Abstract Hypercholesterolemia was induced in adult male rhesus monkeys with a high-fat diet containing an elevated cholesterol level (0.5%). Plasma lipoproteins were chromatographically separated into four size populations (regions) that were subdivided by density until fractions with single electrophoretic mobilities were obtained. The region III lipoproteins (LDL) contained 80% of plasma cholesterol and were present in the highest concentration of all fractions. Their molecular weight was increased over that of controls so that each particle averaged 1.8 times the number of cholesteryl ester molecules as did control LDL. Region II lipoproteins, a heterogeneous group, were present in next highest concentration. Most were cholesteryl ester-rich, β-migrating lipoproteins that overlapped the VLDL and LDL density ranges; apoB was the predominant apoprotein. One region II subfraction had preβ₂ migration and the density range 1.050 < d < 1.10. Another subfraction, cholesteryl ester-rich VLDL including only about 1% of plasma cholesterol, had preβ₁ migration and apoB and apoC as the predominant apoproteins with no apoprotein E. Region I lipoproteins were larger sized, slow β-migrating cholesteryl ester-rich VLDL that included 5% of plasma cholesterol. ApoB and apoE were the predominant apoproteins. Region IV lipoproteins (HDL) contained 4% of the plasma cholesterol; their concentration was decreased to about 1% of the control level. Atherogenic features of the diet-induced dyslipoproteinemia included the increased plasma concentrations and cholesteryl ester contents of the region I, II, and III lipoproteins in addition to the decreased HDL concentration.

Supplementary key words agarose gel chromatography · agarose electrophoresis · apoproteins · cholesteryl esters · hypercholesterolemia

We have previously characterized the plasma lipoprotein spectrum present in rhesus monkeys (1). In that study, animals were fed a semipurified diet designed to approximate the typical North American diet in terms of calorie distribution, but which contained a low cholesterol level (0.05 mg/kcal). In the present study, the same basal diet was used except that the level of cholesterol was increased 20-fold. Hyperlipoproteinemia that results from the higher level of dietary cholesterol is marked in rhesus monkeys. Characterization of diet-induced dyslipoproteinemia has received much attention recently in several different species (2–8). In general, cholesteryl ester enrichment of lipoproteins has been noted to occur often in association with appearance of abnormal lipoprotein classes. The specific characteristics are of interest since dyslipoproteinemia usually occurs concomitantly with exacerbation of atherosclerosis. In the present study, we describe the distribution and composition of the spectrum of lipoprotein classes present in a group of dyslipoproteinemic male rhesus monkeys that were subsequently found by morphologic evaluation to have developed extensive atherosclerosis.

In control animals of the previous study (1), lipoprotein fractions were purified using the properties of size, density, and electrophoretic mobility as criteria with which to define individual lipoprotein fractions. The lipoprotein fraction present in highest concentration was the HDL (region IV lipoproteins), which had as the major apoprotein, apoA-I. The fraction present in next highest concentration was LDL (region III lipoproteins), which contained apoB as the primary apoprotein. It was necessary to use gel filtration chromatography in addition to density centrifugation to separate the LDL from larger preβ-migrating lipoproteins, a portion of which floated in the d 1.019–1.063 g/ml range. This lipoprotein fraction, operationally termed the II_B100 lipoproteins, was similar in size and composition to human Lp(a) and significant concentrations were found in many control diet-fed rhesus monkeys. Lower concentrations of larger VLDL

Abbreviations: CE, cholesteryl esters; HDL, high density lipoproteins; LDL, low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins; TMU, tetramethyl urea.
and LDL lipoproteins (region I and II lipoproteins) were identified and characterized including a cholesteryl ester-enriched, β-migrating VLDL. The β-migrating, intermediate sized d 1.006–1.050 g/ml lipoproteins appeared to represent cholesteryl ester-enriched remnants; this fraction contained less than 5% of the total plasma cholesterol. In the cholesterol-fed animals of the present study, almost all of the plasma lipoprotein fractions were found to have modified chemical compositions, size, and plasma concentrations.

METHODS AND MATERIALS

Animal colony

A group of six adult male rhesus monkeys were fed the test diet, which is the same semipurified diet (45% of calories as lard, 20% of calories as protein) as the control diet of reference 1, except that the cholesterol level was raised to 1 mg/kcal with crystalline cholesterol. All animals maintained or gained weight throughout the course of study and received this experimental diet for at least a year prior to initiation of the lipoprotein studies. The details of feeding, handling, and blood sampling were the same as previously reported (1).

