Characterization of plasma low density lipoproteins of nonhuman primates fed dietary cholesterol[1]

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Abstract LDL from animals of three nonhuman primate species, *Macaca mulatta, Macaca fascicularis*, and *Cercopithecus aethiops*, were studied. A standard preparation of ^{125}I-LDL was added to isolated lipoprotein mixtures just prior to separation of plasma lipoproteins by agarose gel chromatography. A relative size index, r_I, was determined by dividing the elution volume of the iodinated LDL by the elution volume of the sample LDL, both volumes being determined simultaneously during chromatographic elution. Comparison of r_I with molecular weights measured by flotation equilibrium analysis in the analytical ultracentrifuge showed a linear relationship across a molecular weight range of $2.5–8.0 \times 10^6$, $r = 0.985$. A regression equation describing this relationship was used to calculate molecular weights of LDL from a group of *M. fascicularis* that were fed cholesterol-containing diets. In these animals, plasma cholesterol concentration ranged from 100 to over 700 mg/dl and was highly correlated with LDL molecular weight and with the micromolar concentration of the LDL. Using multiple regression analyses, the two variables of plasma LDL could be shown to account for 94% of the variation in plasma cholesterol concentration in the *M. fascicularis* of this study. Micromolar concentration and molecular weight of LDL were not correlated with each other, suggesting that in *M. fascicularis* at least two independent types of controls are operative in the response of plasma LDL to dietary cholesterol. The increase in LDL molecular weight was associated with a large increase in cholesteryl ester content and concomitant smaller increases in protein, phospholipid, and free cholesterol. As molecular weight increased, these components appeared to be added to the LDL particles together as discrete increments of fixed composition. The data are consistent with a spherical model of LDL structure with a core of cholesteryl ester and triglyceride and a 21.3 Å-thick coat of phospholipid, free cholesterol, and protein.

Supplementary key words agarose gel chromatography · analytical ultracentrifugation · ^{125}I-LDL · LDL molecular weight · cholesteryl ester · plasma cholesterol · rhesus monkeys (*Macaca mulatta*) · grivet monkeys (*Ceropithecus aethiops*) · cynomolgus macaques (*Macaca fascicularis*)

Low density lipoproteins have been extensively studied because of the apparent correlation between increased concentrations of these lipoproteins and premature coronary atherosclerosis in man and in experimental animals. Classically, the lipoproteins have been separated on the basis of density and have been named accordingly; thus, the name low density lipoproteins (LDL) refers to the group of macromolecules that float in the preparative ultracentrifuge in the density range 1.019–1.063 g/ml (1). However, studies in experimental animals (2–4) and in humans (5, 6) have indicated that heterogeneity exists among the lipoproteins in this density range. Such heterogeneity probably reflects structurally distinct subclasses of LDL and may indicate the presence of metabolic heterogeneity resulting from LDL synthesis or catabolism.

We have been among those investigators to utilize agarose gel chromatography to separate and analyze plasma lipoproteins (7–10). In general, the lipoprotein fractions purified by gel filtration exhibit the same properties as those isolated by preparative ultracentrifugation (11). We have used gel chromatography to analyze the effects of dietary cholesterol on plasma lipoproteins in several nonhuman primate species (12, 13). A consistent finding has been marked size heterogeneity among LDL of different animals. The present paper describes the method we have developed to monitor molecular weight during preparation of LDL using agarose gel chromatography; it provides further information on the properties of the LDL of cholesterol-fed nonhuman primates as these properties are defined by analytical ultracentrifugation and gel chromatography. Strong rela-

Abbreviations: EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoproteins; ILDL, intermediate-sized low density lipoproteins; LDL, low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate: S_f, Svedburg flotation constant; TCA, trichloroacetic acid; V_e, elution volume; VLDL, very low density lipoproteins; V_t, total column volume.

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tionships between plasma cholesterol concentration and the size and molar concentration of LDL were identified.

METHODS AND MATERIALS

Male and female rhesus monkeys, (*Macaca mulatta*) fed semipurified diets containing 45% of calories as lard and 0.05 or 0.3 mg cholesterol/kcal, male cynomolgus macaques (*M. fascicularis*) fed semipurified diets containing 45% of calories as mixed fats (equal proportions of crisco, lard, and corn oil), and 0.05 or 0.6 mg cholesterol/kcal, and male grivets (*Cercopithecus aethiops*) fed a semipurified diet containing 45% of calories as mixed fats and 0.75 mg cholesterol/kcal were studied. These groups were selected for analyses to give a range of LDL sizes and molecular weights. Animals were fasted for 18–24 hr prior to blood collection. Blood was drawn into tubes containing Na_2EDTA, final concentration 1 mg/ml, and plasma was promptly isolated. The solvent density of plasma was increased to d 1.225 with solid KBr, and lipoproteins were isolated by preparative ultracentrifugation and separated by agarose gel chromatography on 4% agarose (Bio-Gel A-15m, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA) as described by Rudel et al. (11). Elution was continuously monitored at 280 nm as percent transmission and fractions were collected at 20 or 30 min intervals.

The material eluted from the column in the LDL peaks was combined for further analysis. Agarose electrophoresis was carried out according to the method of Noble (14), and only β-migrating material was found in the LDL peak from agarose gel chromatography for animals of each of the diet groups of this study. Immunologic evaluation by immunodiffusion (15) of many LDL samples revealed a single precipitin band against anti-LDL and anti-whole serum, and no cross-reaction with anti-HDL.

All anti-sera were prepared in our laboratory in rabbits using antigens isolated from rhesus and African green monkeys. Cholesterol determinations were performed according to the method of Rudel and Morris (16). Phospholipid phosphorus was assayed (17) after perchloric acid digestion and protein was determined by the method of Lowry et al. (18) using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as the standard. Lipids were extracted in chloroform–methanol 2:1, and the phases split with 1 part of dilute H_2SO_4 to 20 parts of extract. Aliquots of the lower layer were applied to silica gel H thin–layer chromatography plates and lipids were separated using hexane–ethyl ether–glacial acetic acid 70:30:1. Spots were visualized using iodine vapor and marked. Triglyceride, cholesteryl ester, and free cholesterol spots were scraped off and extracted with chloroform–methanol 2:1. Triglycerides were assayed by the method of Sardesai and Manning (19); free and ester cholesterol were assayed as indicated above. Total LDL concentrations were calculated after summing the individual constituents. Statistical evaluations were performed as indicated throughout this study based on the statistical methods described by Snedecor and Cochran (20).

