatty acid methyl esters were prepared by transesterification with acidic methanol, essentially according to the procedure described by Stoffel et al. (1959). Internal standard heptadecanoic acid was added to the dry lipid extract before methanolysis. All operations were conducted under an atmosphere of nitrogen. The methyl esters were injected into a Hewlett-Packard F and M Model 402 gas chromatograph equipped with a flame ionization detector. A U-shaped glass column, 6-ft long, with an inner diameter of 3 mm, was packed with 10% SP-2300 cyanosilicon on 100–120 Supelcoport. The initial column temperature was 160 °C, and the temperature rise was programmed at 2 °C/min. The injection port temperature was 290 °C and the detector was at 270 °C. Peaks were identified by comparing their positions with those obtained from standard mixtures of known methylated fatty acids (Nu-Chek Prep., Elysian, Minn.). Areas were usually obtained by electronic integration (Mini Lab Integrator, Columbia Scientific Ind., Austin, Texas); but when not applicable, areas were obtained manually, either by cutting and weighing, or by triangulation.

Protein content was determined by Lowry's method, with bovine serum albumin used as standard (Lowry et al., 1951). Amino acid and carbohydrate analyses of apo-LDL were carried out as previously described (Fless and Scanu, 1975).

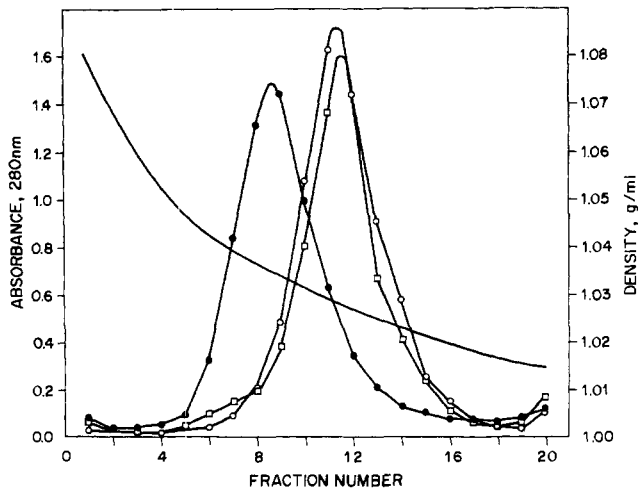

FIGURE 1: Sodium bromide gradient 0–10% in 0.025% Na_2EDTA and NaN_3 (pH 7.0). Rhesus LDL was spun at 38 000 rpm for 66 h in the SW-40 rotor of a Beckman Model L2-65B centrifuge at 25 °C. Fractions were collected from the bottom and monitored for absorbance at 280 nm. (●) n-LDL, pooled from monkeys numbers 4 and 5; (○) hyp-LDL, pooled from five monkeys on a coconut-oil-supplemented diet; (□) hyp-LDL, pooled from four monkeys on an "average" American diet. The densities of the fractions were obtained from refractometric measurements of a control gradient at 20 °C.

TABLE I: Chemical Composition of Rhesus LDL.[a]

	n-LDL[b] (wt %)	hyp-LDL (wt %) "Average" American Diet[c]	hyp-LDL (wt %) Coconut Oil Diet[d]
Protein	23.2 ± 1.0	19.8 ± 0.7	19.0 ± 1.3
Phospholipid	25.2 ± 1.0	25.9 ± 1.0	23.6 ± 1.5
Free cholesterol	11.0 ± 1.2	9.5 ± 1.2	9.1 ± 1.4
Cholesteryl esters	35.7 ± 2.0	44.0 ± 2.3	47.0 ± 2.5
Triglyceride	4.8 ± 0.2	0.9 ± 0.2	1.2 ± 0.2

[a] Mean ± SEM of three different analyses done in triplicate. [b] The chemical composition of n-LDL from the same two monkeys was analyzed previously (Fless and Scanu, 1975). The discrepancy between the two analyses resulted from technical difficulties attending the use of the automated instrument utilized in the earlier study, which gave inaccurate data. [c] Pool of four monkeys. [d] Pool of five monkeys.

