Biosynthesis of Low Density Lipoprotein by Cell-free Preparations of Rat Intestinal Mucosa*

Jacques I. Kessler, June Stein, D. Dannacker, and P. Narcessian

From the Laboratory for Gastrointestinal Research, McGill University Clinic, Royal Victoria Hospital, and Laboratory for Nutrition and Metabolic Research, Jewish General Hospital, Montreal, Canada

SUMMARY

The capacity of cell-free preparations of rat small intestinal mucosa to synthesize and release lipoproteins was investigated. Palmitate-1-14C was administered intragastrically and 30 min later labeled cell-free fractions from homogenates of intestinal mucosa were prepared. The fractions (whole homogenate, mitochondria, and microsomes) were incubated in fortified media containing 3H-labeled amino acids, and lipoproteins of various densities were separated, after the addition of carrier rat plasma, by density centrifugation, by polyanion precipitation, and by immunoprecipitation. All fractions incorporated radioactive into the lipid and protein moieties of the medium lipoproteins. The activity of the microsomal fraction accounted for most of the activity of the whole homogenate. Presence of postmicrosomal supernatant or pH 5 fraction, GTP, ATP, and ATP-generating system was essential for optimal activity of the microsomal fraction. The ratio of 14C:3H of microsomal (solubilized by LiCl) and medium low density lipoprotein (LDL) isolated by ultracentrifugation, by precipitation with polyanions, or by immunoprecipitation remained within reasonably narrow limits.

The identity of microsomal and medium LDL with that of plasma LDL was demonstrated by immunoelectrophoresis, double immunodiffusion, and peptide mapping.

The results indicate that the cellular equivalent of the microsomal fraction may be the major site of synthesis and association of the lipid and protein moieties of LDL. They further indicate that the intestinal mucosa may contribute significantly to the level of plasma LDL and that through the incorporation of LDL apoprotein into chylomicrons and very low density lipoprotein it can be of central importance in the transport of exogenous and endogenous lipid.

The transport of lipid from the intestinal mucosa to the lymph is accomplished in the form of lipoproteins (1, 2). There is ample evidence that the bulk of exogenous lipid is incorporated into lymph chylomicrons (3) and to a lesser extent into very low density lipoprotein (4), although an extensive endogenous contribution has also been demonstrated (5, 6). There is uncertainty regarding the source of the endogenous lipid, which conceivably may originate in the intestinal mucosa, from lipids in the bile (7, 8), or from plasma filtered in the lymph. Analogous uncertainty exists about the source of low density lipoprotein and high density lipoprotein demonstrated in thoracic lymph of several species (9–11) because the thoracic duct drains lymph not only from the intestine but also from the liver and the lower extremities. Furthermore, since lymph is generally considered to be a plasma filtrate containing all plasma proteins (9–11), the intestinal source of the protein moiety of lymph lipoproteins can also be questioned. A number of studies (12–16) involving incorporation of radioactive amino acids into lymph lipoproteins in vivo, or into medium lipoproteins of intestinal mucosa incubated in vitro, have suggested that the intestine may be a source of the protein moiety of lipoproteins. The interpretation of these results, however, is complicated by the fact that in the experiments in vivo thoracic duct lymph was collected, and also by the possibility of adsorption of highly labeled contaminating proteins to the surface of the fat particles during incubation of the intestinal mucosa (17). Recently, convincing evidence for the intestinal source of VLDL and LDL (4, 18, 19) of rat mesenteric lymph was presented. However, since mesenteric lymph may contain a significant amount of filtered plasma proteins, the possibility that the protein moieties of VLDL and LDL may have originated from sources other than the intestinal mucosa has not been completely excluded. The demonstration by Windmueller and Levy (19) of β-lipoprotein in mesenteric lymph of rats at a time when hepatic β-lipoprotein synthesis was effectively blocked by orotic acid (20) would seem to exclude this possibility. However, Lee (21) has recently shown in normal plasma and in plasma of patients with abetalipoproteinemia the presence of the protein moiety (protein B) of β-lipoprotein, with antigenic properties different from those of complete β-lipoprotein. The presence in rat plasma of a lipid-binding apoprotein has been

1 The abbreviations used are: VLDL, very low density lipoprotein; LDL, low density lipoprotein; that fraction of the total lipoproteins, after removal of chylomicrons, with a buoyant density less than 1.006 and pre-β electrophoretic mobility on paper. LDL, low density lipoprotein; the fraction with a buoyant density between 1.006 and 1.063 (d > 1.006 < 1.063) and a electrophoretic mobility on paper. HDL, high density lipoprotein; the fraction with a buoyant density between 1.063 and 1.21 (d > 1.063 < 1.21) and a electrophoretic mobility on paper.
shown previously, and although its identity to B protein has been inferred (21, 22), but not definitely shown, the possibility remains that the antisera against complete γ lipoprotein used by Windmueller and Levy (19) may not have determined its presence in eric acid-fed rats.

