Biosynthesis of Lipoprotein by Rat Intestinal Mucosa*

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SUMMARY

Lymph chylomicrons are at the lipid-rich end of the spectrum of the lipoproteins. The nature of their protein moiety and its biosynthesis have not yet been fully elucidated. Observations are reported on the incorporation of radioactive leucine into a "lowest density lipoprotein" by mucosal cells of the small intestine isolated from the rat during absorption of fat. The amino acid incorporation exhibited the characteristic energy requirement and sensitivity to inhibitors of other systems for biosynthesis of proteins. The protein moiety was not homogeneous, but appeared to consist of a small number of species that could be separated by thin layer electrophoresis in starch granules. Similar, but nonidentical, electrophoretic patterns were obtained with the residual soluble intracellular protein of the intestinal mucosa and with chylomicrons of intestinal lymph.

The lowest density lipoprotein was isolated from a homogenate of the mucosal cells. After removal of lipids by extraction with organic solvents, the protein moiety exhibited several unusual properties in addition to its affinity for lipids: (a) a predominance of apolar amino acids, (b) resistance to fractionation procedures involving ionic properties, (c) considerable solubility in organic solvents, and (d) peculiarities of the peptide map that were related to the foregoing features. Peptide maps were obtained after proteolytic digestion of the protein moieties of the lowest density lipoprotein fraction, the residual soluble protein, and lymph chylomicrons by means of two-dimensional electrophoresis and chromatography. Unusual maps were obtained in which most of the peptide fragments failed to move upon electrophoresis over a broad pH range, but were readily resolved by chromatography in organic solvents. Strong, although imperfect, resemblance was noted among the peptide maps of all three protein fractions. Preliminary studies of low and high density lipoproteins of rat serum revealed different, more complex peptide maps.

No definitive conclusion could be drawn about the specificity of the protein which becomes associated with lipid during absorption through the intestinal mucosa. A speculative interpretation of the aforementioned similarity in peptide maps led us to suggest that lipids in transit become associated with some of the more abundant soluble proteins of the mucosal cells and that the resulting lipoprotein complexes appear in the chylomicrons of intestinal lymph.

Thin layer methods of chromatography and electrophoresis were extensively employed. Their convenience and great sensitivity permitted detailed studies to be carried out on submilligram quantities of material.

Lymph chylomicrons appear to contain a small amount of protein and thus may be included in the category of "lipoproteins" (3-6). The amount of protein present, its identity, and the locus of its biosynthesis continue to be unsettled points (7-10).

There is evidence, first reported by Bragdon in 1958, that the chylomicron protein is at least partly synthesized by the intestine (5). In 1959 Rodbell and Fredrickson (7) and Rodbell, Frederickson, and Ono (8) characterized three proteins which were associated with lymph chylomicrons of the dog and human. One of these proteins was believed to be identical with the protein moiety of plasma high density lipoprotein. Indirect experiments have also suggested a relationship between high density lipoprotein and chylomicrons (11-13). A second protein may have been the same as one found in the very low density (triglyceride-bearing) fraction of the plasma lipoproteins. These two proteins were believed to be synthesized by the intestine on the basis of data on incorporation of radioactive amino acids. Except for a single experiment in vitro with isolated mucosal cells of the dog, contribution of the proteins by the liver through its lymphatic drainage could not be excluded because the cannulated thoracic duct probably transported both hepatic and intestinal lymph. Biosynthesis of the major serum lipoproteins by the liver has been well established by experiments in vivo and in vitro (14, 15).

More recently, clinical observations in patients with inborn errors of lipoprotein metabolism have raised provocative questions concerning the role of lipoproteins in the intestinal absorption of fat. Patients with Tangier disease, who appear to have a hereditary deficiency of α- or high density lipoprotein, have no impairment of intestinal fat absorption (16). On the other hand, the deficiency of β-lipoprotein, which was not implicated in fat absorption by the biochemical experiments cited above, involves a severe impairment in the absorption of triglycerides of long chain fatty acids (17). The fat appears to enter the intestinal
Mucosal cells from oil-fed rats were incubated for 24 hours at 37°C in a Dubnoff shaker under a gas phase of 95% O₂-5% CO₂. The incubation medium contained 5 ml of Krebs-Ringer-bicarbonate buffer (pH 7.5) (20), 5 mg of glucose, 3 mg of penicillin, 0.05 mg of streptomycin, and 10 μC of L-leucine-14C (specific activity, usually 240 μC per mmole). At the end of incubation, 1 mg of nonradioactive leucine was added. Control vessels were incubated, but label was added at the end, after the carrier leucine. Tissue and medium were homogenized and fractionated in the preparative ultracentrifuge (see the text).