TABLE II: Phospholipid Composition of Rhesus LDL.[a]

	n-LDL[b] (wt %)	hyp-LDL (wt %) "Average" American Diet[c]	hyp-LDL (wt %) Coconut Oil Diet[d]
Sphingomyelin	15.2 ± 0.3	15.1 ± 1.0	13.9 ± 0.3
Phosphatidylcholine	60.6 ± 1.2	67.9 ± 3.2	66.7 ± 4.0
Lysophosphatidylcholine	4.8 ± 0.2	4.0 ± 0.6	5.2 ± 0.8
Phosphatidylserine plus phosphatidylinositol	8.3 ± 0.3	5.8 ± 0.9	8.2 ± 1.3
Phosphatidylethanolamine	11.1 ± 0.3	7.2 ± 1.3	5.9 ± 1.5

[a] Mean values ± maximum deviation from the mean of two determinations. [b] Pool of monkeys 4 and 5. [c] Pool of four monkeys. [d] Pool of five monkeys.

Results

Assessment of Purity. Rhesus LDL from hyperlipidemic monkeys fed an "average" American diet or the coconut-oil-supplemented diet were homogeneous as judged by acrylamide–agarose gel electrophoresis, gel filtration on Sepharose 4B, and density gradient centrifugation. Although the hyperlipidemia induced in the monkeys by the coconut-oil-supplemented diet was more severe than that with the "average" American diet, all of the physical parameters of LDL examined were very similar to each other within the experimental error of the analyses and were, therefore, treated together. Hyp-LDL eluted as a homogeneous peak from Sepharose 4B with a K_d of 0.38 and exhibited a single component by density gradient ultracentrifugation in NaBr, although with a lower hydrated density than n-LDL (Figure 1). By sodium dodecyl sulfate gel electrophoresis, the fractions from the central portion of the gradients did not penetrate the gels and were considered to represent apo-B. Additional peptides (mol wt <60 000) were observed in the peripheral fractions of the gradient and were estimated to represent less than 5% of the total protein applied to the gel.

Chemical Composition. The amino acid and carbohydrate composition of apo-LDL from the hyperlipidemic monkeys was identical with that reported previously for the normolipidemic animals (Fless and Scanu, 1975). As shown in Table I, the lipid–protein composition of hyp-LDL was significantly different from that of n-LDL. Hyp-LDL had a smaller weight percentage of protein and triglyceride, but a significantly higher percentage of cholesteryl ester, which accounted for most of the increase in the lipid–protein ratio of the hyperlipidemic particles. The content of free cholesterol relative to the LDL protein remained constant, while the phospholipid–protein ratio increased slightly. The phospholipid composition given in Table II shows no major differences between hyp-LDL and n-LDL.

The results of the fatty acid analyses of the various lipid classes in n-LDL and hyp-LDL are shown in Table III. The diet had little effect on the fatty acid composition of the total phospholipid except for linoleic acid, which was higher in coconut-oil-fed animals, at the expense of oleic and arachidonic acids. In the cholesteryl ester fraction, palmitic, oleic, and linoleic acids were essentially the only constitutents. The amount of palmitic acid and the sum of oleic and linoleic acids were almost the same in all three groups. There was a difference, however, in the monkeys fed the "average" American diet. The major fatty acids found in the LDL triglycerides from all three groups of monkeys were palmitic and oleic. However, the coconut-oil-fed monkeys had a more heterogeneous composition and contained more short-chain fatty acids than the other two groups. This was also evident in the composition of cholesteryl esters and is likely to be a reflection of the feeding of coconut oil, which is rich in short-chain fatty acids.

