Separation and characterization of plasma lipoproteins of rhesus monkeys (*Macaca mulatta*)

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Abstract A group of 14 adult male rhesus monkeys was maintained on a low cholesterol–high fat diet. Periodically, animals were fasted and blood samples were taken for characterization of the plasma lipoproteins. Complete separation of individual plasma lipoprotein classes was not achieved by traditional sequential ultracentrifugation techniques. Rather, initial separation of lipoprotein classes according to size was effected and density centrifugation was used subsequently for further separation. At least six lipoprotein fractions were identified, each of which was unique as defined by the properties of size, density (d), and electrophoretic mobility. These lipoprotein fractions were characterized by determination of chemical compositions and apoprotein patterns. The lipoproteins present in highest concentration in these monkeys were designated as region IV lipoproteins. This fraction had α-migration on agarose electrophoresis, $1.063 < d < 1.225$, and the size, composition, and apoprotein pattern characteristic of HDL. No fewer than three fractions were identified with densities that overlapped the $1.019 < d < 1.063$ range. Of these, the fraction designated as region III lipoproteins was present in highest concentration, had β-migration by agarose electrophoresis, a predominant B apoprotein, and a chemical composition and size characteristic of LDL. Two larger subfractions, identified as region II lipoproteins, were separated from each other at a density of 1.050 g/ml. Agarose electrophoresis showed that the fraction with $d < 1.050$ had a migration intermediate between β and pre-β. The chemical composition and apoprotein pattern were consistent with the possibility that these lipoproteins were remnants of VLDL catabolism. The fraction with $d > 1.050$, had pre-β mobility and a size and composition similar to the Lp(a) lipoprotein in plasma of human beings. At least two VLDL subfractions, identified as region I and II_A lipoproteins, were found although both were present in very low concentrations. Region I lipoproteins were larger and contained relatively more cholesteryl ester and more of the apoproteins that migrated with the mobility of apo-B and arg-rich apoprotein in SDS–polyacrylamide gel electrophoresis. Some of the region I lipoproteins were β-migrating by agarose electrophoresis. These results suggested the possibility that a β-migrating VLDL was present in these normal animals.

Supplementary keywords agarose gel chromatography · agarose electrophoresis · apoproteins · cholesterol · cholesteryl esters · high density lipoproteins · low density lipoproteins · polyacrylamide gel electrophoresis · very low density lipoproteins

Recently nonhuman primates have become popular as animal models for atherosclerosis research (1). Major contributing factors to this popularity have been the close phylogenetic relationship of these animals to human beings and their susceptibility to diet-induced atherosclerosis (2). In order to determine the most appropriate nonhuman primate model for human atherosclerosis, one of the aspects that must be considered is the plasma lipoprotein spectrum. In several species of nonhuman primates, we are conducting detailed studies of the distribution and composition of individual lipoprotein classes. In the present study, rhesus monkeys were selected because this species was readily available and is one of the most frequently used in atherosclerosis research.

Characteristics of the plasma lipoproteins of rhesus monkeys have been studied by a number of groups (3–8), but the number of animals included in each of these studies was small and detailed information about the properties of the complete spectrum of lipoproteins in rhesus monkeys is not complete. In our studies, animals were fed a diet that contained a distribution of calories among fat, carbohydrate, and protein approximating that of the typical North American diet, i.e., 40:40:20, respectively. The level of cholesterol in this diet was low, 0.05 mg/kcal.

Abbreviations: d, density in g/ml; HDL, high density lipoproteins; LDL, low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, trihydroxyaminomethane; VLDL, very low density lipoproteins.

Separation of individual classes of lipoproteins was carried out based on the size, density, and electrophoretic mobility of the lipoproteins. A detailed description of the individual classes was then made based on chemical composition and apoprotein pattern. Subsequent reports will describe companion studies utilizing rhesus monkeys fed the same basic diet but with an increased amount of cholesterol.

MATERIALS AND METHODS

Adult male rhesus monkeys weighing 7–14 kg were fed a semipurified diet containing a cholesterol level of 0.05 mg/kcal and 25% (w/w) lard. At least 300 g of the diet, which had a caloric density of 3.8 kcal/g, was offered each day. All animals maintained or gained body weight throughout the study. Animals, fasted for at least 18 hr prior to blood collection, were caught in squeeze cages and ketamine was administered intramuscularly (10 mg/kg) to facilitate handling and sample collecting. Blood was drawn from the femoral vein and placed in tubes containing Na_2-ethylenediaminetetraacetate (EDTA) at a final concentration of 1 mg/ml and 5,5′-dithio(bis)-2-nitrobenzoic acid (DTNB) at a final concentration of about 0.4 mg/ml. Within an hour after the blood was taken, plasma was isolated at 4°C by centrifugation at 1,000 *g* for 20 min.

An aliquot of plasma was taken for cholesterol and triglyceride determinations (9), and agarose electrophoresis was performed according to the method of Noble (10). On another aliquot, lipoproteins were isolated and separated by a modification of the method of Rudel et al. (11). The solvent density of plasma was raised to 1.225 g/ml by adding solid KBr, 0.3517 g/ml. Ten ml of this solution was placed in an SW-40 ultracentrifuge tube and overlayered with 3.5 ml of a 1.225 g/ml solution prepared by adding solid KBr to 0.9% NaCl containing 0.01% EDTA. The tubes were then centrifuged at 40,000 rpm (200,000 g_{av}) for 40 hr at 15°C using the SW-40 rotor in the Beckman L5-50 ultracentrifuge. Under these conditions, all lipoproteins floated to the top of the centrifuge tube and were removed in a 1.5 ml volume after the tube was sliced. Mean recovery (±SD) of lipoprotein cholesterol in the top fraction for these experiments was 96.2 ± 4.2%. The lipoprotein concentration was brought to a known volume (usually 2 ml) with 0.9% NaCl containing 0.01% EDTA. An aliquot was removed for cholesterol analysis, and an aliquot was applied to an agarose chromatography column. The column consisted of Bio-Gel A-15m (4%), 200–400 mesh (Bio-Rad Labs, Richmond, CA), and was maintained and run as described previously (11).