The rats were fasted for 18 hours and then were given pellets of chow which had been soaked in olive oil or corn oil. Ninety minutes after the pellets had been eaten (during active fat absorption), the rats either were operated upon under pentobarbital anesthesia or were killed by a blow on the head and cervical dislocation.

To the bottom fraction (4 ml) of the original step were added 4 ml of water (4 ml) was layered on top in the original tube, and centrifugation was repeated for 1 hour at 64,000 x g at 14° to remove a small amount of contaminating protein. The tubes were then cut with a Spincor tube slicer to yield a top fraction of approximately 2 ml containing all of the turbid lipoprotein of lowest density. The bottom fraction of this protein was used for analysis.

Methods

Animal Preparation—Male Sprague-Dawley rats weighing 150 g were used for the isotope incorporation experiments in vitro. Larger animals were used for cannulation of the intestinal lymphatic channels or for preparation of unlabeled protein in quantity. The rats were fasted for 18 hours and then were given pellets of chow which had been soaked in olive oil or corn oil. Ninety minutes after the pellets had been eaten (during active fat absorption), the rats either were operated upon under pentobarbital anesthesia or were killed by a blow on the head and cervical dislocation.

Collection of Intestinal Chyle—The chylous main intestinal lymphatic channel was exposed and cannulated with PE-50 polyethylene tubing. After stable lymph flow was established, 35 μC of 14C-labeled Chlorella hydrolysate were administered into the stomach, followed by 1 ml of corn oil. At the end of the experiment blood was collected by cardiac puncture and the intestinal mucosa was removed as described below.

Preparation of Intestinal Mucosal Cells—The small intestine was irrigated with cold Ringer's or NaCl solution, and the duodenum and jejunum were excised. The mucosal cells were extruded onto an ice-cold glass plate by scraping with a spatula along the serosal surface (19). This preparation appeared to consist largely of whole cells under phase contrast microscopy.

Incorporation of L-leucine into lipoproteins by rat intestinal mucosa cells

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<table>
<thead>
<tr>
<th>Ultracentrifugal fraction</th>
<th>Flotation conditions</th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowest density lipoprotein</td>
<td>1.005 g/ml 0.5 ar X g</td>
<td>0.516 771 mg</td>
<td>0.600 2.7 mg</td>
</tr>
<tr>
<td>Very low density lipoprotein</td>
<td>1.005 g/ml 16 ar X g</td>
<td>1.63 88</td>
<td>1.58 2.5</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>1.06 g/ml 20 ar X g</td>
<td>1.03 20</td>
<td>1.19 0</td>
</tr>
<tr>
<td>High density lipoprotein</td>
<td>1.21 g/ml 40 ar X g</td>
<td>1.01 21</td>
<td>0.90 0</td>
</tr>
<tr>
<td>Residual soluble protein</td>
<td>1.21 g/ml Sediment</td>
<td>3.61 85</td>
<td>3.27 8</td>
</tr>
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</table>

* In most subsequent experiments these three fractions were combined by proceeding directly to density 1.21 after the first fractionation step.

Incorporation of L-leucine into lipoproteins by rat intestinal mucosa cells

EXPERIMENTAL PROCEDURE

Materials

L-Leucine-14C, labeled uniformly or in the carboxyl group, and Chlorella protein hydrolysate, labeled uniformly with 14C, were purchased from New England Nuclear Corporation. Penicillin, actinomyacin D, and chloramphenicol were gifts from Dr. M. L. Lamborg, Dr. A. Aisenberg, and Mr. J. T. Murphy, respectively. Crystalline bacterial protease (Nagarse) was obtained from the Enzyme Development Corporation, New York. Trypsin (trichloroacetic acid-treated) and pepsin were products of Worthington. Pronase, chymotrypsin, and ribonuclease were obtained from Calbiochem.

Incorporation of 14C-leucine into lipoproteins by rat intestinal mucosa cells

We present here observations on the biosynthesis of lipoprotein carried out by isolated mucosal cells of the rat, and a comparison from which they arise. However, the nature of this protein differs significantly from that of the lymph lipoproteins obtained in earlier experiments with whole animals of other species (7–9).

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fractions from the first and second centrifugation steps represented lipoproteins of lowest density (corresponding roughly to lymph or serum chylomicrons) and other lipoproteins, respectively. The second bottom fraction (d > 1.21) represented residual soluble cellular proteins. The two top fractions and 2-ml aliquots of the second bottom fractions were treated with 10 ml of 10% trichloracetic acid solution (containing 0.1 mg of nonradioactive leucine carrier per ml) and were allowed to stand overnight at 4°C.