Hydrodynamic Analysis. The sedimentation and diffusion coefficients of LDL were measured in 0.2 M KCl–0.0125 M phosphate buffer (pH 7.0) containing 0.025% Na_2EDTA. At infinite dilution, the $s_{25,b}{}^0$ of hyp-LDL was 4.58 ± 0.11 S and the flotation coefficient, $s_{25,1.063\ g/ml}{}^0$, was −8.53 ± 0.30 S (Figure 2, A and B). The latter was determined at ρ 1.063 g/ml NaCl (pH 7.0) containing 0.025% Na_2EDTA.

The concentration dependence of the D_{app} values of LDL from hyperlipidemic monkeys is shown in Figure 2C. The translational diffusion coefficient at infinite dilution, $D_{25,b}{}^0$, was 1.83 ± 0.23 × 10^{-7} cm^2 s^{-1}. The translational diffusion coefficient of n-LDL from monkeys number 4 and 5 was redetermined and is shown in Figure 2D. By combining the data

TABLE III: Fatty Acid Composition of Rhesus LDL and the Diets Used in the Study.[a,b]

	n-LDL (wt %), Regular Primate Chow	Hyp-LDL (wt %), "Average" American Diet	Hyp-LDL (wt %), Coconut Oil Diet
Phospholipid			
C16:0	20.0 ± 1.1	23.5 ± 0.8	27.3 ± 1.3
C18:0	19.2 ± 0.7	18.7 ± 1.0	21.5 ± 2.0
C18:1	11.4 ± 0.3	11.0 ± 0.1	8.1 ± 0.7
C18:2	19.8 ± 1.3	21.7 ± 0.5	31.3 ± 0.9
C20:4	10.3 ± 0.5	11.2 ± 0.6	5.6 ± 0.4
Cholesteryl ester			
C14:0			6.2 ± 0.3
C16:0	14.4 ± 0.2	13.1 ± 0.6	14.0 ± 0.8
C18:0	4.9 ± 0.1		
C18:1	23.9 ± 0.2	37.0 ± 2.0	19.1 ± 0.4
C18:2	44.5 ± 0.2	35.5 ± 1.5	47.8 ± 0.5
Triglyceride			
C12:0			5.0 ± 1.3
C14:0			11.8 ± 0.4
C16:0	42.1 ± 2.3	31.4 ± 0.2	30.7 ± 1.0
C16:1		8.0 ± 5.0	7.3 ± 0.3
C18:0		7.1 ± 0.9	5.8 ± 1.5
C18:1	40.1 ± 1.7	34.5 ± 2.0	20.7 ± 5.0
C18:2	12.5 ± 0.9	10.8 ± 3.0	8.3 ± 2.3
Diets			
C 8:0			8.5 ± 0.1
C10:0			5.8 ± 0.2
C12:0			42.5 ± 0.7
C14:0		4.9 ± 0.2	17.3 ± 0.1
C16:0	20.7 ± 0.8	28.0 ± 0.7	10.0 ± 0.1
C18:0	11.0 ± 0.3	11.1 ± 0.5	
C18:1	35.4 ± 0.7	38.8 ± 1.1	8.6 ± 0.4
C18:2	29.8 ± 0.6	10.0 ± 0.2	4.7 ± 0.2

[a] Mean percent ± SEM. [b] For clarity, values of less than 5% were not included in the table, but were taken into account in the calculation of the weight percent of fatty acid for each fraction.