LDL fractions from the column elution were dialyzed against deionized glass-distilled water containing 0.01% Na_2EDTA, pH 7.4. Dialyzed fractions were then lyophilized and subsequently extracted for several hours in chloroform–methanol 2:1. An equal portion of ether was then added to the chloroform–methanol, and the protein was separated by centrifugation. This extraction procedure was then repeated twice to remove all lipid, after which the white protein precipitate was dried under N_2 and stored in a vacuum dessicator. For SDS PAGE the protein was redissolved in 0.06 M $(NH_4)_2\ CO_3$, 8.3 mM SDS, pH 8.1, to a final concentration of about 1 mg/ml. SDS PAGE was carried out in a horizontal slab containing 10% and 5.0% polyacrylamide in the running and spacer gels, respectively. The buffer system used was that of Laemmli (21). Gels were fixed and stained overnight in 50% methanol, 5% acetic acid containing 0.12% Coomasie blue dye. Destaining was carried out for 1–2 days in several changes of 7.5% acetic acid. For PAGE in 8 M urea, apolipoproteins were dissolved in 10 M urea, 2.5 mM sodium decyl sulfate, 0.2 M Tris, pH 8.2, and the concentration of sodium decyl sulfate was then reduced to 1.0 mM by dialysis. Final protein concentration was about 1 mg/ml. Electrophoresis on 7.5% polyacrylamide gels, 5 mm in diameter, containing 8 M urea, was then carried out essentially as described by Davis (22). Gels were fixed and stained overnight in 10% TCA, 10% sulfosalicylic acid, 0.1% Coomasie blue, and were destained in 10% TCA. To quantitate the amount of apo-B versus other apolipoproteins in the samples, separate lyophilized, delipidated apolipoprotein preparations were mixed with 1 part of 0.01 M Tris, 8 M urea, pH 8.9, and 1 part of tetramethyl urea, and then allowed to stand overnight. The protein solubilized by this solution was measured. Since the apo-B is insoluble in tetramethyl urea (23), the percent of apo-B was then calculated after subtracting the amount of soluble protein from the total amount in the tube. In the samples of LDL from *M. fascic-*

ularis in this study, the measured values for apo-B were from 93 to 97%.

For iodination, LDL from plasma of rhesus monkeys fed a diet containing 45% of calories as fat and 0.05 mg cholesterol/kcal were prepared by agarose gel chromatography (11) and then were concentrated and repurified by flotation in the preparative ultracentrifuge at a density of 1.063 g/ml. The concentrated LDL was dialyzed against 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.4. LDL protein concentration was 6–8 mg/ml. The concentrated LDL (usually 2–3 ml) was then mixed at 4°C with equal parts of 1 M glycine buffer, pH 10, containing ^{125}I (purchased as the sodium salt, New England Nuclear, Boston, MA) and then with iodine monochloride prepared as described by MacFarlane (24). The estimated molecular weight of LDL protein was 250,000 and enough ICl was added to attach no more than 1 mole of iodine per mole of protein. The mixture was quickly transferred to a dialysis bag, and dialyzed against 0.14 M NaI, 0.01 M phosphate buffer, pH 7.4, for 24 hr at 4°C. Final purification was achieved by gel chromatography on a Bio-Gel A-15 m (4%) column using 0.15 M NaCl, 0.01% EDTA, pH 7.4, as the eluant. The final preparation routinely contained less than 1% free iodine and greater than 98% of the ^{125}I on the protein. Specific activities were 50,000–100,000 cpm/μg protein. ^{125}I-LDL was stored at 4°C in 0.15 M NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4, at a concentration of 0.1–0.5 mg protein/ml and was stable for several months.

Relative constancy among ^{125}I-LDL preparations was maintained each time by preparation of LDL from the pooled plasma of the same group of five donor monkeys. Careful control of the length of the fasting period before blood collection was essential. Each preparation was standardized by comparison with the previous reference standard; little variation was observed. A total of four separate preparations have been used over a period of 18 months.

The ^{125}I-LDL was used as an internal standard for determination of LDL size during chromatography. To each lipoprotein sample applied to the column, an aliquot of ^{125}I-LDL containing less than 10 μg of LDL protein was added as an elution marker for relative size measurement. The radioactivity in individual fractions collected during elution was measured, and the ^{125}I-LDL elution profile was recorded. The elution volume at the center of the ^{125}I-LDL peak, V_{el}, divided by the observed elution volume at the center of the percent transmission peak for the sample, V_{es}, was termed the relative size index, r_I, and was calculated for each individual LDL sample. In this way, the ^{125}I-LDL served as an internal marker for each column separation and any variations between runs were self-correcting. The relative constancy of elution of marker ^{125}I-LDL was excellent, e.g., 19 observations on one column over a four-month period yielded a V_{el} = 116.3 ± 0.30 ml, mean ± SEM.

TABLE 1. Comparison of different column packings on ^{125}I-LDL elution

Agarose[a] Preparation	N	V_{el}/V_t (Mean ± SD)	Significance[b]
No. 33	24	0.6446 ± 0.0069	$P > 0.10$
No. 33	16	0.6485 ± 0.0077	$P < 0.001$
No. 93	9	0.6150 ± 0.0045	$P < 0.001$

[a] Bio-Gel A-15m, Bio-Rad Laboratories.
[b] Based on Student's t test (20).

We became aware early in this work that significant variation in lipoprotein separation occurred among different lots of commercial agarose beads. **Table 1** shows a comparison of V_{el}/V_t for the marker ^{125}I-LDL on two columns poured with the same lot number of Bio-Gel A-15m compared to a column containing agarose of a different lot number. Significant differences existed between columns of agarose with different lot numbers, indicating a difference in behavior of ^{125}I-LDL on these two agarose preparations. On the other hand, when agarose gels with different lot numbers have been compared, agreement between r_I and molecular weight of LDL has been excellent. For instance, the data in Fig. 4 include r_I values derived from four different column packings. Thus, another advantage to the use of the ^{125}I-LDL marker to determine relative LDL size was that the problem of different batches of agarose beads having slightly different elution behavior was overcome. This is probably due to the fact that the marker ^{125}I-LDL has to penetrate the gel during chromatography and elutes in a position relatively unaffected by gel differences. In our experience, the regions in chromatographic separations most affected by agarose gel differences are near V_o and V_t.