The site of association of the lipid and protein moieties of lipoproteins is not known. From morphological observations, it would appear that lipoprotein completion may occur at some point in the luminal half of the cell within the endoplasmic reticulum and Golgi apparatus (23). Glyceride synthesis has been associated with the smooth surfaced membranes of the apical vesicles of the mucosal cells (24). The synthesis of protein, however, has been associated with the rough endoplasmic reticulum, which, during the process of fat absorption, is predominantly located in the basal portion of the cells. This discrepancy in the sites of synthesis of the lipoprotein components suggested the possibility that lipoprotein completion may occur outside the epithelial cells of the intestine (25).

This study was undertaken with the purpose of elucidating whether or not cell-free preparations of intestinal epithelium can synthesize the lipid and protein moieties of lipoproteins and of identifying the site of lipoprotein completion. The results indicate that the micosomal fraction may be the major site of synthesis and association of the protein and lipid moieties of LDL.

**EXPERIMENTAL PROCEDURE**

**Methods**

**Preparation of Cellular Fractions**

Female Sprague-Dawley rats, from Quebec Breeding Laboratories, LaPrairie, Quebec, weighing 200 to 250 g, were deprived of food overnight but allowed free access to water. Two milliliters of olive oil containing 8 μCi of palmitic acid-1°C (specific activity 19.6 μCi per μmole) were administered intragastrically by gavage and 30 min later the rats were killed by ether anesthesia. The abdominal cavity was exposed and the small intestine rinsed with 300 ml of ice-cold 0.15 % NaCl. The upper two-thirds of the small intestine, measured from the ligament of Treitz, was removed and the mucosa was extruded by gentle pressure between the thumb and index finger. This provided a reasonably homogenous preparation of monolayers of epithelial cells with virtually no lamina propria. The extruded mucosa of three to five animals was weighed and added to 9 volumes of Medium A of Littlefield and Keller (26) modified to contain 0.3 M sucrose, 1% bentonite to diminish ribonuclease activity (27) and 0.1% BaSO₄ to precipitate mucus material (28), and homogenized (three down and three upward strokes) in a Potter-Elvehjem homogenizer with a motor driven Teflon pestle (600 rpm). The homogenate was filtered through a double layer of cheesecloth and centrifuged at low speed (10,000 × g) to remove cell debris and mucus. The cell-free fractions (whole homogenate, supernatant) were obtained according to the procedure of Hübscher, West, and Brindle (29). The mitochondrial and microsomal fractions were resuspended to original volume in postmicrosomal supernatant obtained from intestinal mucosa or livers of control (no radioactivity administered) animals. In a number of experiments, a pH 5 fraction obtained by the method of Hongland, Keller, and Zamecnik (30) was substituted for the postmicrosomal fraction. In these experiments the microsomal pellet was resuspended to original volume in pH 5 fraction dissolved in Littlefield's Medium A (0.3 M sucrose and 1% bentonite added) to a volume equal to that of the postmicrosomal fraction from which the pH 5 fraction was obtained. Aliquots from each fraction were taken for assay of radioactivity, protein (31), DNA (32), RNA (33), cytochrome c oxidase (34), and glucose-6-phosphatase (35).

**Incubation of Whole Homogenate and Mitochondria**

Aliquots (2 ml) of the respective fractions were incubated for 90 min in air at 37° with 2 ml of a solution of the following composition: KCl, 0.075 M; MgCl₂, 0.01 M; KI₂CO₃, 0.015 M; glucose, 0.015 M; nicotinamide, 0.02 M; EDTA, 3 × 10⁻⁴ M; nicotinamide adenine dinucleotide, 2.5 × 10⁻³ M; adenosine 5'-phosphate, 2 × 10⁻³ M, and a mixture of uniformly labeled amino acids (20 μCi of 3H per ml).

**Incubation of Micromosomal Fraction**

Aliquots (2 ml) of the microsomal fraction were incubated for 30 min at 37° with 2 ml of Littlefield's Medium A containing ATP, 8 μmoles; P-enolpyruvate, 40 μmoles; pyruvate kinase, 200 μg; GTP, 1 μmole; 0.3 M sucrose, 1% bentonite, and a mixture of amino acids labeled with H (20 μCi per ml) or with C (5 μCi per ml). The final pH of all the reaction mixtures was 7.4. Control samples were incubated at 0° and their subsequent treatment was identical to the samples incubated at 37°.