Lipoprotein isolation procedures

Isolation of plasma lipoproteins, initially by ultracentrifugation at a density of 1.225 g/ml with subsequent separation by agarose column chromatography, has been described (9). Lipoprotein distribution was routinely monitored by the recovery of cholesterol among isolated fractions. For eight separate plasma samples, 99.0 ± 0.6% (mean ± SEM) of the plasma cholesterol was recovered in the initial centrifugation at d 1.225 g/ml, and 92.8 ± 1.1% was recovered among fractions separated chromatographically. LDL molecular weight determinations were carried out during preparative chromatography using 125I-labeled LDL, according to the method of Rudel, Pitts, and Nelson (10).

Within populations of lipoproteins initially separated according to size, the density distribution was also determined. In these cases, samples were centrifuged sequentially at the appropriate densities, and the recovery of cholesterol was monitored. In most cases, ultracentrifugation served as the final purification and concentration step.

Lipoproteins were also isolated from plasma by sequential ultracentrifugation at successively increasing densities, namely 1.006, 1.019, 1.063, and 1.225 g/ml, using discontinuous gradients in the SW-40 rotor by the procedure described previously (1). The d < 1.006 g/ml fraction was heterogeneous as evidenced by the presence of three bands after agarose electrophoresis and was further subfractionated. In some studies, this fraction was first chromatographically separated into large (region I) and small (region II) components (see Fig. 2); then the fractions containing smaller components were pooled, concentrated, and subjected to a density gradient centrifugation. In other cases, the entire d < 1.006 g/ml fraction containing the slow β-migrating region I lipoproteins was used. A discontinuous gradient was prepared in which equal volumes of a solution of d 1.020 and 1.010 g/ml, and distilled water were successively layered over the sample (made to d 1.020 g/ml) in the SW-40 rotor tube. Samples were then centrifuged for 90 min at 200,000 g, 15°C, after which separate fractions were obtained by collecting through a needle from a hole punctured in the bottom of the tube. The β-migrating lipoproteins were found at the bottom of the tube, the preβ-migrating lipoproteins were found in the center of the tube, and slow β-migrating material was found at the top of the tube.

Lipoprotein compositional analyses

Determinations of the lipid composition of isolated lipoproteins were performed as described previously (1). Protein content of isolated lipoprotein fractions was determined directly by the method of Lowry, et al. (11), using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as the standard. Turbidity in the larger lipoprotein fractions was removed by extraction with hexane after the color reaction was complete. Agarose electrophoresis of whole plasma and of isolated lipoproteins was carried out in 0.5% agarose (Bio-Rad Labs, Richmond, CA) essentially according to the method of Noble (12). Gels were stained at 37°C for 30 min in 60% ethanol saturated with oil red O and fat red 7B. Apoprotein analyses were carried out on isolated lipoprotein fractions that had been dialyzed against distilled H2O containing 0.01% disodium ethylenediaminetetraacetate (EDTA) and lyophilized. Delipidation was carried out with chloroform–methanol 2:1, and after with ethyl ether. Resolubilization and polyacrylamide gel electrophoresis (PAGE) techniques have been described previously (1). The amount of apoB in each lipoprotein fraction was determined after precipitation of this apoprotein with tetramethylurea (TMU), as described by Kane (13). Monospecific antisera were used to identify apoA-1 and apoE (also called arginine-rich apoprotein)1 in individual lipoprotein classes. In some cases,

1 Antisera to patas monkey apoE and to human apoA-1 were obtained through the generosity of Dr. Robert Mahley of the NIH.
intact lipoprotein preparations were iodinated with $^{125}$I using the iodine monochloride technique of MacFarlane (14), as previously described (10). Individual apoproteins were then separated using PAGE to determine if apoproteins became iodinated in proportion to their relative amount on the lipoprotein particle.

RESULTS

Lipoprotein distribution

The dyslipoproteinemia induced by dietary cholesterol in rhesus monkeys was characterized initially by the distribution according to size. Typical lipoprotein elution profiles obtained by agarose column chromatography are shown in Fig. 1. In comparison with a profile from an animal fed the control level of cholesterol, much more material of the larger sizes (regions I, II, and III) was present in animals fed the higher level of cholesterol, and much less material was present in region IV. The amounts of cholesterol and protein in lipoproteins of each of the elution regions were then determined and the results are shown in Table 1. Data from animals fed the control level of cholesterol are shown for comparison. Lipoproteins in region III of test diet-fed animals were present in highest concentration and, for both protein and cholesterol, values were significantly higher than for those of control animals. The cholesterol concentration of the lipoproteins in regions I and II was also markedly elevated in test diet-fed animals. At the same time, the protein concentration of these lipoproteins was not increased over control levels to the same extent as was that of cholesterol, indicating that these lipoproteins were enriched in cholesterol. In contrast, the amount of both cholesterol and protein in region IV lipoproteins from test diet-fed animals was decreased to about $\frac{1}{3}$ of the control level, and no change in the relative amount of cholesterol to protein was apparent.