Protein was determined on the fractions by the method of Lowry et al. (21). Turbidity and solid KBr, if present, were eliminated by centrifugation at 3000 rpm before the samples were read in the spectrophotometer. Readings were performed at 750 μm, or at 500 μm for larger amounts of protein. Crystalline bovine serum albumin was used as the standard.

Aliquots of the suspension of precipitated proteins containing 1 mg of less of protein were filtered on Millipore filters (diameter 24 mm; pore size, 0.8 μ) by the method of Loftheld and Eigner (22). The filters were washed with 20 ml of the above trichloracetic acid solution and then with 15 ml of chloroform-methanol (2:1) to remove lipids. The filters were dried in air and placed on the bottom of vials; then 5 ml of sodium mixture (containing, per liter of toluene, 5 g of 2,5-diphenyloxazole and 0.3 g of β-bis 1,2′(8′-phenyloxazoly)benzene were added. The samples were counted in a Packard liquid scintillation counter. The results were expressed in terms of counts per min per mg of protein. The amount of protein was measured as described above because it proved impossible to obtain reproducible measurements directly upon the Millipore filters. As will be shown below, the amount of protein is overestimated in this way. A rather constant proportion of the material giving the color reaction apparently consists of peptides of relatively low molecular weight, which are soluble in trichloracetic acid and pass through the Millipore filter but are retarded by Sephadex G-25. The correction factor for the true protein concentration varies with experimental conditions from less than 0.5 to about 0.7, but would be fairly constant within a group of similar samples.

**Purification of Lipoprotein and Residual Protein on Sephadex Columns**—Preliminary experiments with thin layer chromatography of lowest density lipoprotein and residual protein on Sephadex G-25 (23) revealed evidence for the presence of low molecular weight peptide material in both fractions (24, 25). It was considered desirable to eliminate such peptides before treatment of the proteins with proteolytic enzymes. Each fraction was therefore passed through a column 0.9 cm in diameter which contained 7 g (dry weight) of Sephadex G-25 (coarse beads) with distilled water as eluent. Fractions were collected manually and observations were made of turbidity, protein or peptide, radioactivity, and the colored zones (when appropriate) in the eluate.

**Digestion with Proteolytic Enzymes**—The material of the first peak from the Sephadex column was highly turbid (resembling chylomicrons) in the case of the lowest density lipoprotein fraction. The first peak of the residual proteins was optically clear. Solutions of these materials, containing 0.5 to 5 mg of protein, were lyophilized on glass beads approximately 75 μm in diameter (Prismo Safety Corporation, Huntingdon, Pennsylvania) in a procedure analogous to that presented by Gunatafson (26). Lipids were extracted from the protein-laden beads by shaking three times with chloroform-methanol (2:1, v/v). The beads were then incubated with the protease Nagarse (27) (20 to 50 μg per mg of protein) in water solution for 18 hours at 37°C with gentle shaking. No turbidity suggesting bacterial contamination was noted.

The peptides released by the enzyme were extracted from the beads with water, and the solution was concentrated under a stream of N₂ gas. The peptides were finally obtained in a very small volume of water. Any insoluble matter was centrifuged, and the yield of peptides was estimated by the method of Lowry et al. (21).

**Peptide Mapping and Localization of Radioactive Peptides**—Thin layers (0.375 to 0.50 mm) of Whatman Chromata cellulose (without binder, type CC41) were prepared on glass plates (20 x 20 cm) with a Desaga apparatus (Reinkemeyer Instruments). After brief drying in air, the plates were heated for 30 min at 105°C. An estimated 25 to 100 μg of peptides were applied to the plates in 1-μl portions with drying in air until the total aliquot was added. The plates were then sprayed with the buffer for electrophoresis (guarding the sample application zone) and placed in a cell (E-C Apparatus Corporation, Philadelphia). Wicks of Whatman G F/B glass fiber paper were applied to the ends of the plate, and a clean glass plate or the cell cover was placed on top, separated by spacers from the thin layer plate. Coolant at 15°C was circulated through the lower cooling plate. A regulated potential of 800 volts (16.5 volts per cm with a current of approximately 25 ma) was applied for 20 to 40 min. The usual electrophoretic buffer consisted of pyridine-acetic acid-water (1:10:189) at pH 3.5 (28). In some instances, pyridinium-acetate buffer at pH 6.5 and triethylamine-formate buffer at pH 9.5 were used. The volatile buffers were subsequently removed by a stream of air.