**Isolation of Medium Lipoproteins**

**Ultracentrifugation**—At the end of the incubation, 5 ml of recold 5°C casein hydrolysate was added and the tubes were immersed in crushed ice. Except when stated, 2 ml of pooled rat plasma was carefully layered under the contents of each incubation tube. The tubes were filled to the top with 0.15 NaCl, capped, and centrifuged in a Spinco model L preparative ultracentrifuge for 24 hours at 40,000 rpm (approximately 165,000 × g) and 4° using the 40 rotor. The upper 1.5 cm of the tube which contained lipoproteins with d < 1.006 was sliced and discarded. The remaining solution was separated from the microsomal pellet, the density was adjusted to 1.063 by addition of proper amount of NaCl and KBr solutions containing 2% casein hydrolysate, and the solution was centrifuged for 24 hours at 40,000 rpm and 4° in the Spinco 40 rotor. Lipoproteins with d < 1.063 were recovered from the upper 1.5 cm of the tube by slicing, layered under a solution of d = 1.063 and recentrifuged as before. This procedure was repeated twice. The final lipoprotein solutions were dialyzed for 24 hours at 4° against two changes of 0.5% casein hydrolysate in 0.15 % NaCl. The dialyzed solutions were then treated twice with 2 volumes of the following solutions: 10% trichloracetic acid, ethanol-ether (3:1) at 60° for 5 min, 10% trichloracetic acid at 90° for 15 min, ethanol-ether (3:1), ethanol, and finally ether. The precipitate after each treatment was recovered by centrifugation and finally was dissolved in 1 N NaOH. The washings with organic solvents were pooled and evaporated at 40° in a vacuum oven. The lipid was then taken up in chloroform and made up to volume.

**Polynesian Precipitation**—Low density lipoproteins in incubation media free of microsomes and lipoproteins with d < 1.006 were precipitated according to the method of Burstein and Salmin (36) using manganese chloride and heparin solutions containing 2% casein hydrolysate. A precipitate was allowed to
form for 15 min at 4° which was then separated by centrifugation. Each precipitate was treated twice with the following solutions: ethanol-ether (3:1) at 60° for 5 min, ethanol-ether (3:1), ethanol, and finally ether. The precipitates and the lipid extracts were dissolved in 1 N NaOH and in chloroform, respectively.

Immunoprecipitation—Low density lipoproteins in incubation media, free of d < 1.006 and of microsomes, were precipitated by addition of appropriate amounts of specific anti-LDL (d < 1.006 > 1.063) antisera. Addition of the specific antisera was preceded by treatment of the medium with bovine albumin followed by rabbit antirabbit albumin antisera and the resulting immunoprecipitate was removed by centrifugation. This treatment was repeated twice in order to exclude the possibility of any contamination of the specific antibody-LDL precipitate by unrelated labeled proteins which may have coprecipitated or non-specifically adsorbed on the precipitate. All antisera contained casein hydrolysate to a final concentration of 0.5%. The isolated antibody-LDL precipitates were treated as described for the lipoproteins isolated by polyanion precipitation.

Isolation of LDL from Incubated Microsomes—The microsomal pellets separated after the first centrifugation of the incubation medium were resuspended in Littlefield’s Medium A containing 0.3 M sucrose, 1% bentonite, and 0.5% casein hydrolysate and recentrifuged for 30 min at 40,000 rpm and the LDL in the supernatant was isolated by ultracentrifugation, by polyanion precipitation, or by immunoprecipitation as described above.

Immunoochemical Methods

Preparation of Antisera—Low density (d < 1.006 > 1.063) lipoproteins were isolated from pooled sera from 20 to 30 rats by preparative ultracentrifugation at the appropriate density as described before. The fractions were recovered by slicing off the cellulose nitrate tubes, pooled, and dialyzed for 24 hours at 4° against two changes of 0.15 M NaCl. The lipoproteins were concentrated by dry dialysis against Sephadex G-50 to a final protein concentration of 6.4 mg per ml and used to immunize rabbits (3 to 5 kg, body wt) by the Freund’s adjuvant technique (38). The antigen was stored at 4° and prepared in an equal volume of complete Freund’s adjuvant just before injection. The total amount, administered in five divided doses at intervals of 1 week, was approximately 30 mg. Antiserum against bovine albumin, crystallized, Pentex, Inc., Kankakee, Illinois, was prepared by the same technique except that bovine albumin to a final dose of 80 mg was administered instead of LDL. Blood was collected by cardiac puncture 1 week after the last injection; the serum was separated by centrifugation and frozen after the addition of 0.01% merthiolate.

Characterization of Antisera—The reactivity of the antisera obtained is shown in Table I. Because of cross-reactivity of antisera R1 and R2 with HDL and bovine albumin, only antisera R1 was used in the experiments. The antisera were characterized by immunoelectrophoresis on agarose-coated slides (39) and by double immunodiffusion on Ouchterlony plates (40) using appropriate amounts of the antigen, purified LDL and HDL, albumin, and normal rat serum. The precipitation lines caused by LDL or HDL were shown to stain for lipids with Oil Red O, and the amount of cholesterol in immunoprecipitates obtained by mixing LDL with decreasing amounts of corresponding antisera was proportional to the amount of protein. Antisera R1 was capable of detecting LDL in 1:64 dilution of normal rat plasma. For immunoelectrophoresis and double-immunodiffusion studies of LDL of microsomal incubation media and LDL-solubilized microsomes, no carrier plasma was added to the media, and the supernatants, after separation of d > 1.006 lipoproteins and microsomes, were concentrated by dry dialysis against Sephadex G-50 to an approximate volume of 0.5 to 0.8 ml.