Lipoprotein fractionation

In addition to size separation, we have used density differences as a means to characterize the lipoproteins of test diet-fed monkeys. The size–density relationships for the lipoproteins separated initially by density are shown in Fig. 2. Two size populations are apparent in the $d < 1.006$ g/ml lipoproteins, which represent the largest particles isolated. The size of the $1.006 < d < 1.019$ g/ml lipoproteins was intermediate between that of the largest $d < 1.006$ lipoproteins and the $1.019 < d < 1.063$ g/ml fraction. Considerable size overlap was observed among the $d < 1.006$, the $1.006 < d < 1.019$, and the $1.019 < d < 1.063$ g/ml fractions. The average size of the $1.019 < d < 1.063$ g/ml fraction was the same as for the region III lipoproteins, as indicated by coincidence at the center of the peaks. Very little overlap occurred between the $1.019 < d < 1.063$ and $1.063 < d < 1.225$ g/ml fractions.

<table>
<thead>
<tr>
<th>TABLE 1. Influence of dietary cholesterol on plasma lipoprotein distribution in rhesus monkeys</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>N</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Control*</td>
</tr>
<tr>
<td>Test</td>
</tr>
<tr>
<td>Control*</td>
</tr>
<tr>
<td>Test</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data from reference 1.<br>_<sup>b</sup> All values are mean ± SEM.<br>_<sup>c</sup>Average plasma triacylglycerol concentration, 44 mg/dl.<br>_<sup>d</sup>Significantly different ($P < 0.01$) from control.

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Fig. 1. Agarose column chromatography elution profiles of the plasma lipoprotein spectrum of a representative control and a test diet-fed rhesus monkey. Lipoproteins were isolated from 5 ml of plasma by centrifugation at 1,225 g/ml, after which they were placed on a Bio-Gel A-15 m chromatography column, 1.5 x 90 cm, and eluted at the rate of 6 ml/hr with 0.9% NaCl, 0.01% EDTA, 0.02% Na$_2$SO$_4$, pH 7.4, at 4°C. Two-milliliter fractions were collected in a fraction collector. In the test animal sample, two size populations are apparent in the $d < 1.006$ g/ml lipoproteins, which represent the largest particles isolated. The size of the $1.006 < d < 1.019$ g/ml lipoproteins was intermediate between that of the largest $d < 1.006$ lipoproteins and the $1.019 < d < 1.063$ g/ml fraction. Considerable size overlap was observed among the $d < 1.006$, the $1.006 < d < 1.019$, and the $1.019 < d < 1.063$ g/ml fractions. The average size of the $1.019 < d < 1.063$ g/ml fraction was the same as for the region III lipoproteins, as indicated by coincidence at the center of the peaks. Very little overlap occurred between the $1.019 < d < 1.063$ and $1.063 < d < 1.225$ g/ml fractions.

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Electrophoretic mobility was also used to characterize the lipoprotein fractions (see Fig. 3). Three electrophoresis bands were seen when the $d < 1.006 \text{ g/ml}$ (VLDL) fraction from plasma of fasted test diet-fed monkeys was subjected to agarose electrophoresis. The lipoproteins of region I, which were the largest of the isolated fractions, had a slow $\beta$ mobility on agarose electrophoresis. Some material remained at the origin during electrophoresis. This may represent particles too large to penetrate the agarose gel matrix or possibly breakdown which occurred during concentration of the column fractions. In region II lipoproteins with $d < 1.006 \text{ g/ml}$, fractions with pre$\beta_1$, and $\beta$ migration were found. It was possible to separate the pre$\beta_1$ from $\beta$ migrating VLDL by centrifugation in a density gradient as described in the methods section.

Region II showed further heterogeneity with respect to density (Fig. 2). Lipoproteins migrating $\beta$ were found in both of the density ranges, $1.006 < d < 1.019$ and $1.019 < d < 1.050 \text{ g/ml}$. In addition, a fraction representing the pre$\beta_2$ migrating material of whole plasma was isolated in the density range $1.050 < d < 1.10 \text{ g/ml}$. In order to remove the $\beta$-migrating material that was also present in this density range, we subjected this fraction to a second chromatographic separation on Bio-Gel A-15m and pooled the larger fractions from this separation to obtain the purified pre$\beta_2$ lipoproteins shown in Fig. 3.

Region III lipoproteins were all $\beta$-migrating and represented particles in the $1.006 < d < 1.063 \text{ g/ml}$ (LDL) density range. Subfractionation at $d < 1.019 \text{ g/ml}$ was carried out, although it may represent an artificial separation of this population of lipoproteins. Region IV lipoproteins all had $\alpha$ migration and were isolated in the $1.063 < d < 1.225 \text{ g/ml}$ (HDL) density range.