Lipoproteins were also separated by a series of sequential centrifugations at progressively increasing densities by a modification of the procedure of Havel, Eder, and Bragdon (12). The fractions were isolated in the SW-40 rotor. Solvent density of plasma was adjusted with solid KBr when necessary. The low density fractions $d < 1.006$, $1.006 < d < 1.019$, and $1.019 < d < 1.063$, were harvested after 18 hr of centrifugation at 200,000 g_{av}. The fraction containing HDL, $1.063 < d < 1.225$, was harvested after 40 hr of centrifugation at 200,000 g_{av}. Sequential density centrifugation in the 9-cm long SW-40 rotor tubes was carried out using a discontinuous density gradient. The solvent density of the bottom 10 ml of plasma was at least 0.015 g/ml higher than the top 3.5 ml of overlayer solution, which was made to the final cut-off density. This was necessary due to salt redistribution during the run and has been found to minimize cross-contamination. In some cases, for lipoprotein fractions initially separated by size, it was necessary to use preparative ultracentrifugation to isolate subfractions. The discontinuous gradient was also used in these cases.

After separation, cholesterol distribution was determined among lipoproteins by measuring the amount of cholesterol in the fractions. Recovery (±SD) of lipoprotein cholesterol from the column was 96.7 ± 6.5%. Lipoprotein classes eluted from the column often were concentrated before further analysis. When density classification of a size population was being determined, concentration was effected at the appropriate density in the preparative ultracentrifuge. Because of the large volume decrease required in the region I and II_A samples, concentration was performed either by centrifugation at d 1.006 or in dialysis bags immersed in 50% ultrapure sucrose or Dextran 70 powder (Pharmacia Chemical Co., Piscataway, NJ). In other cases, the Amicon MMC concentrator with UM-10 membranes (Amicon Corp., Lexington, MA) was used. After concentration by centrifugation or in dialysis bags, dialysis of the samples was carried out for 24 hr against the column eluate solution. Agarose electrophoresis and chemical analysis were then performed. Total cholesterol was determined by the method of Rudel and Morris (13), and phospholipid phosphorus was determined by the method of Fiske and SubbaRow (14). Protein was routinely determined by the method of Lowry et al. (15), using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as

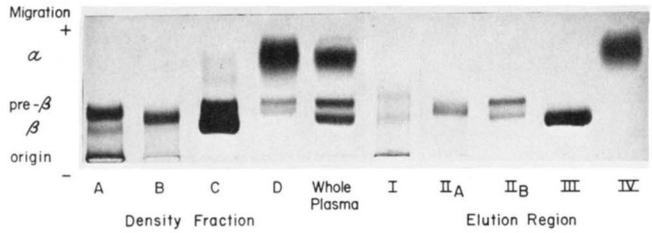

Fig. 1. Agarose electrophoresis of plasma lipoproteins from rhesus monkeys. Lipoprotein fractions isolated by sequential density centrifugation are designated: A, $d < 1.006$; B, $1.006 < d < 1.019$; C, $1.019 < d < 1.063$; and D, $1.063 < d < 1.225$. Lipoprotein fractions isolated from the agarose chromatography column are designated by the elution regions shown in Fig. 2. In this study, 150 ml of pooled plasma from five animals was used. A 30-ml aliquot was subjected to sequential ultracentrifugation and a 120-ml aliquot was used for chromatographic separation of lipoproteins. The concentrations of individual fractions, relative to whole plasma, were: A, 90×; B, 90×; C, 20×; D, 20×; I, 20×; II_A, 30×; II_B, 30×; III, 15×; IV, 15×. A 20-μl aliquot of each was applied to the electrophoresis strip. Samples were stained in an oil red O–fat red 7B mixture.

the standard. A comparison of absorbance at 280 nm with the Lowry protein method was carried out on individual fractions from the column. The comparison showed linearity for fractions throughout the II–III region and across region IV, although regression equations describing the correlations were not the same for region IV as for region II–III lipoproteins. It was possible, with these equations, to use direct absorbance measurements of these lipoprotein classes eluted from the column to estimate the protein concentration.

Lipids were extracted with chloroform–methanol 2:1 and separated on TLC as described earlier (16). Triglyceride determinations were performed according to the method of Sardesai and Manning (17). Free and ester cholesterol were determined as described above. Average recovery of free and ester cholesterol from the plate was >95%. For calculation of results, the determined value for phospholipid phosphorus was multiplied by 25 to obtain the amount of phospholipid, and the value for ester cholesterol was multiplied by 1.7 to obtain the amount of cholesteryl esters in the sample.

Apoprotein analysis was carried out on separated lipoprotein fractions. Samples were dialyzed against distilled water containing 0.01% EDTA; they were then lyophilized and delipidated with chloroform–methanol 2:1 as previously described (16). Apoproteins were then resolubilized in 8 M urea, 0.01 M Tris, pH 8.9. We noted an apparent sharpening of the apoprotein pattern if the samples were allowed to stand at room temperature overnight before electrophoresis. Polyacrylamide gel electrophoresis in 8 M urea, 7.5% gels, was performed according to the method of Davis (18). Just prior to use, urea was deionized on an ion exchange resin. In this system, most of the apoB is soluble. The amount of apoB in the apoprotein samples was determined as previously described (16) based on the insolubility of apoB in tetramethylurea (19).

For SDS PAGE, delipidated apoprotein samples were resolubilized in 0.025 M Tris–0.2 M glycine buffer (pH 8.3) containing 0.02 M sodium dodecyl sulfate. The SDS used in these studies was recrystallized from ethanol. Resolubilization of samples containing apoB was carried out at 50°C for 2–4 hr with continuous stirring. Apoprotein separation by SDS PAGE was carried out in a horizontal slab containing a 5% polyacrylamide spacer gel and either a 10% or 12.5% polyacrylamide running gel (16).