After the electrophoretic run had been completed, ascending chromatography was performed in the second dimension in 1-butanol-acetic acid-water (4:1:1). When the buffer had evaporated, the plates were sprayed with 0.4% ninhydrin in 10% aqueous 2-propanol containing 5% (v/v) collidine, and the color was developed by warming the plates in a gentle flow of steam for 30 minutes. Peptides containing radioactive leucine were located by scraping the cellulose in the ninhydrin-positive spots and appropriate blank areas with a razor blade. The samples were transferred to counting vials and overlaid with 5 ml of toluene scintillation fluid. A sample of protein hydrolysate was dissolved in citrate buffer at pH 5 and placed in a cell (E-C Apparatus Corporation, Philadelphia). Slight quenching by ninhydrin-peptide colors was not significant.

**Total Hydrolysis and Amino Acid Composition of Lipoprotein Proteins**—The material of the first, turbid peak from the Sephadex column treatment of the lowest density fraction was extracted with organic solvents by the method of Peterson (9) to remove lipids. The protein residue was transferred to a glass ampoule with water and 88% formic acid, and the solvents were removed on a rotary evaporator. For about 1.5 mg of protein, 5 ml of redistilled constant boiling HCl were added. The mixture was frozen and evacuated with an oil pump for 5 min; partial thawing was allowed. The ampoule was then sealed under vacuum and heated in a sand bath at 110°C for 40 hours.

The ampoule was opened, and its contents were transferred to a tube in which three extractions with 5 ml of hexane were carried out for removal of residual lipid. The HCl was removed on the rotary evaporator and several additions of water were likewise removed. The residue was taken up in a small volume of water.

A sample of protein hydrolysate was dissolved in citrate buffer...
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Figure 1. Incorporation of amino acids into the ultracentrifugal lipoprotein fractions of lymph in a rat with cannulated intestinal lymphatic channels. After continuous lymph flow was established during fat absorption, 35 μC of 14C-labeled Chlorella protein hydrolysate were administered into the stomach at the time indicated by the arrow. Curve 1 is the time course of specific radioactivity of lymph chylomicron protein; Curve 2, very low density lipoprotein of lymph (d < 1.005); Curve 3, low plus high density lipoprotein of lymph (d 1.005 to 1.21); Curve 4, residual proteins sedimenting at d 1.21.

**TABLE II**

Incorporation of 14C-labeled Chlorella hydrolysate amino acids into lymph, serum, and intestinal mucosal fractions in vivo

Intestinal lymph (1.4 ml) was collected from an operated rat between 30 and 60 min after intragastric administration of 35 μC of uniformly labeled amino acid mixture. At the end of the collection period, serum and a homogenate of intestinal mucosal cells were obtained and fractionated as indicated below. Protein content and radioactivity were determined as in "Methods."

<table>
<thead>
<tr>
<th>Ultracentrifugal fraction</th>
<th>Flotation conditions</th>
<th>Protein</th>
<th>Specific radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph</td>
<td>Density (g/ml)</td>
<td>Time (hr)</td>
<td>Force (X)</td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>1.005</td>
<td>0.5</td>
<td>26,000</td>
</tr>
<tr>
<td>Very low density protein</td>
<td>1.005</td>
<td>16</td>
<td>105,000</td>
</tr>
<tr>
<td>Residual soluble protein</td>
<td>1.21</td>
<td>Sediments</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>1.005</td>
<td>0.5</td>
<td>20,000</td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>1.005</td>
<td>16</td>
<td>105,000</td>
</tr>
<tr>
<td>Very low density protein</td>
<td>1.005</td>
<td>16</td>
<td>105,000</td>
</tr>
<tr>
<td>Residual soluble protein</td>
<td>1.21</td>
<td>Sediments</td>
<td></td>
</tr>
</tbody>
</table>

Table II continued...

**TABLE III**

Incorporation of 14C-leucine into lipoproteins by mucosal cells from fasted or fed rats

Rats were fasted for 18 hours and were killed either at once or 90 min after eating a pellet of chow soaked in olive oil. Incubation conditions were as described in Table I.

<table>
<thead>
<tr>
<th>Ultracentrifugal fraction</th>
<th>Fasted rats</th>
<th>Fed rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (mg)</td>
<td>Specific activity (cpm/mg)</td>
</tr>
<tr>
<td>Lowest density lipoprotein (40 hours; 20,000 X g; d = 1.005)</td>
<td>0.318</td>
<td>12,150</td>
</tr>
<tr>
<td>Other lipoprotein (40 hours; 105,000 X g; d = 1.21)</td>
<td>1.36</td>
<td>2,003</td>
</tr>
<tr>
<td>Residual soluble protein (sediments at d = 1.21)</td>
<td>5.18</td>
<td>8,920</td>
</tr>
</tbody>
</table>

at pH 2.2 and subjected to chromatography on a Beckman/Spinco amino acid analyzer. Another sample, equivalent to 0.1 mg, was applied to a plate of silica gel G (20 X 20 cm; 0.5-mm layer thickness), which had been prepared by drying in air at room temperature (30). This was subjected to ascending chromatography in a first dimension solvent of 1-butanol-acetic acid-water (4:1:1) and a second dimension solvent of phenol-water (75:25) with 20 mg of KCN (31). The amino acid pattern was demonstrated with half-strength ninhydrin spray, and