Preparation of Peptides from Low Density Lipoprotein for Peptide Mapping—Purified LDL, with an approximate amount of 12 mg of protein, was obtained from complete microsomal reaction mixtures as described before, except that the microsomes were not prelabeled and the incubation system contained 0.06 M of a mixture of 14C-labeled amino acids. A comparable amount of purified LDL was obtained from plasma of rats injected with 100 μCi of 14C-labeled amino acids. The purified LDL fractions were partially delipidated by extraction with the n-heptane method of Gustafson (41) using insoluble potato starch. Soluble contaminants in the starch were removed by repeated washings with 1% NaCl and water until the supernatant reacted negatively to iodine. After evaporation of the heptane, the protein-phospholipid residue was separated from the starch by extractions with 0.15 M NaCl (adjusted to pH 8.6 with NH₄OH) until no protein could be detected in the supernatant. Further delipidation was achieved by forcing the heptane-extracted LDL into 30 volumes of ethanol-acetone (1:1 v/v). The mixture was left standing for 24 hours at 4°. The precipitate was collected by centrifugation and washed twice with ethanol-acetone, twice with acetone, and once with ether. The dried precipitate was treated with 1 ml of formic acid and 0.1 ml of 30% hydrogen peroxide, and the oxidation was allowed to proceed for 30 min at 25°. The oxidized protein was precipitated with 40 volumes of ether at 0° and washed twice with ether to remove residual formic acid. After drying, the precipitate was subsequently treated as described by Bungenberg de Jong and Marsh (42). The lyophilized peptides were dissolved in a minimal volume of buffer and separated by electrophoresis and thin layer chromatography on Silica Gel G plates as described by Sargent and Vadlamudi (43). Samples containing approximately 500 μg of peptides were applied on the lower right or left corners of the plates, sprayed with buffer (pyridine-acetic acid-water, 25:1:225 v/v, pH 6.5), and placed in the electrophoretic chamber so that they were facing each other. Whatman No. 3 MM paper extending into the buffer compartments to establish electrical contact. With this arrangement the silica...
Fig. 1. Protein, DNA, and RNA content and activity of cytochrome c oxidase and glucose 6-phosphatase of cell-free preparations from rat small intestine epithelium. The values represent the mean ± standard deviation of the fractions of four intestinal homogenates.

gel surfaces formed a narrow humid chamber and no cooling was necessary. Electrophoresis was carried out for 6 hours at room temperature with a current of 500 volts. Ascending chromatography was carried out at right angles to the direction of electrophoresis in a solvent system of butanol-acetic acid-water (3:1:1 v/v). The spots were visualized by spraying with a ninhydrin solution (0.3 g of ninhydrin, 100 ml of butanol, 3 ml of acetic acid). The ninhydrin-positive spots and appropriate blank areas were scraped into counting vials to which 15 ml of Bray's solution (44) was added. The quenching produced by the ninhydrin color was of no significance.

Determination of Radioactivity—Lipid radioactivity was assayed in aliquots of chloroform extracts, transferred to counting vials, and after evaporation of the chloroform, 12 ml of toluene containing 0.01% p-bis[2-(5-phenyloxazolyl)]benzene (POPOP) and 0.3% 2,5-diphenyloxazole (PPO) was added. For determination of protein radioactivity, aliquots of 1 n NaOH-dissolved precipitates were added to 15 ml of Bray's solution. The samples were counted in a Packard liquid scintillation spectrometer (model 3375) with an approximate efficiency of 70% for 14C and 30% for 3H in toluene-based scintillation solution and of 60% for 14C and 20% for 3H in Bray's solution. The samples were corrected for quenching by the channels ratio method or by addition of appropriate internal standards.

Materials

Phosphoenolpyruvate, GTP, ATP, pyruvate kinase, NAD, and adenosine 5'-phosphate were obtained from Sigma Chemical Company (St. Louis, Missouri). Trypsin (twice crystallized), chymotrypsin (three times crystallized), and pancreatic ribonulease (twice crystallized with ammonium sulfate and once with ethanol) were products of Worthington Biochemical Corporation. Palmitic acid-1-14C and reconstituted 14C- or 3H-algal protein hydrolysate were purchased from New England Nuclear Corporation. Cab-O-Sil, 2,5-diphenyloxazole and p-bis[2-(5-phenyloxazolyl)]benzene were obtained from Packard Instruments. Sephadex G-50 was a product of Pharmacia. Bentonite was purchased from Fisher Scientific, and purified according to the procedure of Fraenkel-Conrat, Siger, and Tsugita (45). All organic solvents were doubly distilled before use.

RESULTS

Characterization of Cell-free Preparations

It has been shown that in the fractionation of mucosal homogenates, a heavy cross-contamination of subcellular fractions occurs because of the presence of mucus. In our preparations, a significant reduction in the mucus content was obtained after an overnight fast of the donor animals and by the initial BaSO4 precipitation of the mucus material in the homogenate. Although no attempt was made to substantiate the purity of the fractions by electron microscopy, the results in Fig. 1 indicate that, with the exception of the nuclei plus brush borders fraction, reasonably clean preparations of mitochondria, microsomes, and particle-free supernatants were obtained.