RESULTS

Lipoprotein distribution was first studied by fractionation of plasma lipoproteins with sequential density centrifugation. The fractions isolated in this way were characterized by agarose electrophoresis (**Fig. 1**). The $d < 1.006$ material showed primarily pre-β mobility, although small amounts of β-migrating material could be detected. The material that remained at the origin probably represented denatured material which resulted from the 90-fold concentration of this fraction. The $1.006 < d < 1.019$ material also migrated as a pre-β band although the relative distance of migration was less than for the $d < 1.006$ material. The major band of the $1.019 < d < 1.063$ lipoproteins was a β-migrating band, although a significant amount of pre-β migrating material often was also present. When a highly concentrated solution of $1.019 < d < 1.063$ lipoproteins was examined, faint bands in the α-region were seen. In the $1.063 < d < 1.225$ fractions, α-migrating material was predominant, although pre-β migrating material was also consistently seen.

Due to the heterogeneity seen by electrophoresis in the lipoprotein fractions separated on the basis of density, size analysis of density fractions was carried out by agarose column chromatography. **Fig. 2** shows the column elution profiles of the individual density fractions and, for comparison, the elution profile of the complete lipoprotein spectrum isolated by an initial centrifugation at d 1.225. All of the $d < 1.006$ lipoproteins eluted as large lipoproteins in the I and II_A region of the elution profile. The decrease in transmittance seen in this fraction is a function of turbidity, not of lipoprotein protein concentration. The $1.006 < d < 1.019$ lipoproteins eluted as smaller lipoproteins in the II_A and II_B regions of the

column elution profile. The $1.019 < d < 1.063$ lipoproteins eluted as two size populations, one in the II_B region and one in the III region of the elution profile. The $1.063 < d < 1.225$ lipoproteins eluted primarily as lipoproteins in region IV, although detectable amounts of material were seen in regions I and II_B.

The results of the size analyses of lipoproteins previously isolated via sequential ultracentrifugation suggested the applicability of initial separation of lipoproteins by agarose column chromatography. The chromatographic elution profile is shown in Fig. 2 for lipoproteins isolated simultaneously from a separate aliquot of the pooled plasma used for sequential density centrifugation. Fractions from the designated elution regions of this chromatographic separation were combined, concentrated, and subjected to electrophoresis. The results are shown in Fig. 1. For lipoproteins from region I, both a β and a pre-β band were detected. Lipoproteins from region II_A were mostly pre-β in migration, although the distance of migration of these lipoproteins was less than for the pre-β lipoproteins of regions I and II_B. A β-migrating band was also found in region II_B. All lipoproteins of region III were β-migrating and all of region IV were α-migrating. At this stage of analysis, it appeared that pure β-migrating, $1.019 < d < 1.063$ lipoproteins, classically known as low density lipoproteins (LDL), and

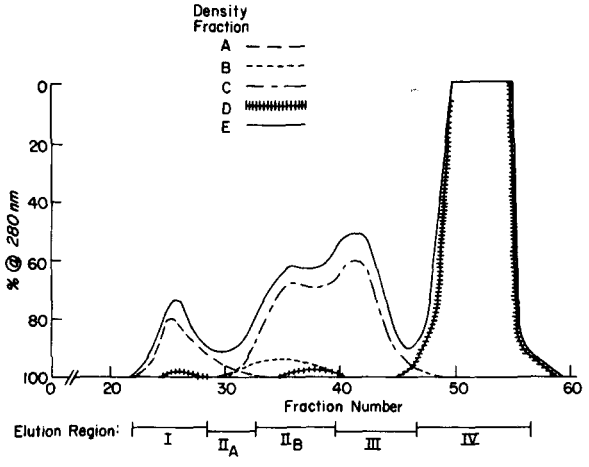

Fig. 2. Agarose column chromatography of plasma lipoproteins isolated by ultracentrifugation from plasma of rhesus monkeys. The density range for individual fractions indicated A through D was the same as for Fig. 1. The total lipoprotein fraction (E) represents the lipoproteins ($d < 1.225$) isolated from 10 ml of pooled plasma from two animals. The fractions eluted from the column within the elution regions designated I, II_A, II_B, III, IV were combined for further analyses. In some cases, fractions in the intermediate region between III and IV were pooled separately. The volume of individual density fractions applied to the column was adjusted to approximate a volume of plasma equivalent to that used for the separation of $d < 1.225$ lipoproteins.

TABLE 1. Plasma lipoprotein distribution in rhesus monkeys determined after chromatographic separation

Lipoprotein Elution Region	N	Plasma Concentration (mg/dl) I	II	III	IV
Cholesterol	7[a]	0.9 ± 0.2	25.7 ± 5.2	53.7 ± 6.5	97.8 ± 6.7
Protein	5	0.4 ± 0.7	23.7 ± 6.7	40.6 ± 3.4	310.7 ± 10.8

[a] Average plasma cholesterol concentration, 191 ± 8.5 mg/dl; average recovery of plasma cholesterol in lipoprotein fractions, 93 ± 3%; average plasma triglyceride concentration, 23 ± 8 mg/dl. All values mean ± SEM.

α-migrating, $1.063 < d < 1.225$ high density lipoproteins (HDL), were most satisfactorily isolated as region III and IV, respectively, of the agarose column chromatography elution profile. Preparative ultracentrifugation alone did not provide fractions of equal purity.

Essentially quantitative recovery of lipoproteins after chromatographic separation made determination of lipoprotein distribution possible. **Table 1** shows the results from chromatographic separations of lipoproteins from the plasma of seven different rhesus monkeys. Both cholesterol and protein concentrations are shown for comparison. The lipoproteins present in highest concentration were those eluted in region IV (HDL). The lipoproteins eluted in region III (LDL) were next highest in concentration. Although little material was found in region I, there were significant amounts of lipoproteins that eluted in region II. Since this region did not represent a single lipoprotein class, the material of this region from several plasma samples was pooled and subjected to further separation.