Table 2 lists the features used to identify each of the separated lipoprotein fractions. A combination of size, density, and electrophoretic mobility has been used in defining separate fractions, and the designations shown in the last column, though cumbersome, appear to be the least redundant yet systematic way to define the isolated subfractions. The percentage of plasma lipoprotein cholesterol in each of the fractions was estimated. Variations may be expected from sample to sample, subject in part to losses incurred during purification procedures and to individual animal differences.

### Lipoprotein chemical compositions

The chemical compositions of the isolated lipoprotein fractions were determined and the results are shown in Table 3. The region I lipoproteins were only 2.1% protein, and 70.4% of the lipid was cholesteryl ester. The TC/Pro ratio was 23.5, which is high compared to that of other lipoprotein fractions. This composition was different from any of the other isolated lipoprotein fractions. The percentage of protein in the II$_{60\alpha}$, II$_{60\beta}$, and the II$_{50}$ lipoproteins progressively increased. The II$_{60\beta}$ lipoproteins were the
fraction highest in percentage of triacylglycerol although this value was only 21%. Essentially no difference was found in the lipid composition of the \( \Pi_{50} \), \( \Pi_{50} \), and \( \III \) lipoproteins, although the lower TC/PL ratio of the region \( \III \) lipoproteins was significantly different. The major component of these lipoproteins was cholesteryl ester. The \( \II_{100\preceq} \) fraction had a higher percentage of protein than any of the other region \( \II \) or \( \III \) lipoproteins, but still contained cholesteryl ester as its major lipid component.

The region \( \IV \) lipoproteins were divided at the peak into front and back subfractions. As can be seen in Table 3, there was a higher percentage of protein in the \( \IV \) back compared to \( \IV \) front lipoproteins, although the percentage composition of the lipid complement was similar. Interestingly, the percentage of free cholesterol was the only significant difference between the lipid percentages of \( \IV \) front and back lipoproteins. This appeared to account for the significant EC/TC ratio difference.

### Table 2. Characteristics of separated lipoprotein fractions of hyperlipoproteinemic rhesus monkeys

<table>
<thead>
<tr>
<th>Lipoprotein Region</th>
<th>Agarose Electrophoretic Mobility</th>
<th>Density Range (g/ml)</th>
<th>Average Diameter (Å)</th>
<th>% of Plasma Cholesterol Distribution (%)</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>slow β</td>
<td>d &lt; 1.006</td>
<td>&gt;650</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>preβ₁</td>
<td>d &lt; 1.006</td>
<td>400</td>
<td>1</td>
<td>( \II_{\text{preβ₁}} )</td>
</tr>
<tr>
<td>II</td>
<td>β</td>
<td>d &lt; 1.006</td>
<td>300</td>
<td>1</td>
<td>( \II_{\beta} )</td>
</tr>
<tr>
<td>II</td>
<td>β</td>
<td>1.006 &lt; d &lt; 1.019</td>
<td>300</td>
<td>3</td>
<td>( \II_{\beta} )</td>
</tr>
<tr>
<td>II</td>
<td>preβ₂</td>
<td>1.050 &lt; d &lt; 1.10</td>
<td>300</td>
<td>1</td>
<td>( \II_{100\preceq} )</td>
</tr>
<tr>
<td>III</td>
<td>β</td>
<td>d &lt; 1.019</td>
<td>245</td>
<td>13</td>
<td>( \III_{19} )</td>
</tr>
<tr>
<td>III</td>
<td>β</td>
<td>1.019 &lt; d &lt; 1.063</td>
<td>245</td>
<td>66</td>
<td>( \III_{19} )</td>
</tr>
<tr>
<td>IV</td>
<td>α</td>
<td>1.063 &lt; d &lt; 1.225</td>
<td>&lt;125</td>
<td>4</td>
<td>( \IV )</td>
</tr>
</tbody>
</table>

* Based on agarose column elution and chemical composition, as described by Sata, Havel, and Jones (26).
* Values were determined on a plasma pool that had a cholesterol concentration of 795 mg/dl. More cholesterol was generally found in d < 1.006 g/ml lipoproteins when plasma concentrations exceeded this value.
* This system is based on the procedural detail. Since size separation was carried out first, the size region is indicated first. Where electrophoretic mobility is needed to further distinguish between two fractions of the same size region and density, this information is also added. Where an abbreviated designation is given, e.g. region \( \III \) lipoproteins, the reader can assume no further sub-fractionation was carried out.