The distribution of the labeled lipid in the cell-free preparations is shown in Table II. The nuclei plus brush borders fraction, which was the most heavily contaminated, contained 40.1% of the radioactivity. The microsomal fraction had 38.0% of the radioactivity, whereas in contrast to the nuclei plus brush borders fraction, 67.1% of it was found in esterified form. The radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.

Radioactivity in homogenate after filtration.

In Table II, the radioactivity in the fractions of 1 g of intestinal mucosa.

The fractions were homogenized and the lipids extracted in chloroform-methanol, 2:1 v/v. Lipid classes were separated by thin layer chromatography on Silica Gel G using a solvent system of n-hexane-diethyl ether-acetic acid-methanol, 90:20:2:3 (v/v). The percentage of esterification was calculated from the radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.

Radioactivity in respective fractions obtained from 1 g of intestinal mucosa.

The fractions were homogenized and the lipids extracted in chloroform-methanol, 2:1 (v/v). Lipid classes were separated by thin layer chromatography on Silica Gel G using a solvent system of n-hexane-diethyl ether-acetic acid-methanol, 90:20:2:3 (v/v). The percentage of esterification was calculated from the radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.

TABLE II

<table>
<thead>
<tr>
<th>Fractions</th>
<th>14C in Total Lipid</th>
<th>Esterification</th>
<th>Distribution of 14C in Esterified Lipid Fractions*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>MG</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>100.0</td>
<td>139,590</td>
<td>61.9</td>
</tr>
<tr>
<td>Nuclei plus brush borders</td>
<td>40.1</td>
<td>55,980</td>
<td>37.2</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>16.9</td>
<td>23,590</td>
<td>54.0</td>
</tr>
<tr>
<td>Microsomes</td>
<td>38.0</td>
<td>53,000</td>
<td>67.1</td>
</tr>
<tr>
<td>Supernatant</td>
<td>3.1</td>
<td>4,310</td>
<td>66.2</td>
</tr>
</tbody>
</table>

* Radioactivity in respective fractions obtained from 1 g of intestinal mucosa.

† The fractions were homogenized and the lipids extracted in chloroform-methanol, 2:1 (v/v). Lipid classes were separated by thin layer chromatography on Silica Gel G using a solvent system of n-hexane-diethyl ether-acetic acid-methanol, 90:20:2:3 (v/v). The percentage of esterification was calculated from the radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.

Radioactivity in homogenate after filtration.

In Table II, the radioactivity in the fractions of 1 g of intestinal mucosa.

The fractions were homogenized and the lipids extracted in chloroform-methanol, 2:1 v/v. Lipid classes were separated by thin layer chromatography on Silica Gel G using a solvent system of n-hexane-diethyl ether-acetic acid-methanol, 90:20:2:3 (v/v). The percentage of esterification was calculated from the radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.

Radioactivity in respective fractions obtained from 1 g of intestinal mucosa.

The fractions were homogenized and the lipids extracted in chloroform-methanol, 2:1 (v/v). Lipid classes were separated by thin layer chromatography on Silica Gel G using a solvent system of n-hexane-diethyl ether-acetic acid-methanol, 90:20:2:3 (v/v). The percentage of esterification was calculated from the radioactivity in the total lipid and free fatty acid fractions. MG, monoglyceride; DG, diglyceride; TG, triglyceride; PL, phospholipid; CE, cholesterol ester.
The distribution of the radioactivity and the extent of esterification, however, remained within reasonably narrow limits.

Incorporation of Lipid and Amino Acid Radioactivity into Lipoproteins by Cell-free Preparations of Rat Intestinal Epithelium

The results in Table III indicate that lipid and amino acid radioactivity was incorporated into lipoproteins of various densities by cell-free preparations of intestinal epithelium. The relative specific activity of the lipoproteins with \( d > 1.006 \) was highest. Although all the fractions incorporated labeled lipid and amino acids, it can be seen that most of the activity of the whole homogenate can be accounted for by the activity of the microsomal fraction. It should be noted that the specific activity is calculated from the protein content of the fractions with various densities, which were separated after the addition of 2 ml of pooled rat plasma. The protein content of the fractions isolated in the absence of rat plasma was too low for a meaningful determination of the true specific activity.

In subsequent experiments only the microsomal system was employed for studies of the biosynthesis of LDL. Preliminary experiments showed that the activity of the microsomal system was proportional to the amount of microsomal fraction added to the system, and that the incorporation of lipid and amino acid radioactivity into medium and microsomal LDL proceeded linearly over a period of 60 min.

Demonstration of Lipoprotein Synthesis de Novo

The possibility that the incorporated radioactivity represented adsorption of label on rat plasma lipoproteins or exchange of lipoprotein components with microsomal and medium radioactivity rather than synthesis de novo was considered and excluded as follows.