Lipoproteins from region II_A (Fig. 2) were centrifuged at d 1.006 for 16 hr at 200,000 *g*. Over 80% of the lipoproteins (as monitored by cholesterol determination) floated at this density. By agarose electrophoresis (**Fig. 3**), a single pre-β migrating band was found for these lipoproteins. The relative distance of migration was apparently increased slightly by the recentrifugation (compare Fig. 1 and Fig. 3). The lipoproteins from region II_B were combined and recentrifugation was carried out at d 1.050 for 18 hr at 200,000 *g*. The lipoproteins of $d < 1.050$, designated II_{B50}[1] lipoproteins, con-

[1] The designations II_{B50} and II_{B100} were chosen to indicate that the lipoproteins had the same size, as determined by elution position on column chromatography, but were different in density, such that I_{B50} were the II_B lipoproteins with a density less than 1.050 g/ml, i.e., a density less than "050", whereas the II_{B100} were II_B lipoproteins with a density less than 1.100 g/ml but greater than 1.050 g/ml.

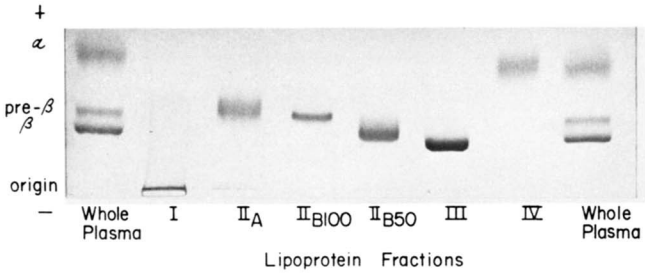

Fig. 3. Agarose electrophoresis of plasma lipoproteins separated from plasma of rhesus monkeys. Fractions are designated by the Roman numeral that indicates the chromatographic elution region of the lipoprotein fraction and, in the case of region II lipoproteins, an indication of density. One hundred and twenty milliliters of plasma pooled from four animals was the starting material. For each fraction, 20 μl of solution was placed in the sample well and cholesterol concentrations (μg/ml) were: whole plasma, 1,590; I, 213; II$_A$, 455; II$_{B100}$, 551; II$_{B50}$, 954; III, 1,560; IV, 750.

tained an average of 40% of the lipoprotein cholesterol of the II$_B$ material. After rechromatography on a Bio-Gel A-15m column, the peak fractions were combined, concentrated, and the electrophoretic mobility was determined (Fig. 3, II$_{B50}$). The broad band was intermediate in distance of migration between β and pre-β. After centrifugation of this fraction at a density of 1.019 g/ml, the $d < 1.019$ material appeared to have a slightly faster migration by agarose electrophoresis than did the $d > 1.019$ lipoproteins, although these subfractions were not separated completely into two different bands.

The remaining II$_B$ lipoproteins, $d > 1.050$ (representing about 60% of the lipoprotein cholesterol of the II$_B$ lipoproteins), were floated at d 1.10, and after removal of the last traces of β-migrating material by chromatography on a Bio-Gel A-15m column, were shown to have a pre-β migration by agarose electrophoresis (Fig. 3, II$_{B100}$). A lipoprotein with similar size, density, and electrophoretic characteristics of the II$_{B100}$ fraction was also found in the $1.063 < d < 1.10$ region of plasma during sequential centrifugation studies.

Properties of the intact lipoprotein classes are tabulated in **Table 2**. At least two separate pre-β migrating lipoprotein fractions were found, the II$_A$ and the II$_{B100}$. The pre-β migrating lipoproteins of region I were presumed to be similar to those of II$_A$ and different from the β-migrating lipoproteins of region I. The diameters of the lipoprotein particles from regions I and II were estimated using the relative elution index determined chromatographically. Reference points in establishing the regression line were sizes of various LDL (16) and of HDL (20). The relationships between chemical composition and size, as first demonstrated by Sata, Havel, and Jones (21), were consistent with these estimates. The cholesterol distribution represents mean values for pooled plasma samples and is shown only for reference; individual animal variation was pronounced.

Based on the distribution of cholesterol among the pre-β migrating lipoproteins (Table 2) and the low plasma triglyceride concentrations (Table 1), we suspected that the strong pre-β band often seen by agarose electrophoresis of whole plasma of some rhesus monkeys represented mostly II$_{B100}$ lipoproteins. Plasma lipoprotein analyses, represented by the data of **Fig. 4**, supported this hypothesis. The whole plasma electrophoresis pattern of animal no. 128 showed a much more intense pre-β band than that for no. 131. At the same time, the amount of the II$_B$ region lipoproteins was the major difference in the lipoprotein elution profiles. The II$_B$ lipoprotein cholesterol concentrations were 18 vs. 61 mg/dl, while the region III cholesterol concentrations in these two animals were comparable, 62 vs. 54 mg/dl for animals no. 131, and no. 128, respectively. Thus,

TABLE 2. Characteristics of plasma lipoproteins of control rhesus monkeys

	Plasma Lipoprotein Fractions (Elution Regions)					
	I	II$_A$	II$_{B50}$	II$_{B100}$	III	IV
Agarose electromobility	pre-β	pre-β	"β"	pre-β	β	α
Density range	$d < 1.006$	$d < 1.006$	$1.006 < d < 1.050$	$1.050 < d < 1.100$	$1.019 < d < 1.063$	$1.063 < d < 1.225$
Relative size index (r_1)[a]	1.72			1.19	1.00	0.821
Diameter (Å)	600	370–600		280–370	210	100
Plasma cholesterol[b] distribution (%)	0.5	2	5	8	32	52

[a] Mean values for six animals, as determined from the relative elution position at the center of the sample peak compared to that of a standard ^{125}I-labeled LDL preparation according to the method of Rudel et al. (16).

[b] Cholesterol distribution was determined on two separate pools of four different plasma samples and the mean values are shown.