### Table 3. Chemical composition of plasma lipoprotein fractions isolated from hyperlipoproteinemic rhesus monkeys

<table>
<thead>
<tr>
<th>Lipoprotein Fraction</th>
<th>% of Mass</th>
<th>% of Total Lipid</th>
<th>W/W</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid</td>
<td>Protein</td>
<td>FC</td>
</tr>
<tr>
<td>I</td>
<td>97.9</td>
<td>2.1</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>± 0.5</td>
<td>± 0.5</td>
<td>± 0.7</td>
</tr>
<tr>
<td>( \II_{\text{preβ₁}} )</td>
<td>90.2</td>
<td>9.8</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>± 0.4</td>
<td>± 0.4</td>
<td>± 0.2</td>
</tr>
<tr>
<td>( \II_{\beta} )</td>
<td>88.4</td>
<td>11.6</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>± 0.7</td>
<td>± 0.7</td>
<td>± 1.1</td>
</tr>
<tr>
<td>( \II_{50} )</td>
<td>86.1</td>
<td>13.9</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>± 1.7</td>
<td>± 1.7</td>
<td>± 0.9</td>
</tr>
<tr>
<td>( \II_{100\preceq} )</td>
<td>76.9</td>
<td>23.1</td>
<td>12.9</td>
</tr>
<tr>
<td>( \III )</td>
<td>82.9</td>
<td>17.1</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>± 1.9</td>
<td>± 1.9</td>
<td>± 0.7</td>
</tr>
<tr>
<td>( \IV ) front</td>
<td>54.4</td>
<td>45.6</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>± 2.3</td>
<td>± 2.3</td>
<td>± 0.6</td>
</tr>
<tr>
<td>( \IV ) back</td>
<td>47.1</td>
<td>52.9</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>± 3.9</td>
<td>± 3.9</td>
<td>± 0.7</td>
</tr>
</tbody>
</table>

* Determined in duplicate on two samples, each pooled from three animals.
* Determined in duplicate on a pooled sample from four animals.
* Determined on individual samples from six animals.

All values are means (±SD). Abbreviations: FC, free cholesterol; PL, phospholipids; CE, cholesteryl esters; TG, triacylglycerols; TC, total cholesterol; EC, ester cholesterol; Pro, protein.
Lipoprotein apoprotein patterns

The apoprotein patterns from test and control animals are compared using SDS PAGE in Fig. 4A. The major apoprotein of control animal lipoproteins of regions I, II, and III remained in the sample well and near the interface between the stacking and separating gels, which is behavior characteristic of apoB. This was also characteristic of the region i, II, and III lipoproteins of the test diet-fed animals. Among

Fig. 4. A. A comparison of SDS PAGE apoprotein patterns of lipoprotein fractions isolated from pooled plasma samples of control and test diet-fed rhesus monkeys. Designations for individual fractions from test animals are explained in Table 2 and, for control animal samples, in reference 1. The region III lipoproteins were separated into a fraction from the front of the peak (III,) and the back of the peak (III,) to ascertain if heterogeneity within this fraction could be identified. The position of migration for individual apoproteins is identified at the left of the gel. Lipoprotein samples containing 100–150 μg of protein were delipidated, redissolved in 100 μl of 0.025 M Tris–0.2 M glycine buffer (pH 8.3) containing 0.02 M SDS, and 40 μl of this solution was placed in the sample well. Electrophoresis was carried out at 10°C for 5 hr at a constant current of 40 mA, after which the slab was stained with Coomasie blue, then destained in 7.5% glacial acetic acid containing 5% methanol. A 10% polyacrylamide separating gel containing 0.1% SDS, 0.4 M Tris, pH 8.8, with a 5% spacer gel was used in this experiment. B. SDS PAGE apoprotein patterns for individual lipoprotein fractions isolated from pooled plasma of test diet-fed rhesus monkeys. Designations for individual fractions are described in Table 2. Details are as given above for A, except that a 12.5% polyacrylamide separating gel with a 5% spacer gel was used in this experiment.
the apoproteins present in lesser amounts, consistently there appeared to be more protein migrating in the region of apoE and less in the region of apoA-I in the I, II, and III lipoproteins of test animals compared to those of control animals. The region II and III lipoproteins of test animals also contained more protein in the apoC region. Apoprotein patterns appeared similar for region IV lipoproteins of test and control animals.

In Fig. 4B, the apoprotein patterns for each of the separate fractions described in Fig. 3 and Table 2 are shown. There were differences among the d < 1.006 g/ml lipoproteins. A major difference was the amount of apoE present in the I vs. IIapoE-β fraction. In the latter fraction, essentially no protein was present in the apoE region and relatively more protein migrating in the apoC region was found. Essentially no protein migrating as apoA-I was found in either fraction. Some protein migrating in the albumin region was consistently seen if these lipoproteins were isolated by centrifugation at d < 1.006 g/ml and then separated by density gradient centrifugation. Further washing caused a loss of albumin and other apoproteins.