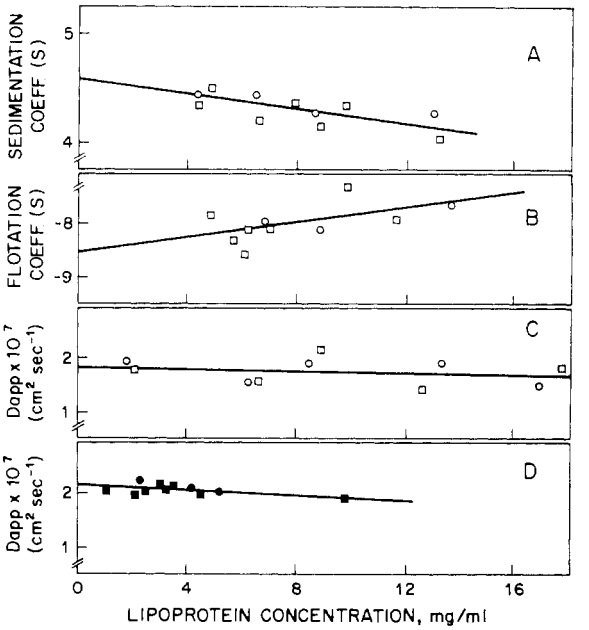

FIGURE 2: (A) Apparent sedimentation coefficient of hyp-LDL plotted as a function of lipoprotein concentration. The solvent was 0.2 M KCl–0.125 M phosphate (pH 7.0) containing 0.025% Na_2EDTA. The rotor speed was 44 000 rpm at 25 °C. (B) Concentration dependence of the flotation coefficient of hyp-LDL in ρ 1.063 g/ml NaCl containing 0.025% Na_2EDTA (pH 7.0). The rotor speed was 44 000 rpm at 25 °C. (C) Concentration dependence of the apparent diffusion coefficient of hyp-LDL. Measurements were made in the same solvent as in A. Rotor speed was 3600 rpm at 25 °C. (D) Concentration dependence of the apparent diffusion coefficient of n-LDL. Conditions were as in C. (○) hyp-LDL from coconut-oil-supplemented monkeys; (□) hyp-LDL from monkeys fed an "average" American diet; (■) n-LDL from monkeys numbers 4 and 5 fed normal purina monkey chow; (●) n-LDL from monkeys 4 and 5, examined 18 months previously (Fless and Scanu, 1975).

TABLE IV: Physical Parameters of Rhesus LDL from Normal and Hyperlipidemic Monkeys.

Physical Parameters		Rhesus LDL Normal[a]	Rhesus LDL Hyperlipidemic
s_{25b}^0	(S)	7.09 ± 0.15[b]	4.58 ± 0.11
$s_{25,1.063}^0$	(S)	−5.75 ± 0.28[b]	−8.53 ± 0.30
$D_{25,b}^0 \times 10^7$	($cm^2\ s^{-1}$)	2.17 ± 0.05	1.83 ± 0.23
$\bar{\nu}_2$	(ml/g)	0.969 ± 0.002	0.973 ± 0.002
$M_w \times 10^{-6}$		3.01 ± 0.12	3.52 ± 0.21
f/f_0[c]		1.08	1.21
δ_1[d]	(g of H_2O/g of LDL)	0.25	0.75
Diameter[e]	(Å)	222	268
K_d[f]		0.43	0.38

[a] Pool of monkeys 4 and 5. [b] Value taken from Fless and Scanu (1975). [c] Maximum asymmetry. [d] Maximum hydration. [e] Diameter of a hydrated, spherical, hydrodynamic particle. [f] Distribution coefficient of LDL on Sepharose 4B.

from the present and previous investigation (Fless and Scanu, 1975), we arrived at a new $D_{25,b}^0$ of 2.17 ± 0.05 × 10^{-7} cm^2 s^{-1}. The fact that the D_{app} values obtained in the two studies (same monkeys used in both investigations) fall on the same line (Figure 2D) indicates that the diffusion coefficient is invariant with time (the interval was eighteen months). A summary of the ultracentrifugal results is given in Table IV.