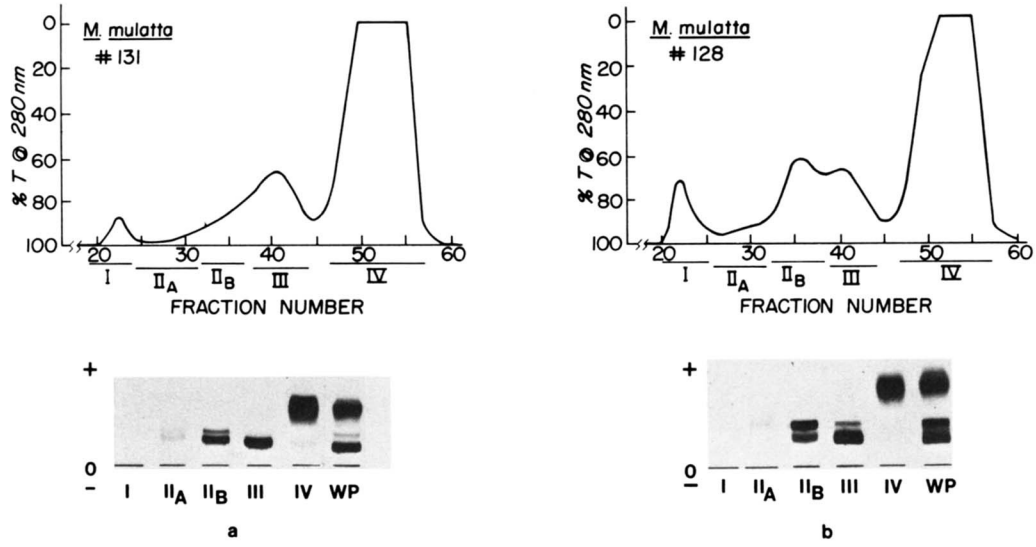

Fig. 4. Agarose chromatography and electrophoresis of plasma lipoproteins from two representative rhesus monkeys. Applied to the chromatography column in each case was an aliquot (1.6 ml of 2 ml) of a solution containing all the lipoproteins isolated from 10 ml of plasma by centrifugation at d 1.225. The fractions eluted in the regions indicated by Roman numerals were combined, concentrated to a final concentration about 2.5 times that of whole plasma, and 20 μl of each was placed in the wells indicated by the same letter. Whole plasma (20 μl) from which lipoproteins were isolated was placed in the wells designated WP.

the heavy pre-β band in plasma from no. 128 appears to be primarily an indication of pre-β migrating II_B lipoproteins, which have been shown to have a density between 1.050 and 1.10 g/ml (Fig. 3).

Each of the lipoprotein fractions was subjected to chemical analysis. The results are shown in **Table 3**. A distinct difference in composition was seen between the region I and II_A lipoproteins. The II_A lipoproteins contained a higher percentage of protein and triglyceride while the region I lipoproteins contained a higher percentage of cholesteryl esters. Sensitive indicators of differences in chemical composition within a lipoprotein fraction often are ratios of constituents. This usefulness is illustrated here

TABLE 3. Chemical composition of plasma lipoprotein fractions isolated from rhesus monkeys

Lipoprotein	% of Mass		% of Total Lipid				w/w		
	Lipid	Protein	FC	PL	CE	TG	TC/Pro	EC/TC	TC/PL
I[a]	95.4	4.6	5.5	13.0	32.9	48.5	5.13	0.78	1.91
II_A[b]	91.4 ± 1.5	8.6 ± 1.5	5.3 ± 0.6	17.3 ± 0.8	14.5 ± 5.0	63.1 ± 5.4	1.45 ± 0.04	0.61 ± 0.10	0.79 ± 0.09
II_{B50}[b]	82.3 ± 1.2	17.7 ± 1.2	11.0 ± 1.0	26.5 ± 0.6	47.3 ± 5.3	15.2 ± 5.7	1.80 ± 0.04	0.71 ± 0.01	1.47 ± 0.19
II_{B100}[b]	73.7 ± 0.6	26.3 ± 0.6	10.1 ± 0.7	27.1 ± 2.1	49.0 ± 0.5	13.8 ± 4.1	1.09 ± 0.02	0.74 ± 0.02	1.45 ± 0.19
III[c]	79.5 ± 2.0	20.5 ± 2.0	11.3 ± 1.6	29.5 ± 3.1	52.9 ± 3.2	6.3 ± 1.6	1.53 ± 0.18	0.74 ± 0.03	1.44 ± 0.15
IV_{front}[c]	55.3 ± 2.7	44.7 ± 2.7	7.3 ± 0.9	50.4 ± 4.0	37.0 ± 3.1	5.6 ± 1.7	0.36 ± 0.06	0.74 ± 0.01	0.57 ± 0.10
IV_{back}[c]	51.8 ± 5.8	48.2 ± 5.8	6.1 ± 0.6	52.5 ± 3.6	35.8 ± 4.1	5.6 ± 2.0	0.29 ± 0.04	0.77 ± 0.01	0.51 ± 0.10

[a] Determined on a single pooled sample from eight animals.
[b] Determined on two separate pooled samples, each from four animals.
[c] Determined on individual samples isolated from six animals.
All values are means (± SD). Abbreviations are: FC, free cholesterol; PL, phospholipids; CE, cholesteryl esters; TG, triglycerides; TC, total cholesterol; EC, ester cholesterol; Pro, protein.

by the TC/Pro ratio, which was over 5 for region I lipoproteins, but only 1.45 for the region II_A lipoproteins.

The percentage of protein in the II_{B50} vs. the II_{B100} lipoproteins was markedly different. The lipid composition was surprisingly similar. Both of the II_B fractions were higher in triglyceride and lower in cholesterol ester and phospholipid content than were the region III lipoproteins. The percentage of protein in the region III lipoproteins was intermediate between II_{B50} and II_{B100}. The region IV lipoproteins were analyzed as the front of the peak versus the back of the peak. This was done to determine if compositional heterogeneity could be found that would mimic the HDL_2 vs. HDL_3 differences that are consistently found when HDL are isolated by sequential density centrifugation. HDL_2 and HDL_3 differ significantly in size, mol wt 390,000 vs. 197,000, respectively, in rhesus monkeys (5) and our results were consistent with HDL_2–HDL_3 differences, i.e., the larger IV lipoproteins contained less protein and phospholipid than did the smaller IV lipoproteins and the EC/TC ratio was higher in the smaller IV lipoproteins.