The IIapo, IIa, and IIapo lipoproteins all showed a similar apoprotein pattern on SDS PAGE and, upon visual inspection, appeared to contain slightly more protein in the apoA-I region and less in the apoC region than either of the larger lipoproteins. The IIapo lipoproteins showed a pattern different from any of the other fractions. Minimal amounts of protein in the regions of apoA-I, apoE, or apoC were present, and the amount of protein that migrated out of the sample well, through the 5% spacer gel, and into the 12.5% separating gel was noticeably less than for other region I, II, and III lipoproteins. The apoprotein pattern for the region III lipoproteins was essentially the same as that of the other β-migrating lipoproteins. The region IV lipoproteins contained a major band in the region of apoA-I, and had significant amounts of protein in the apoC region as well as some unidentified proteins in the regions larger than apoA-I. It was consistently found with SDS PAGE that region IV lipoproteins had no protein in the apoE region. The protein that migrated just ahead of the apoE region was not apoE, as judged immunologically, although it migrated in the apoE region on 8 M urea PAGE (Fig. 5).

The SDS PAGE analyses indicated that apoB, or its equivalent, was a major apoprotein of each of the lipoproteins in regions I, II, and III. In order to quantitate the relative proportion of apoB among apoproteins of these lipoproteins, we made use of its property of insolubility in TMU. Intact lipoproteins were iodinated, and the distribution of 125I among apolipoproteins was measured after delipidation. The insoluble TMU-precipitable protein, i.e., apoB, was 45, 65, 80, 91, 91, and <1% of total apoprotein mass of the region I, IIapo, IIIapo, IIIapo, IIIapoback, and IV lipoproteins, respectively. The percentage of 125I-labeled protein that behaved as apoB was 41, 81, 89, 92, and 91% for region I, IIapo, IIIapo, IIIapo, IIIapoback, and IV lipoproteins, respectively. These values are similar to the percentages of protein obtained for apoB by TMU precipitation. A comparison of radioactivity and Coomassie blue stain distribution was also made for the urea-soluble apoproteins. Densitometric scanning of the gels similar to those shown in Fig. 5 was carried out, and companion gels were sliced into regions and counted. The percentage distributions of radioactivity and stain were determined (Table 4) and were found to be similar, i.e., higher percentages of radioactivity...
and stain were present in the region of apoE (rm 15–23) in region I lipoproteins than for region III lipoproteins and the percentages in the apoC region (rm 40–70) were higher in region III than in region I lipoproteins.

Region III lipoproteins (LDL)

The region III lipoproteins (LDL) were the fraction present in highest concentration in test animals and were increased over control levels the most of any fraction. As shown in Table 5, this response occurred both as an increase in number of particles (μmolar concentration) and as an increase in particle size and molecular weight (compare the patterns of Fig. 1, region III). The size increase is reflected in the within-particle composition. It can be seen that each LDL particle from test diet-fed animals contained, on the average, 1.8 times as much cholesteryl ester, and more free cholesterol, phospholipid, and protein than did particles of control animals.

DISCUSSION

The data of the present study demonstrate that, in plasma of male rhesus monkeys made hyperlipoproteinemic with dietary cholesterol, many separate lipoprotein classes are present most of which bear chemical differences from their counterparts in control animals. In general, the lipoproteins that were present in elevated concentration in test animals (region I, II, and III lipoproteins) were larger in size and contained more cholesteryl ester as a percentage of total mass. Frequently, this was accompanied by an apparent decrease in average density for the fraction and less protein per unit mass. ApoB was the apoprotein present in highest amounts for these lipoprotein fractions, although some increase in the relatively small amounts of apoE was noted. Thus, the dietary cholesterol-induced dyslipoproteinemia of rhesus monkeys represents, predominantly, increased concentrations of large cholesteryl ester-rich lipoproteins that have apoB as the predominant apoprotein. These lipoproteins originate apparently as a result of an increased need to transport more cholesterol, presumably that derived from the diet.

The finding that cholesteryl ester-rich lipoproteins accumulate in plasma of cholesterol-fed animals is not restricted to rhesus monkeys. Such accumulations have previously been described in rabbits (2), pigs (4), rats (5), and patas monkeys (6), to name just a few. Previous reports of high molecular weight, cholesteryl ester-rich LDL of rhesus monkeys have appeared (8, 15). However, unique to the present study is the isolation, quantitation, and complete chemical characterization of the spectrum of plasma lipoproteins present in dyslipoproteinemic rhesus monkeys.