*We are indebted to Dr. S. C. Hartman for this analysis.*
TABLE IV
Effect of inhibitors on leucine incorporation

In incubation conditions were as described in Table I, except for the presence of inhibitory compounds. Lipoprotein fractions were isolated as indicated in Table II.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Lowest density lipoprotein</th>
<th>Other lipoprotein</th>
<th>Residual soluble protein</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Activity</td>
<td>Inhibition</td>
<td>Activity</td>
</tr>
<tr>
<td>Puromycin</td>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Experiment A</td>
<td>0</td>
<td>28,500</td>
<td>0</td>
<td>6,570</td>
</tr>
<tr>
<td></td>
<td>0.4 $\times 10^{-4}$ M</td>
<td>12,170</td>
<td>57</td>
<td>2,320</td>
</tr>
<tr>
<td>Experiment B</td>
<td>0</td>
<td>771</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>Experiment C</td>
<td>0</td>
<td>670</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Experiment A</td>
<td>6 $\times 10^{-4}$ M</td>
<td>520</td>
<td>226</td>
<td>1,042</td>
</tr>
<tr>
<td>Experiment B</td>
<td>0</td>
<td>416</td>
<td>97</td>
<td>323</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>6 $\times 10^{-4}$ M</td>
<td>300</td>
<td>28</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>2 $\times 10^{-4}$ M</td>
<td>3,260</td>
<td>500</td>
<td>1,110</td>
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<tr>
<td></td>
<td>1 $\times 10^{-5}$ M</td>
<td>3,300</td>
<td>0</td>
<td>2,000</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>0</td>
<td>2,810</td>
<td>20</td>
<td>1,095</td>
</tr>
<tr>
<td>Experiment A</td>
<td>2 $\mu g/ml$</td>
<td>414</td>
<td>26</td>
<td>104</td>
</tr>
<tr>
<td>Experiment B</td>
<td>0</td>
<td>310</td>
<td>25</td>
<td>110</td>
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<tr>
<td>2,4-Dinitrophenol</td>
<td>2 $\mu g/ml$</td>
<td>31</td>
<td>25</td>
<td>110</td>
</tr>
<tr>
<td>Experiment A</td>
<td>0</td>
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</tr>
<tr>
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<td>2 $\times 10^{-4}$ M</td>
<td>31</td>
<td>93</td>
<td>122</td>
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as high a specific activity, although, of course, its protein content was much higher than that of the lipoprotein fractions.

In a short term experiment, lymph (1.4 ml) was collected between 30 and 60 min after administration of the labeled amino acids. At the end of the collection period, serum and a homogenate of intestinal mucosal cells were obtained. The results are presented in Table II. In each tissue the chylomicron protein had the highest specific activity. The high level shown by the lymph chylomicrons in comparison to the mucosa is explained by the collection period, which extended into an earlier phase when the mucosa probably possessed a higher radioactivity than when the tissue was removed. The lower level in the serum chylomicrons may be due to dilution with unlabeled serum proteins or to persistence of unlabeled chylomicrons formed before administration of the labeled mixture.

**Incorporation of Radioactive Leucine into Protein Fractions of Intestinal Mucosa Cells In Vitro**

**Characteristics of System**—The incorporation of labeled leucine into mucosal protein proceeded at a constant rate for at least 2 hours. The total amount of incorporation into all fractions was variable. Values ranged in different experiments from 0.01 to 0.5% of the added radioactivity. Incorporation was consistently higher in 150-g rats than in larger animals.

Glucose was required in the incubation medium when the rats had been fasted or fed pure olive oil by stomach tube, but was not required when they had been fed a pellet of oil-soaked chow. The requirements for penicillin and streptomycin were not clear, but it was felt that their presence helped to keep the incorporation low in control vessels. These antibiotics were present in all the incubation experiments reported.

The distribution of incorporated radioactivity in the protein fractions derived from the successive ultracentrifugations is presented in Table I. In other experiments the specific activity of the residual soluble protein fraction (d > 1.21) was higher than in this case, but the lowest density lipoprotein fraction nearly always exhibited the highest specific activity.