To characterize lipoproteins further, electrophoretic separations were performed on the delipidated apoproteins of each fraction. The results shown in **Fig. 5** are of PAGE carried out in the presence of either 8 M urea or 0.1% SDS. The identity of the protein bands was tentatively established based on the coefficient of relative migration, r_m, as compared with apolipoprotein patterns of human lipoproteins run in the 8 M urea PAGE system. The doublet band at r_m 17, corresponds to human arginine-rich (or apoE) apoprotein. The band was present in each of the separated lipoprotein fractions of regions I, II_A, II_{B50} and III and cross-reacted with antiserum to arginine-rich apoprotein.[2] It was absent from the lipoproteins of regions II_{B100} and IV. An apoprotein band in position 17 was found in region IV lipoproteins but this apoprotein did not cross-react with arginine-rich antiserum or comigrate in the SDS PAGE systems with the arginine-rich apoprotein; it migrated faster and was closer to the apoprotein at 25. The apoprotein of band 25 corresponds to the apoA-I of human lipoproteins, cross-reacted with anti-serum to human apoA-I, and was found as the major apoprotein of region IV lipoproteins. In the urea gels, this "broad band" could be seen to give the appearance of multiple, very closely-spaced bands. The apoprotein with r_m 35, corresponds to the apoA-II of human HDL, and appeared to be present only in region IV lipoproteins. The faster migrating apoproteins (apoC) of rhesus monkey lipoproteins generally did not correspond in distance of migration of 8 M urea PAGE to that of the faster migrating apoproteins of human lipoproteins. In SDS PAGE, the apoprotein at 35 migrated with the other fast-migrating apoproteins.

The protein that remained in the sample well and near the interface of the spacer and running gels exhibited behavior characteristic of apoB, and was the major apoprotein of all lipoproteins, except those of region IV which contained very little, if any, of this apoprotein. In the SDS systems used here, apparent heterogeneity of apoB was observed when some protein entered the top of the running gel and migrated as a series of very closely spaced bands.

The apoprotein patterns of region I and II_A lipoproteins were very similar. The small difference, which was consistent from sample to sample, is best illustrated in the 10% SDS pattern of Fig. 5B. Relatively more of the apoB and apoprotein at 17 was present among apoproteins of I compared to those of II_A lipoproteins. In II_A lipoproteins, relatively more of the fast-migrating bands were present, although the relative distribution among the fast-migrating bands was not different from those of II_B lipoproteins (Fig. 5A).

Essentially all of the apoproteins of region II_B and III lipoproteins remained in the well or at the interface of the two gels in the SDS systems, indicating that most of the protein of these fractions was apoB. In separate analyses, the amount of apoprotein that was not apoB was estimated by tetramethylurea precipitation of apoB and was found to be less than 7% of the total protein in II_B lipoproteins and less than 5% in III lipoproteins. Of the soluble protein present in II_B lipoproteins, it can be seen that small amounts migrate in the 17 and 25 positions, and as fast-migrating apoproteins (Fig. 5B). When II_B lipoproteins were separated into II_{B50} and II_{B100} lipoproteins and then analyzed (Fig. 5C), almost all of the protein in the 17, 25, and fast-migrating regions was found in the II_{B50} sample, whereas most of the apoprotein of the II_{B100} fraction remained at the top of the gel. It was not uncommon to find small amounts of protein in the 17, 25, and fast-migrating regions in the peak III lipoproteins, even after recentrifugation of this fraction at d 1.063 and rechromatography on Bio-Gel A-15m. However, the relative amount of apoprotein in position 25 was variable from preparation to preparation.

[2] Antisera to patas monkey arginine-rich apoprotein and human apoA-I were generously provided by Dr. Robert Mahley of the NIH.