Temperature Dependence of Lipid and Amino Acid Incorporation into Low Density Lipoprotein by Microsomal System

The results in Table V indicate that the ratio of amino acid to lipid radioactivity remained reasonably constant when the LDL in the incubation medium was isolated by three different procedures.

Effect of Different Methods of Low Density Lipoprotein Separation on Ratio between Amino Acid and Lipid Radioactivity

The results in Table V indicate that the ratio of amino acid to lipid radioactivity remained reasonably constant when the LDL in the incubation medium was isolated by three different procedures.

Demonstration of Identity of Plasma Low Density Lipoprotein with Low Density Lipoprotein Synthesized by Microsomes of Rat Small Intestinal Epithelium

The identity between LDL in the medium and that of plasma was demonstrated by the following procedures.

**Table III**

Incorporation of lipid and amino acid radioactivity into lipoproteins by cell-free preparations of rat intestinal epithelium

Mean ± standard deviation of five experiments with cell-free preparations from the intestinal mucosa of five rats.

<table>
<thead>
<tr>
<th>Density</th>
<th>Homogenate</th>
<th>Mitochondrial fraction</th>
<th>Microsomal fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>d = 1.006 &lt; d = 1.063</td>
<td>3015 ± 250</td>
<td>552 ± 50</td>
<td>18.3 ± 3.8</td>
</tr>
<tr>
<td>d = 1.063 &lt; d = 1.21</td>
<td>980 ± 148</td>
<td>155 ± 18</td>
<td>20.4 ± 2.1</td>
</tr>
<tr>
<td>d = 1.21</td>
<td>614 ± 95</td>
<td>108 ± 21</td>
<td>21.1 ± 2.8</td>
</tr>
</tbody>
</table>

- Homogenate after removal of the nuclei plus brush borders fraction. The nuclei plus brush borders fraction was not investigated because of its gross impurity.
- Radioactivity per mg of protein in each lipoprotein fraction. Lipoprotein fractions were isolated by ultracentrifugation at the appropriate densities after 2 ml of pooled rat plasma were added to each incubation system and the radioactivities in the lipid and protein moieties were assayed. All fractions were incubated in duplicate in medium containing cofactors and 3H-labeled amino acids (see "Methods") at 0° and 37°. The values represent the difference between the radioactivities at 37° and 0°.

---

**Requirements of Microsomal Preparation for Incorporation of Lipid and Amino Acid Radioactivity in Low Density Lipoproteins**

The results in Table IV show the requirements of the microsomal preparation for incorporation of lipid and amino acids in low density lipoproteins. Presence of the soluble fraction was essential. Replacement of the soluble fraction by pH 5 enzymes (obtained from an equal volume of soluble fraction), or by soluble fraction isolated from an equal weight of liver, maintained effectively the incorporation of lipid and amino acids. Omission of the microsomes or addition of ribonuclease to the incubation system resulted in a marked inhibition of the activity. Replacement of the intestinal microsomal fraction by kidney microsomes was ineffective in promoting amino acid incorporation into medium LDL. Presence of GTP, ATP, and ATP-generating system was required for optimal incorporation of amino acid and lipid radioactivity. Addition of increasing concentrations of puromycin resulted in a progressive inhibition of the activity. Although inhibition of the incorporation of amino acid radioactivity was associated with an inhibition of the incorporation of labeled lipid, it can be seen from the ratio of amino acid to lipid radioactivity in LDL that the inhibition of amino acid incorporation always exceeded that of labeled lipid. The significance of this finding is not clear, although it may indicate that in the presence of an excess of preformed lipid, the protein moiety of LDL may become associated with a greater amount of lipid. This assumption is consistent with the suggestion of Scanu (46) that the degree of aggregation of LDL apoprotein may depend on the amount of available lipid.
**TABLE IV**

Requirements for incorporation of lipid and amino acid radioactivity in medium low density lipoproteins by microsomal fraction of rat intestinal epithelium

Low density lipoproteins were isolated by heparin-MnCl₂ precipitation after 2 ml of pooled rat plasma were added to each incubation system and the radioactivities in the lipid and protein moieties were assayed. The microsomal fraction was obtained from intestinal mucosa of 18 rats to which 8 μCi of palmitic acid-1⁻⁴C dissolved in 2 ml of olive oil were administered intragastrically 30 min before killing. The isolated microsomal fraction was resuspended in 9 volumes of either soluble fraction (obtained from intestinal mucosa of control rats), modified Littlefield Medium A (see “Methods”), or pH 5 enzyme, obtained from equivalent volumes of supernatant fraction (from control rats) and dissolved to original volume in modified Littlefield Medium A. Four milliliters of resuspended microsomal fraction (97,920 dpm) were added to the appropriate incubation systems, all of which contained ³H-labeled amino acids (see “Methods”). Incubations were done in duplicate at 0° and 37°. The values represent the difference between the radioactivities at 37° and 0°.