High salt flotation velocity experiments were carried out on LDL samples obtained from the middle of the LDL elution peak in a manner similar to the methods used by Fisher, Granade, and Mauldin (25). Samples were concentrated by vacuum dialysis, then dialyzed overnight against KBr solutions either containing 0.1 M Tris-Cl and 10^{-4} M EDTA at pH 7.3 or simply EDTA adjusted to pH 7.2. The density of the solvent was determined with a Westphal balance, occasionally checked by pycnometry. A double sector cell was filled with 0.4 ml of dialysate on one side and 0.4 ml of dialyzed protein

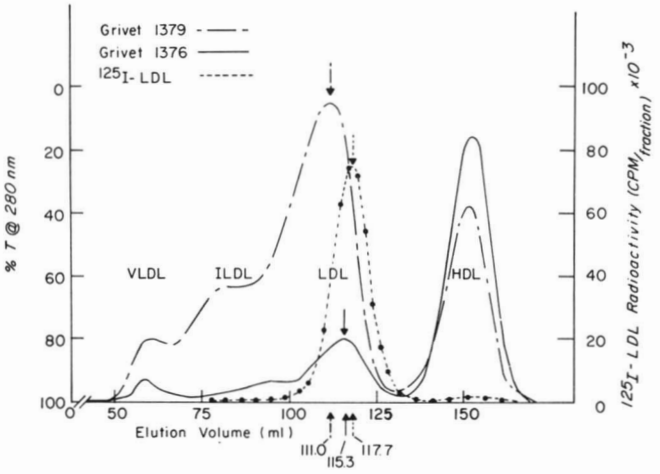

Fig. 1. Agarose gel chromatography elution profiles of plasma lipoproteins isolated from two grivets, *Cercopithecus aethiops*, also showing the position of the marker ^{125}I-LDL added to the sample prior to sample application on the column. The centers of the LDL peaks are indicated by the arrows, and elution volumes (V_e) are indicated. Lipoproteins isolated from 10 ml of plasma were applied to the column; thus 73 mg of lipoprotein cholesterol was applied for grivet No. 1379 and 16 mg was applied for grivet No. 1376. Fractions were collected at 4°C at 20 min intervals and contained about 2.5 ml of eluant per fraction. Turbidity in the VLDL fractions was not corrected for.

on the other; an identical wedge cell contained another sample so that two solutions could be run simultaneously. Flotation was carried out at 25° at 42,040 rpm in a Beckman/Spinco analytical ultracentrifuge (Spinco Div., Palo Alto, CA). Photographs taken at 4–16 min intervals were measured and flotation rates were determined by standard procedures. Flotation equilibrium was carried out as previously described (26) on LDL samples obtained from the middle of the LDL elution peak. Partial specific volumes for individual samples were determined from the variation of S_f with solvent density (25).

RESULTS

Typical elution profiles of lipoproteins separated by agarose gel chromatography are shown in **Fig. 1.** The position of the ^{125}I-LDL has been fixed, and the profiles of two separate animals have been superimposed to illustrate the differences in relative size that are typically seen during elution. In this example, No. 1379 had a plasma cholesterol concentration at the time of study of 732 mg/dl and No. 1376 had a concentration of 158 mg/dl. The elution volumes at the center of the LDL peak for these two animals were different from each other and were different from the ^{125}I-LDL, indicating the size heterogeneity between these samples. In four grivets intermediate to these in plasma cholesterol concentrations (mean 438 mg/dl), LDL eluted from the column between those of No. 1376 and No. 1379 (see Table 2). Such a finding, apparently related to plasma cholesterol concentration, prompted us to further examine this phenomenon using the analytical ultracentrifuge.

The schlieren patterns shown in **Fig. 2** compare the flotation patterns of LDL of grivets No. 1379 and No. 1376 at two densities, 1.063 g/ml and 1.2 g/ml. At each density, the flotation rate for LDL from No. 1379 was higher than for LDL of No. 1376, and the flotation equilibrium molecular weights were 4.5 and 3.6×10^6, respectively (see **Table 2**). The average molecular weight of the LDL of the other four grivets was 4.0×10^6, a value intermediate to those of No. 1376 and No. 1379 as was the case for r_I. These findings suggested to us that the preparative chromatographic procedure could at the same time yield analytical information about LDL size and we extended our observations into larger monkey populations available to us in which the range of plasma cholesterol concentrations could be expected to be quite variable.

A population of 20 male *M. fascicularis* was studied. **Fig. 3** shows a plot of the relative size of LDL versus the whole plasma cholesterol concentration and the LDL cholesterol concentration. In both cases, a statistically significant positive correlation coefficient was found. These results suggested that the variation in LDL size was related to the increased whole plasma and LDL cholesterol concentrations induced by dietary cholesterol in *M. fascicularis*. A similar

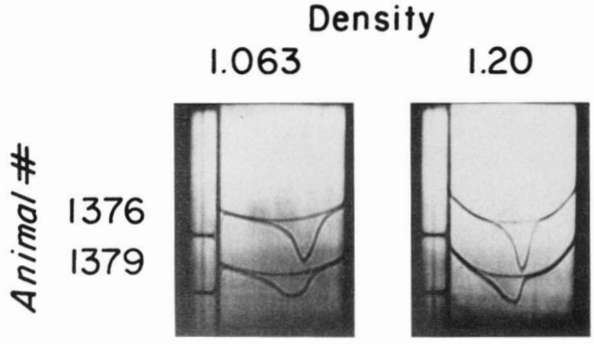

Fig. 2. Analytical ultracentrifuge flotation velocity patterns of LDL from grivets No. 1376 and No. 1379. Material from the center of the LDL peak obtained during preparative agarose gel chromatography was analyzed. On the left are the patterns obtained at 40 min after reaching speed at a solvent density of 1.063 g/ml. On the right are the patterns obtained at 12 min after reaching speed at a solvent density of 1.2 g/ml. Flotation is from right to left at 25°C and the schlieren bar angle is 60°. For No. 1376, $S_{f1.063} = 10.1$ and $S_{f1,2} = 46$; for No. 1379 $S_{f1.063} = 12.1$, and $S_{f1,2} = 51$. In addition, heterogeneity of LDL is clearly apparent in the LDL from No. 1379; that of No. 1376 is relatively homogeneous.

TABLE 2. Comparison of plasma cholesterol concentration and LDL size in grivets (*C. Aethiops*)

Animal	Plasma Cholesterol	Properties of LDL	
		r_I	Mol wt
	mg/dl		($\times 10^{05}$)
1376	158	1.021	3.60
1377	399	1.039	4.01
1378	428	1.033	4.11
1380	455	1.041	3.86
1375	470	1.044	4.06
1379	732	1.059	4.50

conclusion was drawn from the studies of the small population of grivets (Table 2).