Little relationship existed between the incorporation of radioactive amino acid and the richness in absorbed lipid of the isolated mucosal cells, as estimated by their appearance or by measurement of triglyceride by a modification of the method of Van Handel (38). The specific radioactivities of lipoprotein and residual protein fractions also did not differ greatly when fed and fasted rats were compared (Table III). It was of interest that, when approximately equal amounts of mucosal tissue were used from fed or fasted rats, the same amount of protein was found in the ultracentrifugal fraction of lowest density. However, this fraction was markedly turbid when derived from fed rats and was optically clear when derived from fasted rats. Thin layer chromatography on silica gel of a chloroform-methanol extract of the lowest density fraction from fasted rats revealed the presence of cholesterol and cholesterol esters, triglycerides, and lecithin. Small quantities of diglycerides and nonesterfied fatty acids were also tentatively identified. The glycerides showed overwhelming predominance in a similar extract from an oil-fed rat.

The lowest density lipoprotein and residual protein fraction each contained RNA, approximately 3% of the amount of protein. From 7 to 15% of the protein (Lowry reaction) of lowest density lipoprotein and lymph chylomicrons was soluble in chloroform-methanol (2:1).

**Effect of Inhibitors on Amino Acid Incorporation**—Data on the effects of several compounds known to inhibit various aspects of cell metabolism are presented in Table IV. The results are consistent with those obtained in certain other mammalian systems in which protein biosynthesis occurs within intact cells (39, 40). However, no attempt has been made at a thorough study of experimental design or inhibitor concentrations.

**Studies on Homogeneity of Lipoprotein and Residual Protein Fractions**

It would not be expected that the lipoprotein or residual protein fractions isolated from cellular homogenates in the manner...
described would have been found to consist of single homogeneous proteins because no techniques of high resolving power were employed. It was important, however, to learn something about the degree of heterogeneity to provide a basis for interpretation of the succeeding studies of peptide mapping and amino acid composition. In order to minimize the technical complications resulting from a large amount of lipid in the lowest density lipoprotein, these fractions were isolated from fasted animals and were studied by electrophoresis and chromatography after purification by Sephadex column chromatography. The studies are summarized in Fig. 2.

The lowest density lipoprotein and the residual soluble protein exhibited similar behavior in several systems. Thin layer electrophoresis on starch granules gave the best resolution available (Fig. 2A). The lowest density lipoprotein had one major component and a minor one which moved only a short distance toward the cathode (perhaps owing to electroendosmosis), and another minor component moving rapidly toward the anode.

![Fig. 3. Peptide maps on thin layers of cellulose obtained after extraction of lipids and digestion with Nagarse. A, lowest density lipoprotein fraction; B, lymph chylomicrons; C, residual soluble protein fraction sedimenting at $d = 1.21$. In each instance, the first (horizontal) dimension represents movement during electrophoresis at pH 3.5 in pyridine-acetate buffer, and the second (vertical) dimension represents movement during ascending chromatography in 1-butanol-acetic acid-water (4:1:1).]
The residual soluble protein appeared to have the same three components, but in nearly equal quantities, plus a minor cathodal band. Lymph chylomicrons from which lipids had been extracted revealed two components, one apparently corresponding to the fast anodal fraction of mucosa and the other having approximately the mobility of the main slow band of the lowest density lipoprotein.

Thin layer chromatography on DEAE-cellulose (Fig. 2B) showed a single mobile component (of slightly different mobility) for the two mucosal fractions. Some of the residual soluble protein remained at the origin, possibly corresponding to the prominent anodal band seen in electrophoresis. Column chromatography of the lowest density lipoprotein on DEAE-Sephadex A-50 was carried out at pH 8.4 with a gradient of Tris-C1 buffer. The protein was eluted in a single peak beginning at a buffer concentration of about 0.22 M.

Disk electrophoresis in acrylamide gel columns (41) was relatively ineffective because most of the protein of the mucosal and lymph lipoproteins remained within the sample gel, apparently uninfluenced by the applied potential.

Thin layer chromatography on Sephadex G-100 (fine) (Fig. 2C) showed single zones for the two mucosal fractions. Comparison of their mobilities with those of pure proteins permitted estimation of molecular weights of 110,000 for lowest density lipoprotein and 200,000 for residual soluble protein (42, 43). It is possible that the Sephadex zones represent stable aggregates of smaller monomers, since we have observed a reversible aggregation of the lowest density fraction (after extraction of lipid) when the ionic strength was lowered by electrodialysis, and some aggregation with precipitation during storage at 4°C.

### Mapping of Peptides Derived from Proteolytic Digestion of Protein Fractions

**Protein Digestion with Various Enzymes**—Digestion of the lowest density lipoprotein and residual soluble protein fractions, after Sephadex column treatment, was carried out principally with Nagarse. On the basis of estimation of the peptide products with the reaction of Lowry et al. (21), this enzyme gave more extensive hydrolysis of both fractions than did several other proteases. The yield of soluble peptides recovered after delipidation was about 50%; significant mechanical losses occurred in the manipulation of small quantities. Part of the protein was an insoluble “core” which was removed by centrifugation. Further digestion of this “core” with Pronase or Nagarse gave a small additional yield of soluble peptides with practically the same peptide map as the original Nagarse digest.