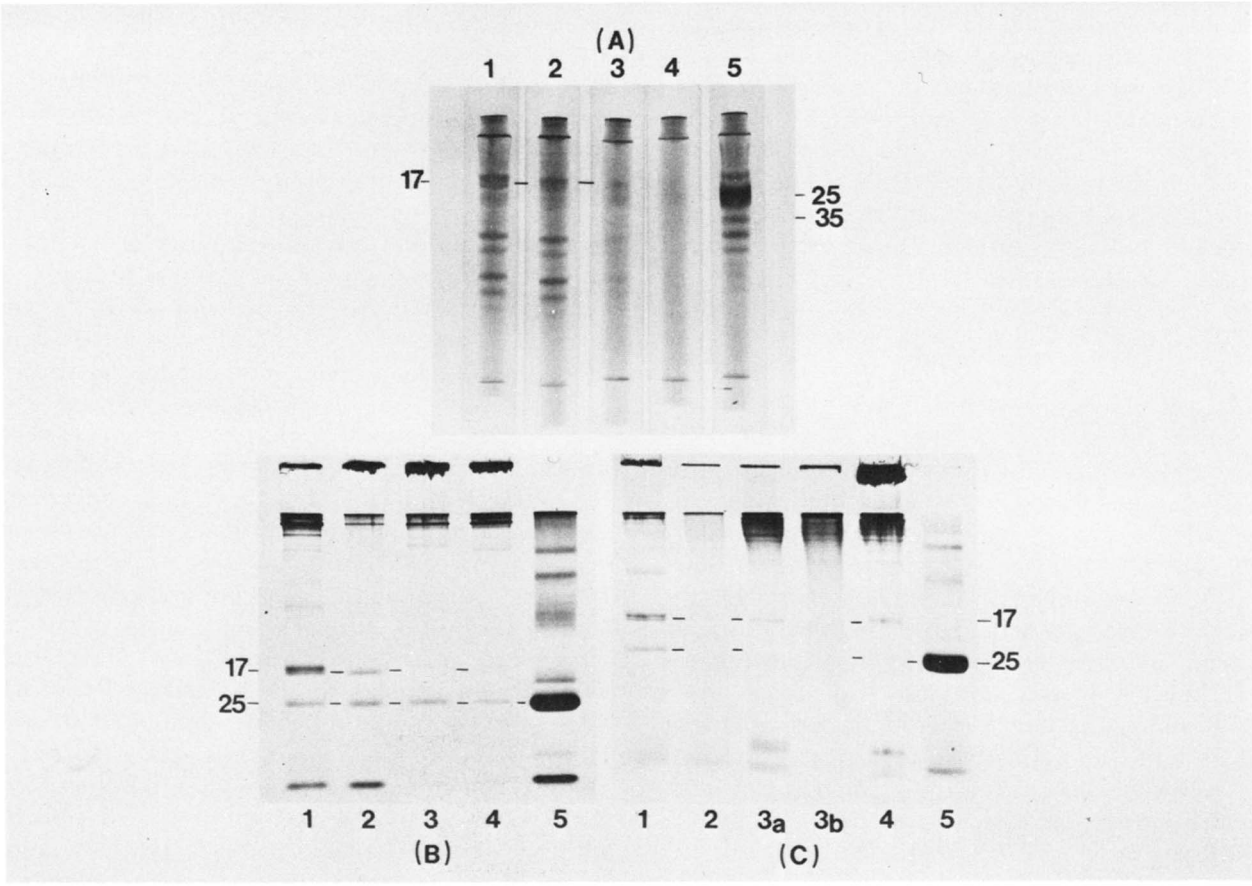

Fig. 5. Rhesus monkey plasma lipoprotein apoprotein patterns as determined by PAGE. The numbers for each sample refer to the following lipoprotein fractions: 1, region I; 2, region II_A; 3, region II_B; 3a, region II_{B50}; 3b, region II_{B100}; 4, region III; and 5, region IV. In (A), electrophoresis was carried out in 7.5% polyacrylamide tube-gels containing 8 M urea, 0.01M Tris, pH 8.9. In (B), electrophoresis was carried out in 10% polyacrylamide slab-gels containing 0.1% SDS, 0.5M Tris, pH 8.8, with a 5% polyacrylamide spacer gel. In (C), electrophoresis was carried out in 12.5% polyacrylamide slab-gels with the same buffer and SDS concentrations as (B). Routinely, lipoprotein samples containing 100–150 μg of protein were delipidated, redissolved in 100 μl of the appropriate buffer (see Materials and Methods) and 40 μl of this solution was applied to each gel. Exceptions were in (C) 2, where only 50 μg of protein was available and (C) 4, where the amount of protein taken for delipidation was 500 μg. The number used to identify a particular apoprotein was determined in the urea gel system. It represents the relative migration index (r_m), as determined by the equation

$$r_m = D_x/D_d \times 100$$

where D_x is distance (in mm) from the center of sample band to the interface of the spacer and sample gels and D_d is distance from the bromphenol blue dye band to the interface. In the apoprotein patterns of human lipoproteins run in this system, $r_m = 17$ for the arg-rich apoprotein, $r_m = 25$ for apoA-I, and $r_m = 35$ for apoA-II.

DISCUSSION

Early in this work, we found that classical ultracentrifugation procedures (12) were not adequate to separate and purify individual plasma lipoprotein classes in adult male rhesus monkeys fed a high fat, low cholesterol diet (Fig. 1). We have used an alternative procedure in which the initial separation was based on the size differences among lipoprotein classes. Several size populations of lipoproteins were obtained (Fig. 2). The characteristic electrophoretic mobility during agarose electrophoresis was monitored for individual fractions. Where indicated, size populations were subfractionated into density classes until fractions were obtained that migrated as a single band on agarose electrophoresis. One exception was the region I lipoproteins which contained both a β and pre-β band on agarose electrophoresis. No attempts to separate these bands were made because of the extremely small amounts of

this material that we could obtain from rhesus monkey plasma. In the case of region III and IV lipoproteins, further subfractionation was not necessary to achieve single band migration on agarose electrophoresis.

Based on the information given in Table 2, it was possible to relate characteristics of the six lipoprotein fractions we isolated to those that have been prepared by other techniques. Individual fractions will be discussed separately below. Both region I and II_A lipoproteins fit the classical definition of VLDL because they have a density less than 1.006 g/ml. The fact that VLDL could be separated into different size populations by the agarose column is not unprecedented. Sata, Havel, and Jones (21) have shown that a spectrum of sizes exists among human plasma VLDL which were separated by agarose chromatography. The II_A lipoproteins had a composition similar to VLDL of other species (22). However, the region I lipoproteins had a surprising compositional difference from the II_A lipoproteins; they contained more cholesteryl ester and less triglyceride. This finding, along with the β-migrating electrophoresis band and the slight increase in apoprotein bands that, by PAGE, behave as apoB and arg-rich apoprotein, suggest that these control diet-fed animals had a β-migrating VLDL with characteristics similar to that described in cholesterol-fed patas monkeys (23), dogs (24), and swine (25). The potential significance of finding such a lipoprotein fraction in control animals relates to the significance of their elevation in cholesterol-fed animals. Rather than being a unique lipoprotein class found only in pathologic states, perhaps they are usually present in the control diet-fed animals, with the elevation in cholesterol-fed animals presumably due to an increased rate of synthesis or decreased rate of catabolism.

At least two different classes that could be separated at density 1.050 g/ml were present in region II_B lipoproteins. Those with $d < 1.050$, termed the II_{B50} lipoproteins, did not exhibit true β or pre-β mobility on agarose electrophoresis but rather were intermediate in distance of migration. The II_{B50} lipoproteins were heterogeneous when examined in the analytical ultracentrifuge.[3] The chemical composition, apoprotein patterns, relative size, density range, and electrophoretic mobility are properties consistent with the possibility that the II_{B50} lipoproteins are intermediates (remnants) in the conversion of the triglyceride-rich VLDL (II_A lipoproteins) to LDL, as suggested by others (26). Kinetic studies are needed to establish the validity of such speculation.