More information about the LDL particle was necessary in order to provide an explanation for this relationship. Molecular weights of LDL could be determined during chromatographic elution if the relative size increase seen in response to dietary cholesterol was proportional to a molecular weight increase. The grivet data (Table 2) suggested that molecular weight could be determined in this way. Accordingly, plasma samples from grivets and cynomolgus macaques throughout the observed plasma cholesterol

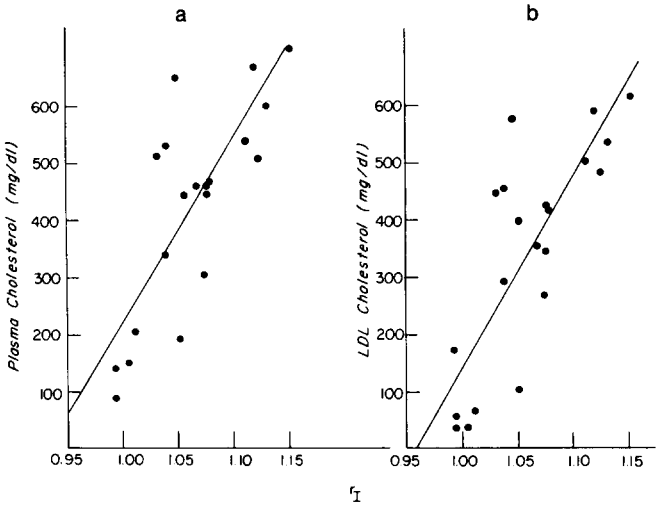

Fig. 3. Comparison of the variation in plasma cholesterol concentration (a) and of LDL cholesterol concentration (b) with that of relative size (r_I) as measured by agarose gel chromatography using marker ^{125}I-LDL, where $r_I = V_e(^{125}\text{I-LDL})/V_e$ (sample LDL). Twenty male *M. fascicularis* monkeys were studied for 16 months; 16 were fed a diet containing 0.5 mg cholesterol/kcal, four were fed a diet containing 0.05 mg cholesterol/kcal. The means of the bimonthly observations on plasma cholesterol concentration are represented here. LDL was isolated by agarose gel chromatography from each animal three times during the study period, and the means of LDL size and LDL cholesterol concentration of these three determinations are represented. The correlation coefficient between relative LDL size, r_I, and plasma cholesterol concentration was 0.78, and for r_I vs. LDL cholesterol concentration was also 0.78, both values being significant at the $P < 0.001$ level.

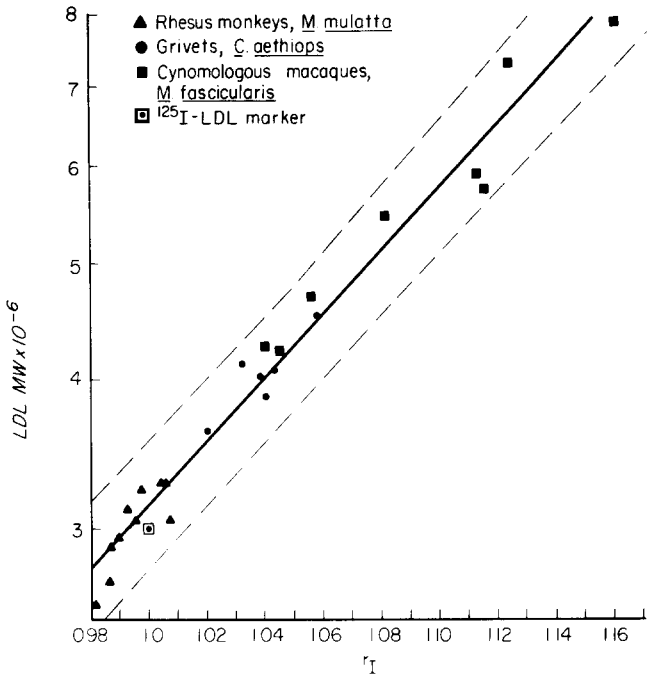

Fig. 4. Comparison of relative LDL size (r_I) as determined by agarose gel chromatography using marker ^{125}I-LDL with molecular weight as determined by flotation equilibrium analysis in the analytical ultracentrifuge. LDL samples isolated from the center of the elution peaks of agarose gel chromatography were used for molecular weight determinations. As indicated, samples from 25 animals representing three species were compared, as was the ^{125}I-LDL used as the marker during gel chromatography. The correlation coefficient was 0.985, and the 90% confidence limits are indicated by the dotted lines, with all data points falling well within this range.

concentration range were then selected for determination of LDL r_I and molecular weight. In addition, samples from rhesus monkeys fed low cholesterol-containing diets were also evaluated. These animals had lower plasma cholesterol concentrations than did the grivets or cynomolgus macaques of this study and were chosen for study based on the assumption that their LDL size would be lower.

Fig. 4 shows the results. A molecular weight range of $2.5–8.0 \times 10^6$ was covered, and the size of LDL expressed as the relative size index, r_I, was highly correlated with the log of molecular weight ($r = 0.987$, $P < 0.001$). This relationship was found to be comparable for each of the three species included in the summary graph shown in Fig. 4. Each species defined a separate segment of the line although overlap between species occurred. The molecular weights exceeding 6×10^6 showed maximum variation from the line, perhaps reflecting the marked heterogeneity in the analytical ultracentrifuge schlieren patterns. The r_I values found for LDL from three normal human beings were 0.992, 1.0, and 1.0, which were