Somewhat smaller yields of soluble peptides were obtained by digestion of the lowest density lipoprotein fraction with trypsin in the presence of urea (7), with or without subsequent addition of chymotrypsin. Single experiments were also carried out with pepsin and papain, which gave similar results.

**Behavior of Peptides in Two-dimensional Electrophoretic Chromatography**—There was a strong resemblance between the peptide maps of the lowest density lipoprotein and residual protein fractions (Fig. 3, A and C). Both maps were unusual in that the majority of the peptides did not move at all in the electric field, or moved only a distance consistent with electrodialysis. The electrophoretic immobility of most of the peptides was observed at pH 3.5, 4.5, 6.5, and 9.5. In the second (chromatographic) dimension, the electrophoretically immobile peptides were resolved into about seven spots distinguishable on the basis of mobility or color after ninhydrin spraying, or both. None of the material remained adsorbed at the origin. The electrophoretically mobile peptides were resolved into small numbers of anodal and cathodal spots at pH 3.5, and their mobility varied appropriately with pH.

The final two-dimensional pattern consisted of a column of peptides that was resolved only in the chromatographic dimension and a small number of peptides that moved in both dimensions. Thus large regions of the map were devoid of any detectable peptides. However, many experimental variations of these techniques failed to produce greater resolution of the peptide mixture. Under similar conditions, proteolytic digests of hemoglobin, ribonucleic acid, and albumin were well resolved over all regions of the map.

### Amino Acid Composition of Lowest Density Lipoprotein Fraction

**Total Amino Acid Composition**—The amino acid analysis of the lowest density lipoprotein is presented in Table V. The presence of a significant amount of material tentatively identified as hexosamine is noteworthy. The very large amount of NH₃ observed cannot as yet be explained; it is far in excess of that which could have been bound in amide linkage with the

---

**Table V**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Content*</th>
<th>Integral No. of residues</th>
<th>Amino acid</th>
<th>Content</th>
<th>Integral No. of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/1.48 mg protein</td>
<td></td>
<td></td>
<td>µmoles/1.48 mg protein</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.291</td>
<td>9</td>
<td>Glycine</td>
<td>0.386</td>
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<tr>
<td>Histidine</td>
<td>0.110</td>
<td>4</td>
<td>Alanine</td>
<td>0.323</td>
<td>10</td>
</tr>
<tr>
<td>Ammonia</td>
<td>4.72</td>
<td></td>
<td>Cysteine</td>
<td>0.246</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>0.230</td>
<td>7</td>
<td>Valine</td>
<td>0.330</td>
<td>11</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.403</td>
<td>13</td>
<td>Methionine</td>
<td>0.100</td>
<td>4</td>
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<tr>
<td>Threonine</td>
<td>0.257</td>
<td>8</td>
<td>Isoleucine</td>
<td>0.242</td>
<td>8</td>
</tr>
<tr>
<td>Serine</td>
<td>0.298</td>
<td>10</td>
<td>Leucine</td>
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<tr>
<td>Glutamic acid</td>
<td>0.450</td>
<td>14</td>
<td>Tyrosine</td>
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<tr>
<td>Proline</td>
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<td>8</td>
<td>Phenylalanine</td>
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<td>8</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>4.514</td>
<td>146</td>
</tr>
</tbody>
</table>

* Protein estimated by method of Lowry et al. (21).

* In estimating number of residues, greatest weight was given to eight amino acids which are most stable during acid hydrolysis, and allowances were made for unstable or slowly released residues.

* Sum of methionine and isomeric sulfoxide peaks.

* Excludes ammonia.
dicarboxylic amino acids, but a part may have been derived from degradation of hexosamine.

A portion of the foregoing lowest density lipoprotein was carried through the acid hydrolysis and then subjected to two-dimensional partition chromatography on a thin layer of silica gel G. Two unidentified ninhydrin-positive spots were noted: one was between aspartic and glutamic acids and the other was between histidine and proline. The latter may be hexosamine.

