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Fat Absorption During Inhibition of Protein Synthesis: Studies of Lymph Chylomicrons

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Abstract The effect of protein synthesis inhibition on the absorption of oleic acid from micellar solution was studied in mesenteric lymph fistula rats. A micellar solution of oleic acid labeled with tracer doses of oleic acid-14C was administered by intraduodenal infusion to rats with indwelling mesenteric lymph cannulas. Protein synthesis was inhibited by intraperitoneal acetoxycycloheximide (ACH), 0.25 mg/kg, 1 hr before lipid infusion. Lymph chylomicrons labeled with oleic acid-14C were collected from control and protein inhibited animals at various times after lipid infusion and subjected to sucrose density gradient centrifugation to determine changes in size. In control animals there was a transient increase in chylomicron size during maximal triglyceride absorption; however, in protein-inhibited animals there was a marked and sustained increase in chylomicron size as late as 4 hr after lipid infusion.

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These studies clearly demonstrate that the intestinal inhibition of protein synthesis is associated with an increase in the size of intestinal lymph chylomicrons and support the concept that protein synthesis is important in the formation and transport of chylomicrons from the mucosal cell into the lymph.

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Introduction

The intestinal mucosa is known to synthesize lipoproteins of density <1.006 (chylomicrons and very low density lipoprotein [VLDL]), and recent studies have shown that the intestine is a major source of these lipoproteins in lymph (1). Intestinal microsomes can synthesize the β-apoprotein portion of these lipoproteins (2) and also are the subcellular sites within the intestinal cell for the esterification of glycerides and the synthesis of phospholipids (PL) (3, 4). These apolipoprotein and phospholipid components are believed to associate with reesterified triglyceride (TG) within the intestinal mucosal cell and the resultant lipid-rich particles are transported into the lymph as chylomicrons or very low density lipoproteins (5). The major evidence for the essential role of β-lipoprotein in the transport of intestinal triglyceride is the inherited disease, abetalipoproteinemia, where inability to synthesize β-apoprotein results in the absence of β-lipoprotein and a complete inability to form chylomicrons (6, 7). The recent demonstration by Goto, Levy, John, and Fredrickson (8) that a specific apoprotein, apolipoprotein-serine, present in β-lipoprotein and chylomicrons, is absent from the sera of patients with abetalipoproteinemia strongly suggests that this apoprotein is required for the assembly and/or release of triglyceride from the intestine into the lymph or plasma.

Sabesin and Isselbacher (9), using inhibitors of protein synthesis, seemed to produce a similar experimental picture to abetalipoproteinemia in rats after puromycin administration. The interpretation was put forth that protein synthesis, specifically β-lipoprotein, was required for normal fat transport out of the intestine.

1 Abbreviations used in this paper: ACH, acetoxycycloheximide; PL, phospholipid; POPOP, P-bis[2-(5 phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; TG, triglyceride; VLDL, very low density lipoproteins.
However, this hypothesis has been questioned by others (10, 11) who found that lipid absorption into lymph of protein synthesis inhibited animals proceeded at almost normal levels and attributed any decreased absorption to decreases in lymph flow produced by the toxic action of the drugs employed. Therefore, the obligatory role of protein synthesis in the formation and transport of chylomicrons is still unclear.

In an attempt to resolve these conflicting results and to determine whether protein synthesis is required or important for chylomicron formation and transport, the effect of acetoxytoclycemohemside (ACH), a potent inhibitor of protein synthesis, was studied on the transport of triglyceride into the lymph of mesenteric cannulated rats. If protein synthesis is required for chylomicron formation and, if as a result of the inhibition of protein synthesis the availability of 3-lipoprotein (apo-protein) is a limiting factor during active fat absorption, this shortage of surface components might be reflected as an increase in chylomicron size. An increase in chylomicron size would permit the transport of more triglyceride in fewer but larger particles and thereby conserve surface apoproteins and phospholipids.

The present studies demonstrate that a significant increase in the size of lymph chylomicrons does occur during the inhibition of intestinal protein synthesis and, together with the observations in abetalipoproteinemia, they suggest that protein synthesis does influence the formation and transport of chylomicrons from intestine into lymph.

**METHODS**

Operative technique. After intraperitoneal pentobarbital anesthesia, the main mesenteric lymphatic duct was cannulated without prior fat feeding as described by Bollman, Cain, and Grindley (12). The duodenum was cannulated for administration of micellar solutions. Postoperatively, animals were maintained in restraining cages without food, and allowed to drink 5% dextrose in isotonic saline ad lib. All animals were studied 16-24 hr after surgery. Each animal was used only once.