<table>
<thead>
<tr>
<th></th>
<th>dpm/mg</th>
<th>%</th>
<th>dpm/mg</th>
<th>%</th>
<th>% dpm/mg</th>
<th>%</th>
<th>% dpm/mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>3261</td>
<td>100.0</td>
<td>6128</td>
<td>100.0</td>
<td>1.87</td>
<td></td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>Soluble fraction omitted</td>
<td>339</td>
<td>10.4</td>
<td>570</td>
<td>9.3</td>
<td>1.08</td>
<td></td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>pH 5 enzymes instead of soluble fraction</td>
<td>3003</td>
<td>92.1</td>
<td>5607</td>
<td>91.5</td>
<td>1.86</td>
<td></td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>Boiled soluble fraction</td>
<td>447</td>
<td>13.7</td>
<td>760</td>
<td>12.4</td>
<td>1.70</td>
<td></td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>GTP omitted</td>
<td>2227</td>
<td>68.3</td>
<td>3600</td>
<td>58.9</td>
<td>1.62</td>
<td></td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>ATP omitted</td>
<td>1781</td>
<td>51.9</td>
<td>2842</td>
<td>46.5</td>
<td>1.46</td>
<td></td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>ATP and ATP generating system omitted</td>
<td>662</td>
<td>22.3</td>
<td>784</td>
<td>12.8</td>
<td>1.48</td>
<td></td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td>Microsomes omitted</td>
<td>319</td>
<td>9.8</td>
<td>411</td>
<td>6.7</td>
<td>1.28</td>
<td></td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>Puromycin added</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 μM</td>
<td>2491</td>
<td>76.4</td>
<td>4038</td>
<td>65.9</td>
<td>1.62</td>
<td></td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>20 μM</td>
<td>655</td>
<td>20.1</td>
<td>864</td>
<td>14.1</td>
<td>1.31</td>
<td></td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>600 μM</td>
<td>258</td>
<td>7.5</td>
<td>282</td>
<td>4.6</td>
<td>1.05</td>
<td></td>
<td>1.05</td>
<td></td>
</tr>
</tbody>
</table>

**Immunoelectrophoresis**—Fig. 2A shows the immunoelectrophoretic identity between plasma LDL and the LDL synthesized by intestinal microsomes in vitro. Although the electrophoretic mobility of the two precipitation bands is identical, the band of the LDL synthesized by intestinal microsomes is diffuse and not well defined. Whether this is due to heterogeneity in the structure or composition is impossible to conclude from our results. It is conceivable, however, that this difference may be accounted for by adsorption of some of the components of the incubation system on the LDL synthesized in vitro.

**Double Immunodiffusion**—Fig. 2B shows precipitation lines of complete identity between rat plasma LDL and the LDL synthesized by the microsomal preparation.

**Peptide Mapping**—The maps of trypsin-chymotrypsin digested peptides of the two LDL-apoproteins is shown in Fig. 3. It can be seen that the electrophoretic and chromatographic mobilities of the corresponding spots are identical. The map of the peptides from LDL-apoprotein isolated from the microsomal system shows three (marked with X) additional spots. The significance of this finding is not clear since it is impossible to decide whether the additional peptide spots indicate structural difference or adsorption of components from the incubation medium.

Fig. 4 shows a reasonably good correlation between the radioactivity in the corresponding peptide spots obtained from plasma LDL-apoprotein and LDL-apoprotein synthesized by the microsomal system. This correlation suggests not only the identity of the two apoproteins but also the possibility that the isolated microsomal system may reflect the function of the intact cells responsible for the synthesis of plasma LDL in vivo.
TABLE V
Ratio between amino acid and lipid radioactivity of LDL isolated by three different procedures from microsomal incubation medium

LDL was isolated by the respective methods from equivalent volumes of pooled supernatants of microsomal incubation media after addition of carrier rat plasma d < 1.006 lipoprotein and removal of the radioactivities in the lipid and protein moieties were assayed.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Amino Acid Radioactivity (dpm/mg)</th>
<th>Lipid Radioactivity (dpm/mg)</th>
<th>Ratio of Amino Acid to Lipid Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation First</td>
<td>5838</td>
<td>2996</td>
<td>2.29</td>
</tr>
<tr>
<td>Ultracentrifugation Second</td>
<td>5928</td>
<td>2650</td>
<td>2.24</td>
</tr>
<tr>
<td>Immuno-precipitation</td>
<td>5642</td>
<td>2432</td>
<td>2.32</td>
</tr>
<tr>
<td>Immuno-precipitation</td>
<td>4172</td>
<td>1846</td>
<td>2.26</td>
</tr>
</tbody>
</table>

*a Radioactivity per mg of protein in LDL.

Fig. 3. Peptide mapping of rat plasma LDL-apoprotein (PLDL) and the apoprotein of LDL isolated from microsomal incubation medium (MLDL). There is complete identity of Peptides 1 through 24. MLDL contains 3 peptides (marked with X) which were not present in PLDL. Broken outlines indicate faint spots.