Molecular Weight Determination. Sedimentation and flotation equilibrium measurements of rhesus LDL were carried out in solutions of NaBr (pH 7.0) of different densities, containing 0.01% Na_2EDTA and 0.005% NaN_3. The plots of log c vs. the square of the distance from the center of rotation were linear at all densities employed. Under conditions of "flotation equilibrium", however, d log c/dr^2 was negative because the buoyancy term $(1 - \bar{\nu}_2\rho)$ was negative. The mean molecular weight of n-LDL from five control monkeys was 3.12 ± 0.21 × 10^6, with a $\bar{\nu}_2$ value of 0.969 ± 0.003 ml/g, both values being obtained simultaneously from the plot of solvent density, ρ, vs. $(T\ d\ log\ c/dr^2)/\omega^2$. The LDL from control monkeys numbers 1, 2, and 3 were analyzed individually, but the LDL from control monkeys 4 and 5 were pooled so that a detailed comparison with the molecular weight obtained previously (Fless and Scanu, 1975) could be made. A typical molecular-weight analysis is shown in Figure 3, which gives the results for the n-LDL of monkeys 4 and 5. The molecular weight of 3.01 × 10^6 obtained in the present investigation for their pooled LDL compares favorably with the value of 3.18 × 10^6 that was calculated by correction of the molecular weight of 2.29 × 10^6 found in the previous investigation with the new and more accurate partial specific volume ($\bar{\nu}_2$ = 0.969 ± 0.002 ml/g). The time interval between the two investigations was 18 months; this indicates that the molecular weight of normal

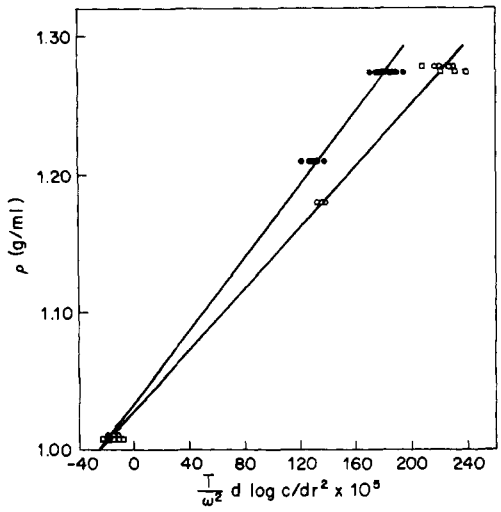

FIGURE 3: Flotation equilibrium data of n-LDL and hyp-LDL, plotted according to the equation $\rho = (-4.606RT\mathrm{d}\log c/\mathrm{d}r^2)/M\bar{v}_2\omega^2 + 1/\bar{v}_2$. The rotor speed was varied as a function of the solvent density; at density 1.28 g/ml, speeds used ranged from 3000 to 3600 rpm; at densities 1.18 and 1.21 g/ml, speeds were varied from 4800 to 5200 rpm; and at densities from 1.008 to 1.017 g/ml, speeds varied from 10 000 to 14 000 rpm. The temperature was usually 25 °C. Each point represents a single determination. (●) n-LDL, pooled from monkeys 4 and 5; (○) hyp-LDL, pooled from five monkeys on a coconut-oil-supplemented diet; (□) hyp-LDL, pooled from four monkeys on an "average" American diet.

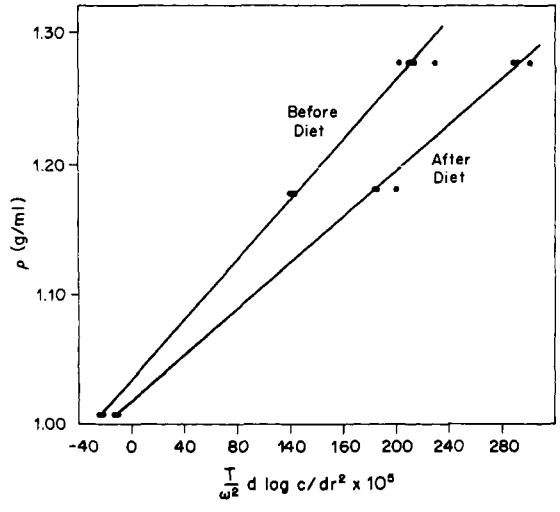

FIGURE 4: Flotation equilibrium data of n-LDL from monkey 3 before and after a 2-month diet consisting of coconut-oil-supplemented Purina monkey show. Conditions were the same as for Figure 3.

rhesus LDL does not change significantly with time. The molecular weights found for n-LDL of monkeys 1, 2, and 3 were 2.92×10^6, 3.18×10^6, and 3.45×10^6, respectively. It is evident from the above results that the molecular weight of n-LDL may vary from animal to animal.