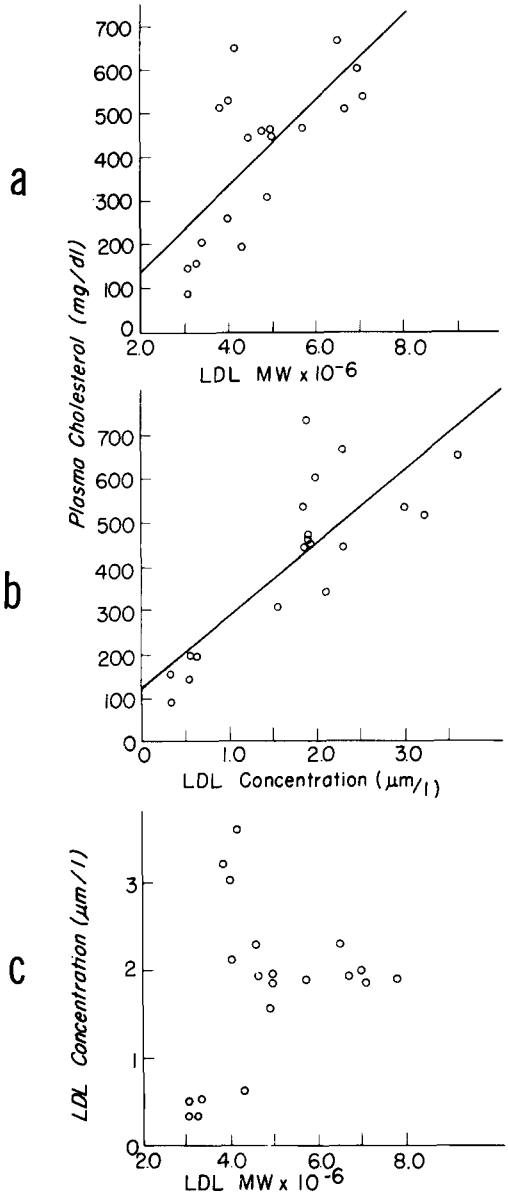

Fig. 5. Relationships between plasma cholesterol concentration and LDL molecular weight (a) and micromolar concentration of LDL (b) and between LDL molecular weight and micromolar concentration (c). Data from the same 20 *M. fascicularis* as described in Fig. 3 are presented. The correlation coefficients and significance levels were (a) r = 0.75, $P < 0.001$, (b) r = 0.81, $P < 0.001$, and (c) r = 0.29, $P > 0.1$.

equivalent to molecular weights near 3×10^6, based on equation (1). These values are in good agreement with published mean values of Nelson et al. (26) and Fisher, Hammond, and Warmke (27), of 2.87×10^6 and 2.95×10^6, respectively, determined by analytical ultracentrifugation and that of Margolis (7) using agarose gel chromatography, 3×10^6.

The relative size index, r_I, was compared to the log of the molecular weight since column elution measurements have generally been shown to be logarithmically related to molecular weight (28). The equation that defines the line described by our data is as follows:

$$\log \text{Mol wt} = 2.6215 r_I - 2.127 \qquad \text{Eq. 1}$$

This equation predicted a molecular weight of the ^{125}I-LDL used in these studies of 3.12×10^6 ($r_I = 1.0$) whereas the observed value was 3.0×10^6. This error is well within the limits of accuracy of either column chromatography or analytical ultracentrifugation.

Interestingly, a linear relationship between the observed molecular weights and r_I could be shown to give an equally satisfactory fit to the data, as defined by the equation:

$$\text{Mol wt} = 27.94\ (r_I) - 24.86 \qquad \text{Eq. 2}$$

A least squares best fit of this line was not significantly different from that of the semi-log plot as shown in Fig. 4. This apparently was due to the fact that a very narrow range of sizes is covered by the data, compared to the overall size-separation capability of the column.

Using Equation 1, the molecular weights of LDL for the same 20 *M. fascicularis* represented in Fig. 3 were calculated. A significant correlation was found between molecular weight of LDL and plasma cholesterol concentration, as shown in **Fig. 5a** ($r = 0.75$, $P < 0.001$). The micromolar concentration was calculated using the molecular weights and total LDL concentrations determined after column chromatography. A significant correlation between μmoles/l of LDL and plasma cholesterol concentration was also found, see Fig. 5b ($r = 0.81$, $P < 0.001$). On the other hand, no significant correlation between μmolar concentration of LDL and mol wt of LDL was found, Fig. 5c ($r = 0.29$, $P > 0.10$), indicating that concentration and molecular weight were independent variables, although both were related to plasma cholesterol concentrations. When multiple regression analysis was applied to these data, it was found that 94% of the variation in plasma cholesterol concentration could be accounted for by these two variables of LDL.

The fact that both micromolar concentration and molecular weight of LDL were highly correlated with plasma cholesterol concentrations, while at the same time being independent of each other, indicated that dietary cholesterol exerted at least two separate effects on LDL metabolism. The increase in micromolar concentration, viewed as an increase in the number of LDL molecules per unit of plasma volume, was anticipated. However, the effect on molecular weight, separate from the increase in LDL concentra-

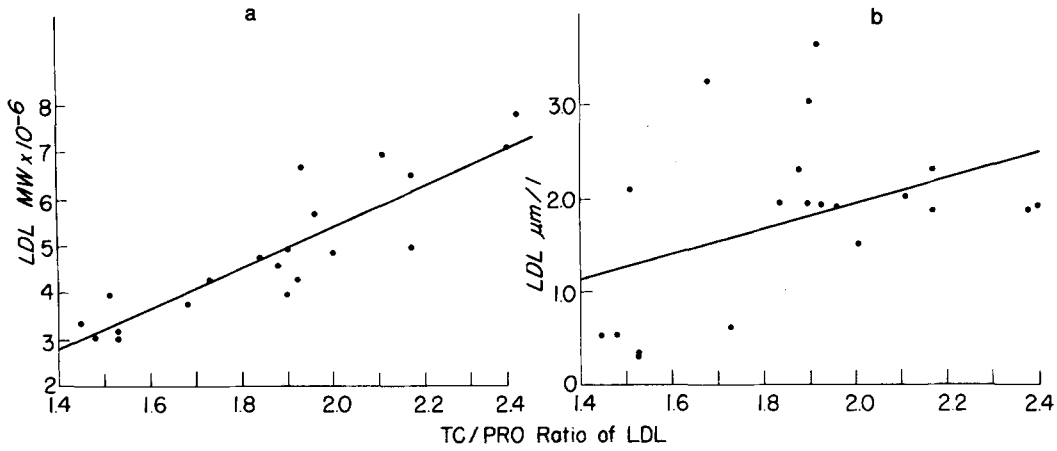

Fig. 6. Relationship between the total cholesterol to protein ratio for LDL compared to LDL molecular weight (a) and micromolar concentration of LDL (b). Data from the same 20 *M. fascicularis* as described for Fig. 3 are represented. The correlation coefficient for (a), r = 0.86 was significant ($P < 0.001$) and for (b) r = 0.48 was just barely significant ($P = 0.05$).

tion, was more unexpected. In an attempt to provide insight into this finding, we examined the chemistry of the LDL particles. One of the most sensitive measurements that can be used to detect modified chemical composition in lipoproteins is the total cholesterol to protein ratio. We determined that the total cholesterol/protein ratio was highly correlated, $r = 0.863$, $P < 0.001$, with molecular weight (**Fig. 6a**), while the correlation with LDL concentration, $r = 0.477$, $P = 0.05$ just reached significance. This indicated that LDL of higher molecular weight were relatively cholesterol enriched, when compared to protein content.