During this work, the inadequacy of the nomenclature system for lipoproteins was apparent, to wit, the identification of three VLDL (d < 1.006 g/ml) subfractions and three subfractions with densities overlapping the 1.019 < d < 1.063 g/ml, or LDL, range. We found it necessary to develop a nomenclature system which was adequate to help during preparation to distinguish between fractions without redundancy (see Table 2). The chemical compositions of many of these fractions were different, i.e., I vs. II60 vs. II60preβ, II60 vs. II100preβ, and II100preβ vs. IIIback. On the other

TABLE 5. Effects of dietary cholesterol on region III lipoproteins (LDL) of rhesus monkeys

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>N</th>
<th>Plasma Cholesterol (mg/dl)</th>
<th>Concentration (μmoll)</th>
<th>Molecular Weight (x 10^8)</th>
<th>Pro</th>
<th>FC</th>
<th>PL</th>
<th>CE</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>153 ± 16</td>
<td>0.53 ± 0.02</td>
<td>3.2 ± 0.1</td>
<td>26</td>
<td>742</td>
<td>968</td>
<td>2038</td>
<td>177</td>
</tr>
<tr>
<td>Test</td>
<td>6</td>
<td>795 ± 37</td>
<td>3.61 ± 0.35</td>
<td>4.8 ± 0.2</td>
<td>33</td>
<td>1194</td>
<td>1350</td>
<td>3671</td>
<td>44</td>
</tr>
</tbody>
</table>

* Calculated from average compositions for Region III lipoproteins (LDL) of each diet group. Abbreviations as given for Table 3. Molecular weights assumed for these calculations were: Pro, 25,000; PL, 775; CE, 660; and TG, 900.

* Mean values ± SEM.
hand, separation of region II lipoproteins that migrated 
\( \beta \) on agarose electrophoresis (II\(_{60} \), II\(_{19} \), and II\(_{60} \)) into
density subclasses did not appear to generate frac-
tions with different chemical compositions or different
apoprotein patterns. Likewise, the lipoproteins of
region III were separated into two density subfrac-
tions, III\(_{19} \) and III\(_{60} \), although resulting chemical
compositions and apoprotein patterns were minimally
different. In comparing compositions of all of the
\( \beta \)-migrating region II and III lipoproteins, the small
differences present could have been primarily related
to the size of the particles. This would account for the
slight difference in core lipid (predominantly CE)
to coat (Pro, FC and PL) ratio. Such a finding is con-
sistent with the possibility that these lipoproteins
represent remnant lipoproteins that were derived
from catabolism of triglyceride-rich precursors with
different cholesteryl ester contents. It remains for
further structural and metabolic studies to help define
whether or not these subfractions of \( \beta \) lipoproteins
represent discrete metabolic entities rather than seg-
ments of a size continuum of lipoprotein particles.

One of the unexpected findings of these studies
was that while the total plasma cholesterol increased
over 4-fold in animals fed the higher levels of dietary
cholesterol, the plasma level of lipoprotein protein
remained almost the same (Table 1). This was due to
the fact that apoprotein-rich region IV lipoproteins
decreased in concentration at a time when increases
occurred in the more cholesterol-rich, apoprotein-
poor lipoproteins of regions I, II, and III. The fact
that the level of total lipoprotein protein changed
little may be coincidental. On the other hand, with
the striking differences in apoprotein content of these
two groups of lipoprotein fractions, the shift in relative
amounts of the two major apoproteins, apoB and
apoA-I, is quite remarkable. We have calculated the
relative changes based on the data of Table 1 and
the data for apoB and apoA-I distribution.\(^2\) The con-
centration of apoB in lipoproteins was approximately
60 mg/dl in control animals compared to 280 mg/dl
in test animals, values proportional to the increase
in cholesterol concentration. On the other hand,
apoA-I levels decreased from about 250 to 80 mg/dl.

Postulation of a metabolic relationship between
this inverse proportionality between apoB and apoA-I
seems reasonable. A relationship between HDL levels
and responsiveness to dietary cholesterol in rhesus
monkeys has been discussed previously (17). In the
future, direct quantitation of individual apoprotein
levels using available immunologic techniques (18)
should provide valuable information on this type of
metabolic relationship and help determine the relative
significance to atherogenesis. Such studies are now
in progress in our laboratory.

We carried out in vitro iodination of intact lipopro-
tines in this study to determine if selective changes
in availability of apoproteins for iodination occurred
among lipoproteins isolated from hyperlipoprotein-
emic animals. However, the distribution among apo-
proteins of radioactivity vs. stain was not significantly
different, suggesting a surface location on the lipop-
protein particle of all apoproteins and relatively similar
conformations of apoproteins at the surface of the
various particles.