The LDL from the monkeys on the "average" American diet and from those on the coconut-oil diet had a molecular weight of $3.52 \pm 0.21 \times 10^6$ and a partial specific volume of 0.973 ± 0.002 ml/g (Fig. 3). The observed difference between n-LDL and hyp-LDL was significant and was not related to any variation from animal to animal, or to variation with time (as was shown above). In a further attempt to rule out variation among animals, we measured the molecular weight of LDL in monkeys 1 and 3 before and during the administration of the coconut-oil-supplemented diet. The feeding of the hyperlipidemic diet to monkey 1 was attended by a significant elevation in LDL molecular weight, which was already evident 3 days after the start of the diet (from a basal value of 2.92×10^6 to 3.19×10^6), reaching a plateau of 3.64×10^6 after just 10 days. In monkey 3, the molecular weight of LDL increased from 3.45×10^6 to 4.41×10^6 after the animal had been on the diet for 2 months, indicating that, even with a relatively high initial basal value, the molecular weight of LDL increased once hyperlipidemia developed (Figure 4). In parallel with the molecular weight, the partial specific volume increased from 0.967 to 0.981 ml/g over this 2-month period.

Discussion

It has previously been shown that the determination of the molecular weight of LDL by flotation and diffusion analysis at solution densities far removed from the particle buoyant density yields results which are more accurate than those in solutions of comparatively low density (Adams and Schumaker, 1969a; Fisher et al., 1971). In these earlier studies, it was found that high-salt solutions do not adversely affect the structure of LDL and that there is no preferential salt binding. Studies by Nelson et al. (1974) subsequently indicated that flotation equilibrium of LDL in solutions of different densities provides even more accurate values of molecular weight since this method does not depend on the shape or hydration of the particle and since it permits the simultaneous determination of the partial specific volume. In addition, calculating the molecular weight from the slope of the graph of ρ vs. $(T\,\mathrm{d}\log c/\mathrm{d}r^2)/\omega^2$ makes the result less sensitive to the partial specific volume. Thus, in the present work we made use of the technique of flotation equilibrium which proved both practical and highly reproducible. Two important results emerged from these studies: (1) the molecular weight varied among the control normolipidemic monkeys, but was relatively invariant with time in the individual animals (difference within 18 months: 0.17×10^6); (2) regardless of the starting basal value, the molecular weight of LDL showed a significant progressive increase in the animals fed an atherogenic diet. Fisher et al. (1975) have reported that the molecular weight of LDL observed in human subjects is essentially invariable over a 2-year period (difference less than 0.2×10^6). They also recognized the existence of molecular weight differences in the LDL of various normolipidemic individuals. Although the number of our observations is limited, the results of our present studies appear to indicate that the LDL of the rhesus monkey has a behavior similar to that of man. Fisher et al. (1975) linked their observations to a genetic basis; our results for the rhesus monkey do not permit us to rule on this possibility at present. Our observations make it appear likely, however, that the size and shape of circulating LDL are under a fine metabolic control which is maintained under normolipidemic conditions.

In our earlier account (Fless and Scanu, 1975), we gave a value of 2.29×10^6 for the weight-average molecular weight of LDL in normolipidemic rhesus monkeys. We now recognize that this value was probably too low, and that a more appropriate figure is $3.12 \times 0.21 \times 10^6$, the value obtained in the current study by use of a more accurate method for determining the molecular weight and partial specific volume. This molecular weight is in agreement with the value of 3.3×10^6 reported by Lee and Morris (1974). The new partial specific volume of n-LDL was 0.969 ± 0.003 ml/g. In addition, contrary to our previous study in which the graphs of $\log c$ vs. r^2 were curved, we obtained only linear plots for our present normolipidemic animals. At this time, we have no explanation

for the apparent discrepancy, and we are pursuing this problem further.