When the percentage composition of LDL of increasing molecular weights was examined **(Table 3),** cholesteryl ester was the only component found to be increased. The percentage of LDL protein actually decreased. When the within-particle composition was calculated (as g/μmole of component) and compared with molecular weight **(Fig. 7),** it was seen that the cholesteryl ester content of LDL increased to the greatest extent. The g/μmole of protein, phospholipid, and free cholesterol in LDL also increased as molecular weight increased, but the magnitude of this increase was considerably less than for that of cholesteryl ester. Triglyceride content did not change with molecular weight and was lower than for any other component, 0.115 ± 0.012 g/μmole, mean ± SEM. These data show that a molecular weight increase in LDL of *M. fascicularis* in response to dietary cholesterol may be the result of an increased cholesteryl ester content of these lipoprotein particles, which occurred concomitantly with increases in protein, phospholipid, and free cholesterol.

Since the protein content (measured as g/μmole of LDL) increased as LDL molecular weight increased, we examined the apolipoprotein composition. **Fig. 8** shows the gel electrophoresis patterns arranged in order of increasing LDL molecular weight. Major qualitative changes among apolipoproteins of LDL did not occur in response to dietary cholesterol in *M. fascicularis*, although the molecular weight range covers $3-7.8 \times 10^6$. A small amount of protein migrated in the position where the arginine-rich apolipoprotein normally is found, and some between-animal heterogeneity is apparent in this region. Detectable amounts of protein are seen at the ion front and probably represent apo-C. The majority of the protein remained in the sample well and at or near the interface between the spacer gel and the running gel, which is behavior typical of apo-B. These data, in combination with the findings that the tetramethyl urea-insoluble apolipoprotein was 93–97% of the total apolipoprotein and did not vary proportionally with LDL molecular weight, suggest that the increase in protein content of LDL that was proportional to molecular weight represented primarily an increase in apo-B.

TABLE 3. Chemical composition of LDL isolated from male *M. fascicularis*

Mol wt	PL	FC	CE	TG	Pro
($\times 10^{-6}$)	*% of total lipoprotein*				
3.17 ± 0.08	21.67[a] ± 0.73	8.59 ± 0.11	42.98 ± 0.10	3.93 ± 0.76	22.74 ± 0.32
4.63 ± 0.12	19.54 ± 0.30	8.83 ± 0.30	49.10 ± 0.87	2.34 ± 0.44	20.23 ± 0.50
6.81 ± 0.14	19.86 ± 0.16	8.41 ± 0.13	51.06 ± 0.59	2.22 ± 0.58	18.45 ± 0.33

[a] Values represent mean ± SD for triplicate determinations on each of four animals.

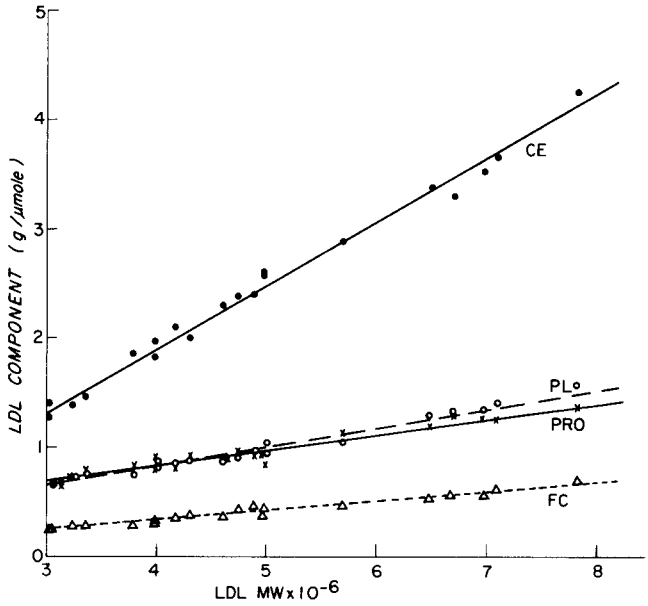

Fig. 7. Comparison of the relationships between LDL molecular weight and the within-particle content of individual components of LDL. Data for LDL of all 20 *M. fascicularis* were used to construct this graph. Molecular weights were determined by column chromatography according to the relationship described in Fig. 4 and the g/μmole of each component was determined from the percentage composition for each LDL preparation. The equations for the lines were: CE = 0.589 MW − 0.457; Pro = 0.140 MW + 0.273; PL = 0.177 MW + 0.118; and FC = 0.087 MW − 0.012, where the amount of component is in g/μmole and MW is in mol wt units × 10^{-6}.

DISCUSSION

The hyperlipoproteinemic response to dietary cholesterol in most nonhuman primates has been identified as an increase in plasma LDL cholesterol concentration (for review see ref. 29). We have further examined this phenomenon in three species of nonhuman primates. Within each species, a wide variation in the response of plasma cholesterol concentration to dietary cholesterol occurred among individuals. Furthermore, when plasma lipoproteins from individual animals were separated by gel chromatography, a heterogeneity in LDL size became apparent that was correlated with plasma cholesterol concentration. In addition, species differences in the relationship between LDL size and plasma cholesterol concentrations were also found. In the *M. fascicularis* of this study, plasma cholesterol concentrations ranged as high as 733 mg/dl, and LDL molecular weights were as high as 7.8 × 10^6. The LDL size response in other macaques, namely *M. mulatta* (30), and *M. nemestrina* (29), appears comparable to the *M. fascicularis* of this study. In contrast to these Asian monkeys, the LDL size response of the African green monkeys (grivets) was much more subtle. The highest plasma cholesterol concentration was comparable to that of *M. fascicularis*, 732 mg/dl, but the maximum LDL molecular weight was 4.5 × 10^6.

In order to accurately quantitate the size modification of LDL induced by dietary cholesterol, we developed a technique of size measurement during preparative gel chromatography that employed a standard ^{125}I-LDL preparation. The choice of ^{125}I-LDL as a marker was made for several reasons: *1*) it was relatively easy to prepare and was stable during subsequent storage; *2*) its elution profile was easy to detect even though only microgram quantities were needed; *3*) its size was near the median of the lipoproteins under study; and *4*) its behavior during agarose gel chromatography was predictable and reproducible. To validate this method of size determination and to convert the relative size index, r_I, to molecular weight, we made comparisons with molecular weight determinations performed by sedimentation equilibrium analysis on the analytical ultracentrifuge (26). The data in Fig. 4 show that agreement between these two methods is excellent. The limitations of the measurement of relative size by gel chromatography are delineated in Fig. 4 by the 90% confidence intervals. In general, molecular weights determined from agarose column elution are accurate to within ±10%. It is possible that small but significant differences in LDL size among control animals may be missed by the chromatographic procedure. In animals fed cholesterol-free diets, between-animal variation in molecular weight is present although the differences among animals are relatively small and are not apparently correlated with plasma cholesterol concentration.