The II_{B100} lipoproteins with $1.050 < d < 1.10$ migrated as a single pre-β band. The relatively high plasma concentration of the II_{B100} lipoproteins was one of the more unexpected findings of this study. In the plasma of two of the 14 monkeys we have examined, there were more of the II_{B100} lipoproteins than there were region III lipoproteins (Fig. 4). We are not aware of a description of such high levels in any other species, although, in plasma of other nonhuman primate species, these lipoproteins are present at easily detectable levels.[4]

Nelson and Morris (4) have recently described a lipoprotein from rhesus monkey plasma with many of the same properties of the II_{B100} lipoproteins described here. The similarities included the chemical composition, relative size measurement, and flotation rate (S_f). Some of the II_{B100} lipoproteins from our studies were sent to Dr. Nelson and an $S_f^{1.063} = 3.3$ was found, a finding consistent with their report. The protein content of the region II lipoproteins isolated by Nelson and Morris was higher than that of the II_{B100} lipoproteins of our study, but so was the protein content of the region III lipoproteins (LDL). In our experience, this result is consistent with the diet differences between the two studies, i.e., their observations were made in animals fed monkey chow, which has a low fat content (<5%) whereas our diet contained 45% of calories as fat.

Apoprotein analysis of the II_{B100} lipoproteins failed to reveal any unique apoproteins that could account for the altered composition and electrophoretic migration. In fact, the II_{B100} lipoproteins appeared to have even less of those proteins that comigrate in the polyacrylamide gel electrophoresis systems with apoA-I, apoC, or arginine-rich apoprotein than did the region II_{B50} or III lipoproteins. The similar behavior by PAGE analysis of the apoproteins of the II_{B100} and III lipoproteins, in light of the obvious differences in the intact lipoprotein particles, suggests that comparison of specific aspects of the apolipoproteins should be fertile ground for future investigation of the characteristics of the apoproteins that control LDL structure.

In rhesus monkeys the high level of the II_{B100} lipoprotein class with a density range overlapping that of LDL and HDL was probably a large part of the reason why the $1.019 < d < 1.063$ lipoproteins and the $1.063 < d < 1.225$ lipoproteins were heterogeneous and contained significant amounts of pre-β

[3] Rudel, L. L., and Nelson, C. A., unpublished observations.

[4] Rudel, L. L., unpublished observations.

lipoproteins (Fig. 1). Since the II_{B100} lipoproteins are larger than either LDL or HDL (III or IV, respectively), the column separation technique was a rapid initial preparative technique with which to obtain electrophoretically homogeneous LDL and HDL.

In studies in the patas monkey by Mahley et al. (23, 27), a lipoprotein termed LDL-II was described which has many of the same properties of II_{B100}. The authors found that small amounts of apoA-I and arg-rich apoproteins were present in LDL-II. This did not appear to be the case in the II_{B100} lipoproteins of the rhesus monkeys used in this investigation. Our results would not appear to be in agreement with their suggestion that this class of lipoproteins is the nonhuman primate counterpart of HDL_c, which these workers have described in dogs (24) and swine (25), if rhesus monkey II_{B100} lipoproteins are similar to the patas LDL-II. If there is a counterpart to LDL-II among rhesus monkey lipoproteins, it would appear to be included in the II_{B50} lipoprotein fraction.

One final comment can be made about II_{B100} lipoproteins. This class appears to share many of the characteristics of the lipoproteins carrying the Lp(a) antigen, which have been isolated and characterized from human plasma by Ehnholm et al. (28). A possible association between increased levels of Lp(a) lipoproteins and increased risk to premature coronary heart disease has been described (29). Rhesus monkeys with high levels of II_{B100} lipoproteins would appear to be valuable experimental models to test this relationship if the apparent similarities between II_{B100} lipoproteins and Lp(a) prove to be real.

The region III lipoproteins are a single lipoprotein class as judged by their β-migration during agarose electrophoresis, and are low density lipoproteins, $1.019 < d < 1.063$. The region III lipoproteins are the major ones of at least three classes of lipoproteins having densities in this range. Heterogeneity within the LDL density range is not unprecedented in other primates (16, 30). The relative size index (r_I) of the region III lipoproteins (LDL) of the rhesus monkeys in the present study was 1.00 (Table 2). Based on the regression equation for the relationship of LDL r_I and the log of molecular weight (16), the LDL molecular weight in the present study was 3.1×10^6. For reasons not known to us, the equation for the established relationship between LDL molecular weight and r_I cannot be applied to the region II_{B100} and IV lipoproteins; the molecular weights are grossly overestimated by use of the equation. The reason presumably relates to the increased protein to lipid ratios of these particles and to three-dimensional configurations within these lipoprotein particles that are different from those of LDL. The relative elution position of lipoprotein fractions other than region III lipoproteins was shown in Table 2 only as a reference point for other studies.

The lipoproteins of region IV were within a single lipoprotein class characterized by α-migration during agarose electrophoresis, and were high density lipoproteins, $1.063 < d < 1.225$. We have not clearly established the existence of the separate subclasses, HDL_2 and HDL_3, although chemical analysis of the larger vs. the smaller of the region IV lipoproteins (Table 3) would be consistent with the possibility that subclasses are present. HDL_2 and HDL_3 in rhesus monkey plasma have been described previously (5).

In the present study the high fat diet, rather than monkey chow, was used in an attempt to simulate the diet of most human beings at risk for complications of premature coronary atherosclerosis. The lipoprotein cholesterol distribution presented here (Table 1) was comparable to that presented by Lee and Morris (3) for rhesus monkeys fed monkey chow, although some difference in LDL composition was suggested. The high levels of HDL cholesterol and low LDL/HDL ratios generally are not found in human beings with comparable plasma cholesterol concentrations. In consideration of the usefulness of rhesus monkeys as models for human atherosclerosis research, the rather large difference in the LDL/HDL ratio should be taken into account, as it has been implicated as being an important factor in human atherosclerosis (31). Another characteristic difference in lipoprotein distribution of rhesus monkeys compared to that in human beings is the low plasma VLDL and triglyceride concentration. The mean plasma triglyceride level in the present study was 23 mg/dl, and the VLDL cholesterol levels were below 5 mg/dl. Normal human levels are 60–100 mg/dl and 12–20 mg/dl, respectively (32). The impact of such differences on atherosclerosis research is unknown; however, they clearly define a potential difference in plasma atherogenicity and should be taken into account.

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