Lipid infusions. Infused lipid was administered in a 20 mM sodium taurocholate solution at pH 7.2. A volume of benzene solution containing 4.97 mg/ml oleic acid and 3.42 mg/ml monolene was evaporated to dryness with 2 µCi oleic acid-14C and then dissolved in a volume of 20 mM sodium taurocholate in isotonic saline equal to the pre-evaporation volume of benzene. The resultant micellar solutions were completely clear. 5 ml of the above micellar solution was infused intraduodenally over a 20 min interval to control and protein synthesis inhibited lymph fistula animals. Lymph was collected at half-hourly or hourly intervals, as indicated, and defibrinated with wooden applicator sticks before ultracentrifugation or density gradient separations.

Inhibition of protein synthesis. Acetoxytoclycemohemside (ACH) was administered intraperitoneally in a 0.9% saline solution at a dose of 0.25 mg/kg, 1 hr before lipid infusion. This dose level and schedule was found to effectively inhibit leucine-14C incorporation into intestinal mucosal protein by at least 80% for at least 3 hr (13).

Density gradient centrifugation. Whole lymph was subjected to density gradient centrifugation by a modification of the method of Pinter and Zilversmit (14). Sucrose was dissolved in samples of defibrinated lymph to a final concentration of 50% w/v and 0.2 ml was layered above 0.5 ml 60% sucrose in a 10 ml glass tube. 5 ml of a continuous 30-37% sucrose gradient of density limits 1.2-1.16 was then carefully formed above each sample. The gradient was formed in a two-chambered gradient maker with one outflow delivered, via gravity, by means of a thin polyethylene tubing. Vigorous mixing of the outflow chamber was accomplished with a motor-driven mixing blade. The gradients were immediately centrifuged in a swinging bucket rotor at 2000 g at the bottom of the gradient in a Lourdes Model 50-R Clinfuge at a constant temperature of 20°C for 2 hr. Serial fractions (approximately 0.5 ml) were pumped out from the bottom of the gradient with a peristaltic pump by means of a thin metal cannula introduced into the bottom of the gradient. Aliquots of each fraction were counted in a Beckman LS250 liquid scintillation counter in 12 ml of a scintillation solution consisting of 2.5-diphenyloxazole (PPO), 70 g, p-bis [2-(5 phenyloxazo- yl)] benzene (POPOP), 0.05 g, naphthalene, 50 g, and 95% ethanol, 145 ml/1 liter dioxane. Since all samples showed similar degrees of quenching, corrections for quenching were unnecessary.

Preparative ultracentrifugation. Whole defibrinated lymph was layered beneath 0.15 mM NaCl in cellulose nitrate tubes and was centrifuged at approximately 3 x 10^6 g-min in a Beckman Spinco SW39 swinging bucket rotor. The lipoprotein particles which rose to the top were separated from the subnatant by means of a tube slicer and were designated "chylomicrons" (15). This procedure was repeated twice, and the washed chylomicron fraction was then extracted with chloroform: methanol and triglycerides determined enzymatically by the method of Eggstein and Kreutz (16). Phospholipid was determined by the method of Bartlett (17). A conversion factor of 25 was used to convert phosphorus to lipid phosphorus.

Electron microscopy. Samples of whole lymph were examined directly after negative staining with 2% phosphotungstic acid (18) using a Philips Model 200 electron microscope. Chylomicron measurements were performed with a graduated ocular from photographic enlargements (X 6) of at least three unselected fields from each lymph sample.

Materials. Sprague-Dawley male rats (200-250 g) CD strain, were purchased from Charles River Labs (Wilmington, Mass.). Oleic acid-14C was purchased from New England Nuclear Corp. (Boston, Mass.) and found to be radiochemically pure after silica Gel G thin-layer chromatography in petroleum ether: ether: glacial acetic acid, 90:15:1.5 and subsequent radioactive scanning. Oleic acid and monolene were obtained from Calbiochem (Los Angeles, Calif.) and their purity checked by thin-layer chromatography using the same solvent system. Taurocholate was obtained from Calbiochem and its purity checked on silica Gel G thin-layer chromatography utilizing tripropionic acid: iso-amyl acetate: water: n-propanol, 15:20:5:10 as the solvent system. Reagents and enzymes for triglyceride determinations were purchased in kit form from Boehringer Mannheim Co., New York. Acetoxytoclycemohemside was generously supplied by the John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc.
Figure 1. Effect of inhibition of protein synthesis on chylomicron density distribution. 5 ml of a micellar solution of oleic acid-\(^{14}\)C was administered intraduodenally to control and protein synthesis-inhibited rats with mesenteric lymph fistulas. Samples of lymph were defibrinated and the distribution of radioactivity in continuous 30-37% sucrose gradients determined (see Methods). Acetoxycycloheximide (ACH) in a dose of 0.25 mg/kg was administered intraperitoneally 1 hr before lipid infusion to inhibit protein synthesis. Times shown refer to times of lymph collection after lipid infusion.