By using regression Equation 1 to calculate the molecular weight of LDL for each of the 20 male *M. fascicularis*, we were able to show significant correlations when both LDL molecular weight and μmolar concentration were compared to plasma cholesterol concentration. Together these two variables accounted for 94% of the variation of plasma cholesterol concentration. This latter calculation was possible since the μmolar concentration of LDL was not significantly correlated with molecular weight, indicating that these responses of LDL to dietary cholesterol in *M. fascicularis* were apparently independent of each other. Such a finding suggests the possibility that at least two separate types of controls are operative in the response of plasma LDL to dietary cholesterol.

Speculation about the significance of this possibility seems in order since little is known about the physiologic control of plasma LDL concentration. The results of the present experiments seem to offer some

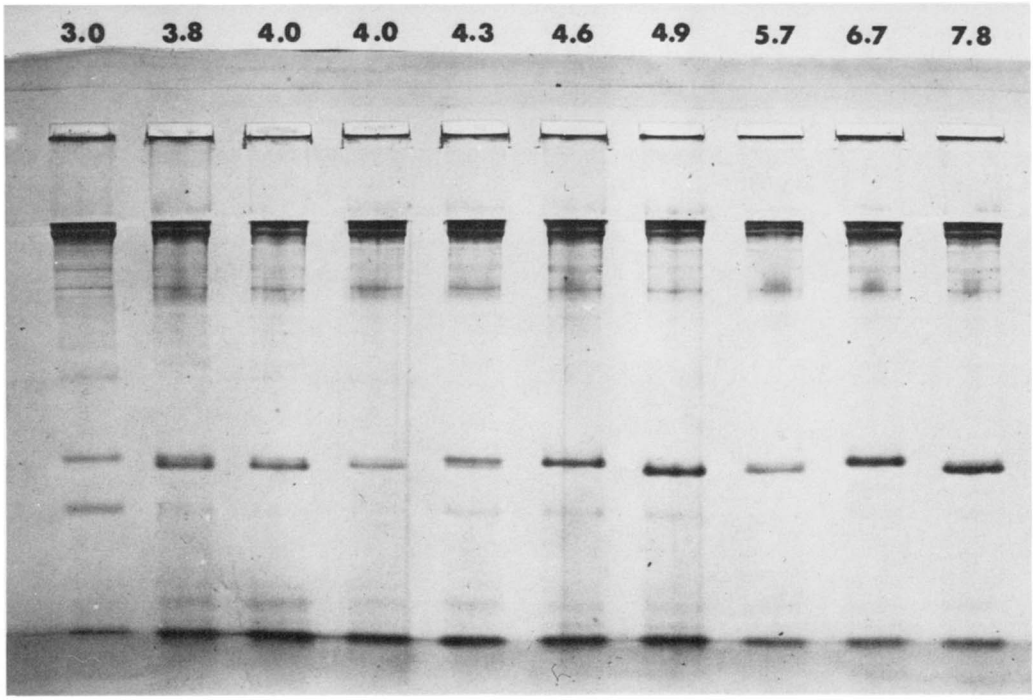

Fig. 8. Demonstration of the SDS–polyacrylamide gel electrophoresis patterns of apolipoproteins isolated from LDL of 10 *M. fascicularis*. The molecular weight (mol wt units × 10^{-6}) of the intact LDL is shown for each sample at the top. Between 40 and 50 μg of protein was applied for each sample, which represents about 2–3 times the amount of protein usually applied. The overloading was done purposely to illustrate the distribution of nonapo-B apolipoproteins of LDL, which represent less than 7% of total protein in all samples.

insight that has not been previously available. In this discussion, we will assume that, by increasing dietary cholesterol, an increased demand to transport cholesterol has occurred and this primarily affects LDL synthesis. Such an assumption is consistent with our kinetic analyses of LDL turnover in rhesus monkeys fed cholesterol (31), which indicated that the absolute rate of LDL catabolism was comparable among animals in which plasma cholesterol concentrations varied. It now seems well established that the primary route of LDL "synthesis" is via VLDL catabolism (32) and we will assume this is the case. We will also assume that the apoprotein of major physiologic importance in LDL is apo-B. Essentially all of the isolated intact LDL migrated in the β position on agarose electrophoresis; thus, we feel that the presence of significant amounts of HDL_c, as described in cholesterol-fed swine by Mahley et al. (4), in our LDL preparations is unlikely. With these assumptions in mind, the following possibilities are discussed.

In animals that increase the micromolar concentration of LDL in response to dietary cholesterol, greater numbers of LDL particles are made and thus more LDL apoprotein is needed. This requires that some animals have the ability to synthesize more apo-B on demand, and would in turn imply that the rate of apo-B synthesis does not occur maximally when low levels of dietary cholesterol are fed. Thus, one aspect of the variability among animals in response to dietary cholesterol could be the limiting rate at which their bodies can synthesize apo-B and the extent to which dietary cholesterol can influence this rate.

In those cases where LDL molecular weight was increased, the data show that not only is the need for apo-B increased but elevated apo-B content of LDL particles occurs concomitant with an increase in lipid content, i.e., LDL particles increase in size due to the addition of discrete increments of lipid and protein. This relationship was suggested by quantitating the changes in LDL molecular weight and chemical composition that occurred in *M. fascicularis* in response to dietary cholesterol. In Fig. 7, the data show that the elevation in molecular weight was related to a proportional increase in individual components, with cholesteryl esters being increased to the greatest extent when compared to the increase in free cholesterol, phospholipids, and protein. The relationships between the constituents that increased with molecular weight appeared fixed; each increased linearly in the proportions described by the slopes of the regression lines. Apparently, dietary cholesterol

resulted in an increase in LDL molecular weight which occurred by the stepwise addition of discrete increments of fixed chemical composition.

The weight relationships were determined from the data of Fig. 7 as the slopes of the lines for LDL molecular weight versus the content in g/μmole of cholesteryl ester, free cholesterol, phospholipid, and protein, respectively, which were 0.589, 0.087, 0.177, and 0.140. The appropriate values were then used to calculate the lipid molar ratios which were 4:1:1 for cholesteryl ester:free cholesterol:phospholipid. For calculation, the molecular weights of cholesteryl esters were assumed to average 660, and for phospholipids, 775. Although the relative weight contribution to the increments by the protein was apparent, the molar contribution is uncertain because of the lack of definitive information about the molecular weight of apo-B, the apoprotein added as molecular weight increased (Fig. 8). By assuming that the molecular weight of apo-B was 250,000, as published by Smith, Dawson, and Tanford (33), the molecular weight of the increment can be calculated to be nearly 1.8×10^6. This molecular weight value for the increment seems too high, based on the observed range of values for LDL molecular weight, but cannot be completely ruled out because heterogeneity within the higher molecular weight LDL was seen using analytical ultracentrifugation.