Region I lipoproteins from test animals were pre-
dominantly slow \( \beta \)-migrating VLDL whereas, in con-
tral animals, only a subfraction of region I lipopro-
tines had this migration. This finding, together with
the differences in percentage of cholesteryl ester (70
vs. 33\%) and triacylglycerol (11 vs. 49\%), and in apoE
predominance, indicate that the region I lipoproteins
of test animals may in fact represent a marked increase
of the \( \beta \)-migrating region I subfraction over that
found in the control animals. This subfraction may
represent the rhesus monkey counterpart to the
\( \beta \)-VLDL of cholesterol-fed patas monkeys, described
by Mahley, Weisgraber, and Innerarity (6), although
the percentages of protein and phospholipid are lower
and the average particle size is larger in the fraction
isolated from rhesus monkeys. The I\(_{60}\)lipoprotein
may be the counterpart of the pre\( \beta \) VLDL of control
animals. It was interesting to note that, when purified,
the I\(_{60}\)lipoprotein contained no detectable apoE
(Fig. 3B) even though it was a cholesteryl ester-en-
riched VLDL (51\% of lipid as CE vs. 21\% as TG,
Table 3).

The I\(_{100}\)lipoproteins we have isolated from
test animals have similar size, density, and electrophoretic properties to the I\(_{100}\) lipoproteins isolated from control animals (1), although they are chemically modified to contain more cholesteryl ester and less triacylglycerol and protein in test animals. The apo-
protein patterns of I\(_{100}\) lipoproteins of test and con-
trol animals were similar in that essentially all of the
apoprotein aggregated and poorly penetrated the
SDS-containing polyacrylamide gel. Also similar was
the relative absence of apoE, apoA-I, and apoC. The
amount of the I\(_{100}\) fraction in test animals indicates
that this fraction is not significantly increased in con-
centration in response to dietary cholesterol. The
similarity between this lipoprotein and Lp(a) of hu-
man beings was discussed previously (1). Many sugges-
tions have appeared that higher levels of Lp(a)
in humans is a risk factor for clinically detected compli-
cations of coronary atherosclerosis (19). The relatively

\(^2\) Based on the PAGE analyses and in agreement with the results
of Edelstein, Lim, and Scanu (16), apoA-I was estimated to be
75\% of the protein of HDL. It was no more than 1\% of any of
the other isolated fractions.

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low levels of this lipoprotein in the hyperlipoproteinemic rhesus monkey suggest that this fraction may not be a major factor in the development of experimentally induced atherosclerosis in this species.

Based on the chemical and physical properties of each fraction, a similar conclusion was reached about region III and IV lipoproteins of test animals as for those of control animals (1), namely that these fractions represent, respectively, LDL and HDL. In this sense, the name LDL refers to the population of β-migrating lipoproteins with an average size centering in region III that has apoB as the predominant apolipoprotein and that has a chemical composition with the core–coat relationships as defined here. The metabolic origin is not implied.

Our data showed heterogeneity among LDL fractions from individual animals. Cholesteryl ester content of LDL particles increased with molecular weight out of proportion to free cholesterol and phospholipid which, in turn, increased proportionally more than protein. The fact that increases occurred in both the particle concentration (μmol/l) and the molecular weight is important in consideration of relationships between the dyslipoproteinemia and atherosclerosis. Not only are arteries exposed to more LDL particles per volume of plasma, but the chemical composition of each LDL particle that gets into the artery wall is modified (Table 4). The recent work of St. Clair and Leight (20), using smooth muscle cells in tissue culture, has suggested that the large molecular weight LDL of rhesus monkeys stimulated cholesteryl esterification and accumulation by the cells out of proportion to their particle number and mass of cholesterol. In other studies carried out in our laboratory in Macaca fascicularis, we have found that LDL molecular weight had a highly significant correlation with the extent of coronary atherosclerosis (21). Thus, the large molecular weight LDL resulting from dietary cholesterol induction appears to be an atherogenic lipoprotein both in terms of the increased concentration (μmol/l) and in the modified composition that is reflected in molecular weight enlargement.

Another shift induced by dietary cholesterol which is likely to be atherogenic is the decrease in plasma HDL concentration, especially since this response occurs at the same time that the LDL changes occur. Recent studies in humans have shown that HDL concentration is important in determining the relative risk to coronary heart disease (22). We have shown in Macaca fascicularis that a significant relationship existed between the plasma HDL concentration and experimentally induced atherosclerosis (21). Other aspects of the induced hyperlipoproteinemia that are atherogenic could be the appearance of apoE-rich lipoproteins, since Mahley and coworkers (23) have shown that apoE has a high affinity for the LDL receptor site described by Brown and Goldstein (24). The latter authors feel that this cell surface receptor is important in controlling atherogenesis (25).

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