Site of Completion of Low Density Protein in Microsomal System

The microsomal system was inadequate for investigation of the site of association of the lipid and protein moieties of LDL. The possibility that completion of LDL could have occurred outside the microsomes could not be excluded with certainty. However, support for the possibility that completion of the LDL in the incubation media may have occurred in the microsomes was provided by the following experiments. Incubated and twice washed microsomes were solubilized with 2 M LiCl, and LDL in the supernatant and of the respective incubation media was isolated by ultracentrifugation, by polyanion precipitation, and by immunoprecipitation. These studies showed that the ratio of amino acid and palmitate radioactivity in LDL isolated by three different procedures remained reasonably constant and comparable to that of the medium LDL. In addition, complete identity between medium and microsomal LDL was demonstrated by double immunodiffusion using plasma LDL antiserum (R3). When the supernatant of LiCl-solubilized microsomes was equilibrated for 18 hours at 4°C with the lipids of unlabeled postmicrosomal supernatant, the ratio of amino acid and palmitate radioactivity of the subsequently isolated LDL remained within 10% of that of the LDL isolated before the equilibration. Since the amount of lipid in the postmicrosomal supernatant was much greater than that of the isolated LDL, the possibility of significant exchange or of association of the lipid and protein moieties of LDL outside the microsomes would appear to be unlikely.

DISCUSSION

This study was designed to investigate whether or not cell-free preparations of small intestinal mucosa could synthesize the lipid and protein moieties of lipoproteins. All the investigated fractions of intestinal mucosal homogenates were able to incorporate lipid and amino acid radioactivity into lipoproteins of different densities (Table III). The activity of the microsomal fraction, however, accounted for most of the activity of the whole homogenate. We decided to limit our studies to the biosynthesis of β-lipoproteins by intestinal microsomal preparations for the following reasons. Reasonably pure preparations of β-lipoprotein can be obtained by density centrifugation and by polyanion precipitation (36, 47). β-Lipoprotein is homogenous with regard to its apoprotein composition (47), and since its presence has been demonstrated in chylomicrons (48) and VLDL (39, 47), demonstration of biosynthesis of β-lipoprotein would provide indirect evidence for the intestinal contribution to the synthesis of chylomicrons and VLDL. Finally, the possibility of contamination of β-lipoproteins by highly labeled, nonrelated proteins is considerably lower than that of the much larger fat particles (17).

The obtained results indicate that intestinal microsomal preparations can synthesize and release the lipid and protein moieties of β-lipoprotein. In addition, the identity of the protein moiety of the LDL synthesized by the microsomal system to that of plasma LDL was demonstrated by peptide mapping and immunochromatographic techniques. These results indicate that the intestinal mucosa may contribute significantly to the level of plasma LDL and that through its participation in the formation of chylomicrons and...
VLDL it may be of importance in the intestinal transport of exogenous and endogenous lipids. The results provide further support for the intestinal origin of thoracic duct (9–11) and mesenteric lymph lipoproteins (4, 18, 19), and therefore eliminate the uncertainties related to the possibility of lipoprotein or apoprotein contribution from sources other than the intestine.

Although there is ample morphological (23, 24, 49) and biochemical (1) evidence that lipid and protein synthesis takes place within the endoplasmic reticulum, the site of lipoprotein completion, whether within or outside the intestinal epithelial cell, is not known. Our results with LiCl-solubilized microsomes, showing release of LDLs, immunologically identical and with 14C:H ratios analogous to that of the medium LDL, strongly support the possibility that completion of this lipoprotein may occur in the cellular equivalent of the microsomal fraction. This possibility is supported by recent electron microscopic and radioautographic studies (50) showing appearance of lipoprotein particles within the cisternae of the endoplasmic reticulum and Golgi apparatus of liver cells. In addition, Mahley, Hamilton, and LeQuire (51) were able to isolate lipoprotein particles from the Golgi apparatus of rat liver, which exhibited morphological, floational, chemical, and immunological similarity with plasma very low density lipoproteins. There are many similarities between lipoprotein synthesis in the liver and the small intestine. The participation of the Golgi apparatus in the process of lipid absorption has been implicated by Pulay and Karlin (52). Following a fatty meal, lipid droplets, morphologically similar to lipoprotein particles in the intercellular spaces and lymphatics (23), were observed in the Golgi apparatus of the mucosal cells of the rat small intestine. Further support for the possibility that the association between the lipid and protein moieties of lipoproteins may occur within the endoplasmic reticulum or Golgi apparatus of the intestinal mucosal cells was provided by the observation of Sабесин et al. (53) that inhibition of protein synthesis produced a block in the transport of intramural lipid to the lymphatics. A similar blockage of intestinal lipid transport occurs in the rare human disease, abetalipoproteinemia, which is attributed to a hereditary defect in the ability for synthesis of the apoprotein of LDL (54) or to a failure in the association between the lipid and protein moieties of LDL (21). The presence of the apoprotein of LDL in chylomicrons (48) and in VLDL (39, 47) and the observation that patients with abetalipoproteinemia cannot form chylomicrons and VLDL (54) suggest that the intestinal synthesis of LDL may have a central role in the transport of dietary and endogenous lipid.

REFERENCES

32. BURTON, K., Biochem. J., 63, 315 (1956).