Since the weight relationships among components of the increment were known, it was possible to calculate[4] the partial specific volume ($\bar{v}$) of the increment, and a value of 0.971 ml/g was found. Another method in which molecular volume was plotted versus molecular weight (34) was used to test the idea that LDL molecular weight increased by increments of fixed composition. A straight line resulted, indicative of an increase in LDL molecular weight by incremental addition. The slope of the line was 0.979 ml/g which represents the $\bar{v}$ of the increment. The close agreement between the two calculated partial specific volumes provides support for the hypothesis that LDL molecular weight is increased by the addition of discrete increments of fixed chemical composition.

It appears, then, that factors controlling the extent of incremental enlargement of LDL should be considered in attempts to understand lipoprotein metabolism. For instance, it would be instructive to know if an analogous response to dietary cholesterol occurs in human beings. The data of Hammond and Fisher (6), from diabetic patients, and of Adams and Schumaker (34), from postheparin plasma of human beings, suggested that incremental enlargement in human LDL can occur although the stimulus for enlargement was not dietary cholesterol and the increment in their studies had a higher $\bar{v}$, namely 1.03 ml/g, than that found in the present study. The contribution of protein to such an increment would appear to be much less than for an increment with $\bar{v}$ of 0.97 ml/g, as found here. The LDL of the human studies contained much more triglyceride (35) than was true for the monkeys of our study. It seems a reasonable possibility that alterations in triglyceride metabolism lead to a separate type of incremental increase in LDL molecular weight than would be the case for alterations in cholesterol metabolism.

The significant individual animal variation in LDL molecular weight response to dietary cholesterol, which in this study was instrumental in detection of the increment, suggests that this response is under genetic control. Such a possibility would appear to us to be consistent with the proposed model of Fisher et al. (36) for genetic control of LDL molecular weight. However, one aspect of this phenomenon on which we differ is on the effect of environmental influence. Clearly, in our nonhuman primate population we were able to demonstrate the LDL molecular weight difference by the environmental stimulus of dietary cholesterol. In contrast, Fisher et al. (36) found a low correlation coefficient when comparing the LDL molecular weights of mothers and fathers, and implied that this establishes that "nutritional and physical environment . . . is not a significant determinant".

There may be a fundamental difference between the nonhuman primates of our study and the human primates of the Fisher study. For instance, these workers selected only those individuals with monodisperse LDL and this was not done in our nonhuman primate populations. On the other hand, dietary control in our nonhuman primates is absolute and differences due to diet can be relatively easily studied. In contrast, for North American human populations, wide differences in dietary habits exist, with the average cholesterol intake approaching 0.3 mg/kcal (37). This level of dietary cholesterol in nonhuman primates is adequate to raise plasma cholesterol concentrations and to draw out LDL size differences.[5] We feel that more data on environ-

[4] Partial specific volumes for lipids used in these calculations were the same as those published by Sata, Havel, and Jones (10) and were: cholesteryl esters, 1.044; free cholesterol, 0.968; phospholipids, 0.970; triglycerides, 1.093. The value used for partial specific volume of the protein was 0.72 and was taken from Scanu, Pollard, and Reader (43).

[5] Rudel, L. L., and M. D. Morris. Unpublished observations.

mental effects in human beings are needed before conclusions are drawn.

The data for LDL in the present study were consistent with a core–coat model for LDL particles that are spherical in structure, analogous to the model described for chylomicrons (38), for VLDL (10), and as suggested for LDL (39, 40). The apparent volumes for the LDL particles of different molecular weights were determined by multiplying the $\bar{v}$ for the individual constituents by the g/μmole of constituent, and summing the products. The apparent volumes of LDL were used to determine diameter of the whole LDL particle by solving the equation for the volume of a sphere ($V = \pi d^3/6$). By solving the same equation for the diameter of the central core of the spherical particle, based on the $\bar{v}$ of cholesteryl ester plus triglyceride, and subtracting this core diameter from that of the whole LDL particle, twice the thickness of the outer coat of the particle was obtained. The thickness was found to be 21.3 Å ± 0.27 (mean ± SEM) for the 20 *M. fascicularis* of this study, and did not change as the LDL molecular weight increased from 3 to 7.8×10^6. The calculated range of diameters for the LDL of these molecular weights was 209–288 Å and the relationship between molecular weight (mol wt units $\times 10^{-6}$) and a diameter (Å) was linear as described by the regression equation, $d = 16.65$ mol wt + 162.10. The value for coat thickness agrees very well with the 21.45 Å thickness calculated for VLDL by Sata et al. (10). In summary, the data suggest that, as the cholesteryl ester content of LDL within the core was increased in response to dietary cholesterol, a proportional amount of surface material was added to maintain a coat of constant thickness. This type of experimental evidence provides strong support for a core–coat model for LDL structure.

Finally, a statement about the significance of the relationship between plasma LDL response and coronary atherosclerosis should be made, although this subject is the topic of a separate publication (41). A significant correlation between LDL molecular weight and a coronary atherosclerosis index was found in a study of 40 *M. fascicularis*, whereas no significant correlation between micromolar concentration of LDL and the coronary atherosclerosis index was found. Such findings tend to emphasize the usefulness of distinguishing these properties of LDL and indicate that metabolism of LDL in some tissues may be related to molecular weight of LDL.

If this point is considered in light of the current model of LDL catabolism proposed by Brown and Goldstein (42), the relative significance of a difference in LDL molecular weight can be evaluated. These workers have proposed that after interacting with a specific cell surface receptor, LDL are internalized. For each lipoprotein molecule bound, the associated cholesteryl ester must be metabolized. When binding occurs for an LDL molecule of 8×10^6 compared to an LDL molecule of about 3×10^6 (the molecular weight range found in this study) approximately three times more cholesteryl ester will be internalized. The ability of individual tissues to metabolize the extra cholesteryl ester may be quite variable and could in turn determine the extent of accumulation. Another possibility is that the increase in cholesteryl ester content is associated with an allosteric modification of the LDL particle surface which could enhance its ability to interact with the receptor and lead to cholesteryl ester accumulation.

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