**Amino Acid Composition of Separated Acidic, Neutral, and Basic Peptide Fractions**—A sample of 400 µg of peptide digestion mixture from the lowest density lipoprotein was separated in the electrophoretic dimension only at pH 4.5. After a guide strip at the edge of the plate was colored with ninhydrin, the acidic, neutral, and basic peptide regions were scraped from the plate and eluted from the cellulose. The three fractions were hydrolyzed with HCl, and the amino acid mixtures were analyzed by the two-dimensional thin layer technique described. Three noteworthy features were revealed: (a) the neutral peptides contained an essentially complete complement of amino acids; (b) 50% of the residues in the acidic peptides were dicarboxylic amino acids, with small amounts of several other amino acids, and 46% of the residues of the basic peptides were basic amino acids, with only a few others present; (c) about 80% of the total leucine plus isoleucine content was in the neutral peptide fraction, with about 10% each in the acidic and basic peptide fractions. The incorporation of radioactive leucine was likewise limited almost entirely to the neutral peptides.

**Peptide Maps of Rat Lymph Chylomicrons and Serum Lipoproteins**

Peptide maps were prepared for lymph chylomicrons (Fig. 3D) and chylomicrons, very low density, low density (β), and high density (α1) lipoproteins of serum (Fig. 4). The pattern obtained from lymph chylomicrons was nearly, but not quite, identical with that of the lowest density lipoprotein of intestinal mucosa.

When our technique was applied to the serum lipoprotein fractions, the results were not so satisfactory nor reproducible as with the mucosa and lymph. Drawings of the peptide maps for each serum fraction are presented in Fig. 4. The chylomicron pattern had a column of electrophoretically immobile peptides which was complex in appearance (resembling a double column). There were two streaked zones moving toward the anode that were similar to those of mucosa and lymph, but additional anodal peptides were also present. The very low density lipoprotein represents movement during ascending chromatography in 1-butanol-acetic acid-water (4:1:1). Dashed lines, continuous lines, and filled outlines represent increasing intensity of ninhydrin staining. Peptide colors differing from the usual violet are indicated in two instances.
exhibited a simple pattern with over-all resemblance to those of the lowest density fraction and lymph chylomicrons. Close examination, however, showed a lack of correspondence of the individual peptides. Low density lipoprotein gave a map with much greater complexity in the anodal region. High density lipoprotein showed a larger number of individual peptides, with greater scatter over the plate, and relatively fewer zones did not move in the electrophoretic dimension.

**DISCUSSION**

The role of the intestine in lipoprotein metabolism is poorly understood. Biosynthesis of protein by the intestinal mucosa appears to be essential for lipid transport, since Sabin and Isselbacher have recently reported that inhibition of protein synthesis in the rat prevents chylomicron formation and alimentary lipemia (44). With regard to the specificity of protein in the chylomicron, previously reported evidence is not definitive. On the one hand, it is suggested that specific chylomicron proteins may be synthesized by the intestinal mucosa (7, 8); on the other hand, non-specific adsorption of proteins into chylomicrons from the environment is favored (9).

In the studies reported here, active biosynthesis of mucosal proteins has been demonstrated in vivo and in vitro by means of the incorporation of labeled amino acids and the effects of inhibitors. The fraction of highest specific activity was found to be associated with lipid during its absorption across the intestinal mucosa. This protein fraction was not homogeneous, but exhibited several unusual properties in addition to its affinity for lipids: (a) a predominance of apolar amino acids (Table V) similar to that which is present in two other lipoproteins, structural protein of the mitochondrial, and myelin proteolipid (45); (b) resistance to fractionation procedures involving ionic properties; (c) considerable solubility in organic solvents; and (d) peculiarities of the peptide map that are related to the first three properties.

Our studies again fall short of settling the dilemma about the specificity of the protein which becomes associated with lipid during transport. Further comment depends almost entirely upon a speculative interpretation of the starch electrophoretic patterns and the unusual peptide maps observed for various fractions. The key to this interpretation is the notion that patterns obtained from the residual protein fraction presumably represent the most abundant soluble proteins of the mucosal cells. There is an incomplete but strong resemblance among the electrophoretic patterns (Fig. 2A) and the peptide maps (Fig. 3) of (a) the residual protein fraction, (b) the lowest density lipoprotein, and (c) the intestinal lymph chylomicrons. It is possible that this resemblance means that lipids in transit through the mucosa become associated with some of the more abundant soluble proteins of the cells and that the resulting lipoprotein complex then appears in the intestinal lymph. One would particularly wish to understand the relationship between the proteins we have described and the endoplasmic reticulum of the mucosa, for this cell organelle is believed to play an important role in intestinal fat absorption (46-48).

**Thin Layer Methodology**

A brief comment is appropriate on the usefulness of thin layer methodology in this work. In the mapping and measurement of radioactivity of peptides obtained from proteolytic digestion and in the semiquantitative assay of amino acid composition, it has been feasible to work with amounts ranging between 25 and 100 μg of material. Without the availability of these methods it would have been necessary to pool samples from substantial numbers of animals, and the procedures would have become much more cumbersome.

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Biosynthesis of Lipoprotein by Rat Intestinal Mucosa
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