For the hyperlipidemic animals, the molecular weight of hyp-LDL (average of nine monkeys) was $3.52 \pm 0.21 \times 10^6$ and the partial specific volume was 0.973 ± 0.002 ml/g. Although the results indicated a significant difference between the LDL of normo- and hyperlipidemic animals, the recognized molecular weight variability of the LDL among control animals made it difficult, at first, to determine the validity of the observed difference. However, the studies in which each animal served as its own control clearly showed that the administration of atherogenic diets caused significant changes in the size, density, and likely hydration of LDL (see Table IV). The frictional ratio of hyp-LDL was found to be somewhat larger than that of n-LDL; this suggests that the particle from the hyperlipidemic animals was either more hydrated, asymmetrical, or both, than the LDL from the control animals. The decrease in the diffusion coefficient from 2.17×10^{-7} to 1.83×10^{-7} cm^2 s^{-1}, the increase in diameter from 222 to 268 Å, and the smaller K_d in Sepharose are all consistent with an increase in size of hyp-LDL. Furthermore, the decrease in hydrated density for hyp-LDL is consistent with its larger partial specific volume, which can be predicted from the results of the chemical analyses (Table I).

These observed differences between normal and hyperlipidemic particles may be accounted for by the changes in chemical composition of hyp-LDL with respect to n-LDL particularly by the large increase in cholesteryl esters, the small increase in phospholipid, and the substantial decrease in triglycerides (Table I). This contrasts with the protein and free cholesterol content, which remained essentially constant irrespective of the levels of hypercholesterolemia. Initially, we considered the possibility that the increase in size of hyp-LDL was due to the association with n-LDL of a second particle having as a protein moiety a polypeptide different from apo-B. Our studies ruled out this possibility since the hyp-LDL, like the n-LDL, contained either only apo-B (particles collected from the central portion of the gradient) (see Figure 1) or less thn 5% by weight of fast-migrating electrophoretic components (fractions in the peripheral portion of the band).

Further evidence supporting the correlation between increased size of hyp-LDL and its relative increase in lipid content was obtained when the molar volume of LDL was plotted as a function of molecular weight, according to the method of Schumaker (1973). The reciprocal of the slope of such a graph provides an average value for the density of the hyp-LDL mass in excess of that of n-LDL. The value obtained was 1.015 g/ml; being so low, it may be taken to indicate that the excess mass consists primarily of lipid. Adams and Schumaker (1969b) and Hammond and Fisher (1971), who also noted variations in size among the various LDL which they studied, attributed them to changes in the lipid content and not to protein mass, which remained relatively constant. At this time, we are unable to specify the type of structural abnormality which attends the changes in chemical composition. Studies by small-angle x-ray scattering (Aggerbeck, private communication) have shown that important structural differences exist between n-LDL and hyp-LDL; the exact nature of these differences is still under investigation. Wissler and collaborators (Fischer-Dzoga et al., 1974) showed that, in cultured arterial smooth-muscle cells from rhesus monkey aortas, the stimulating proliferative effect exhibited by hyp-LDL is not shared by n-LDL. From the above results, it is evident that diet-induced hyperlipidemia is associated with the appearance of LDL particles in the circulation that are abnormal in terms of both structure and function. Although the lipids appear implicated in the observed changes, our studies do not rule out that changes in the structure of apolipoprotein B may have occurred. However, these changes, if present, were probably so subtle that they could not be detected by amino acid or carbohydrate analysis. The same reasoning applies to the fatty acid composition shown in Table III which, per se, cannot readily explain the structural differences between n-LDL and hyp-LDL.

The results obtained with experimentally induced hyperlipidemia in rhesus monkeys invite a comparison with the spontaneous form. Lee and Morris (1974) have observed that the molecular weight of the LDL in rhesus monkeys with spontaneous hyperbetalipoproteinemia is essentially the same as in normolipidemic monkeys. It appears that the administration of atherogenic diets leading to hypercholesterolemia is attended by structural changes in LDL not seen in either normolipidemic states or genetically determined hyperlipidemias. The exact mechanisms whereby diets with a high fat content cause changes in the rhesus monkey LDL and, perhaps, of the other circulating lipoproteins remain open for investigation.

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Properties of Deoxycholate Solubilized Sarcoplasmic Reticulum Ca^{2+}-ATPase†

Marc le Maire, Karl E. Jørgensen, H. Røigaard-Petersen, and Jesper V. Møller*,‡

ABSTRACT: The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum after solubilization with deoxycholate and removal of lipid by gel chromatography exists as a mixture of monomer, dimer, and smaller amounts of higher molecular weight aggregates. The binding capacity of deoxycholate by monomeric and oligomeric forms of the ATPase is 0.3 g/g of protein at pH 8 and ionic strength 0.11. Examination in the analytical ultracentrifuge results in estimates of protein molecular weight of monomer of 115 000 ± 7000 and of Stokes radius of 50–55 Å. The results indicate an asymmetric shape of both delipidated monomer and dimer. Solubilization of ATPase vesicles by deoxycholate at high protein dilutions leads to almost instantaneous loss of ATPase activity. However, ATPase may be solubilized by deoxycholate in presence of phospholipid and sucrose in a temporarily active state. Inactivation appears to be accompanied by delipidation and conformational changes of the protein as evidenced by circular dichroism measurements. Sedimentation velocity examination of enzymatically active preparations of soluble ATPase in presence of phospholipid and sucrose strongly suggests that the major part of enzymatic activity is derived from a monomer with an asymmetric shape. The extent of formation of soluble oligomers by column chromatography was dependent on the exact conditions used for initial solubilization of ATPase. No evidence for differences among monomer and dimer fractions was obtained by isoelectric focusing and amino acid analysis. The results of these studies are compatible with electron-microscopic studies by other authors which suggest that the ATPase has an elongated shape with limited hydrophobic contact with the membrane lipid. A resemblance of delipidated oligomers with the form in which ATPase occurs in the membrane is conjectural at present.

The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum is characterized by tight association with membrane lipids. Studies aiming at a physicochemical characterization of the ATPase are therefore hampered by difficulties in obtaining native ATPase in a molecularly dispersed state. For this reason, detergents have to be used which solubilize the ATPase by forming micellar-like complexes with the protein. In a previous paper we have reported on the properties of detergent-solubilized ATPase, using retention of enzymatic activity as a criterion of a native-like conformation (le Maire et al., 1976). It was found that nonionic detergents under appropriate conditions could maintain the solubilized protein in an enzymatically active state. Those conditions involved initial treatment of vesicles of the ATPase with an effective solubilizer such as dodecyl octaoxyethylene glycol monoether, followed by removal of excess detergent and membrane lipid by gel chromatography in which the eluent contained a relatively low concentration of nonionic detergent. By contrast, long-term exposure of ATPase vesicles to deoxycholate, even at nonsolubilizing concentrations, was found to lead to enzymatic inactivation. From this point of view deoxycholate is a less satisfactory detergent for characterization of ATPase in the solubilized state. However, there is ample evidence in the literature showing that it is possible to reconstitute Ca^{2+}-transporting vesicles under carefully controlled conditions after solubilization with deoxycholate (Meissner and Fleischer, 1974; Warren et al., 1974a).

Thus deoxycholate appears to retain another characteristic function of the protein, viz., the capability of forming an organized structure with membrane lipids. It was therefore considered worthwhile to continue our preliminary characterization studies of ATPase solubilized by deoxycholate (le Maire et al., 1976). The work proceeded in three stages. First, we examined the properties of delipidated and enzymatically inactive enzyme in order to determine such factors as molecular weight, Stokes radius, and the aggregational state. In the second stage, we explored conditions for solubilization of dilute ATPase in an enzymatically active form. It was found that inactivation of the solubilized ATPase could be slowed down by addition of phospholipid and sucrose to the deoxycholate

† From the Institute of Medical Biochemistry, University of Aarhus, Denmark, and the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received March 3, 1976.*
‡ Address correspondence to this author at the Institute of Medical Biochemistry, University of Aarhus, Denmark.