Figure 2. Effect of inhibition of protein synthesis on chylomicron density distribution. 5 ml of a micellar solution of oleic acid-\(^{14}\)C was infused intraduodenally to control and protein-inhibited mesenteric lymph fistula rats. At each indicated time interval after lipid infusion, samples of defibrinated lymph were subjected to sucrose gradient density centrifugation, and the distribution of radioactivity determined in each gradient (Methods). Comparable points within each gradient were analyzed for statistical significance. In the inhibited animals experiments were conducted for an additional hour after lipid infusion (3-4 hr).
RESULTS

Reproducibility of gradient preparations. Duplicate gradients on the same sample of lymph virtually showed identical patterns, differing by less than 5% at any point within the gradient. Whereas all samples of lymph were run immediately after defibrination, samples run the next day gave identical gradient patterns suggesting that significant aggregation of chylomicron particles or changes in size did not occur in vitro. Verification that administered oleic acid-\(^{14}C\) was incorporated into lymph triglyceride both in normal and protein synthesis–inhibited animals was provided by thin-layer chromatography of lymph samples from both groups. At all times during fat absorption at least 90% of \(^{14}C\) counts were recovered in triglycerides in both groups. Therefore, the distribution of radioactivity with each gradient reflects newly absorbed triglyceride.

Density gradient distributions. As shown by Pinter and Zilversmit (14), chylomicrons will distribute within a sucrose gradient at any given set of centrifugal conditions as a function of their density. Larger lipid-containing particles will be less dense and move a greater distance toward the center of rotation. Therefore, there should be a shift of radioactivity towards the top of the gradient if chylomicron size increases. A representative series of gradients from a control animal is shown in Fig. 1. After infusion of 5 ml of a micellar solution of oleic acid labeled with oleic acid-\(^{14}C\) (see Methods), half-hourly lymph samples were collected and the pattern of radioactivity determined. It can be seen that early in the course of fat absorption curve 1), approximately 90% of the radioactivity is distributed in the lower half of the gradient. However, as fat absorption progressed and peaked over the next hour, more radioactivity was present in lighter particles (curves 2 and 3), suggesting that chylomicron size had increased. As fat absorption then decreased (curves 4 and 5), the pattern of radioactivity progressively returned towards earlier values by 24 hr. Thus, there appears to be a progressive increase in chylomicron size and a gradual return during the course of fat absorption in the normal animal.

In contrast, however, a representative protein synthesis–inhibited animal is also shown in Fig. 1. It can be seen that at all time points there was a marked shift in the distribution of radioactivity with more label appearing in the upper half of the gradient, suggesting that an increase in chylomicron size had occurred after ACH administration. It should be noted that this increase in size persisted, even at 4 hr, a time when in the control animal chylomicron size had returned to earlier values.

A detailed analysis of the time course of chylomicron distribution in control and protein synthesis–inhibited animals is shown in Fig. 2. At all time intervals there was a shift of chylomicron distribution in the ACH-treated animals. Comparable points within each gradient at a given time were compared. It will be seen that at each point there was a significant shift of chylomicron distribution towards the top of the gradient in the inhibited group, again suggesting an increase in chylomicron size had occurred. These differences in size distribution became even more marked with time since at 2–3 hr chylomicron size had returned to initial values in the control group, while even as late as 3–4 hr an increased chylomicron size persisted in the inhibited group.

The time course of lipid absorption and lymph triglyceride concentrations in control and protein-inhibited animals is shown in Table I. The mean recovery of label in control animals was 65% compared to 43% in the inhibited group (\(P < 0.005\)). In the control animals, the peak period of lipid absorption was at 1–2 hr reflected by the greatest percentage of absorbed radioactivity and as the highest concentration of lymph triglyceride. This time period was also associated with the largest chylomicron size in the control group (Figs. 1 and 2). However, this correlation between the amount of lipid absorbed in a given time period, the concentration of lymph triglyceride and chylomicron size was not present in the animals treated with ACH. In that group

<table>
<thead>
<tr>
<th>Time after Lipid Infusion</th>
<th>Control</th>
<th>ACH</th>
<th>% Administered (^{14}C) Recovered in Lymph (0–4 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>%</td>
<td>mg/100 ml</td>
<td>65 ± 7.43</td>
</tr>
<tr>
<td>0–1</td>
<td>4</td>
<td>4</td>
<td>182</td>
</tr>
<tr>
<td>1–2</td>
<td>46</td>
<td>23</td>
<td>495</td>
</tr>
<tr>
<td>1–1</td>
<td>33</td>
<td>—</td>
<td>314</td>
</tr>
<tr>
<td>1–2</td>
<td>1–2</td>
<td>34</td>
<td>318</td>
</tr>
<tr>
<td>1–2</td>
<td>10</td>
<td>—</td>
<td>152</td>
</tr>
<tr>
<td>2–3</td>
<td>7</td>
<td>23</td>
<td>116</td>
</tr>
<tr>
<td>3–4</td>
<td>—</td>
<td>16</td>
<td>—</td>
</tr>
</tbody>
</table>

After the intraduodenal infusion of a micellar solution of oleic acid-\(^{14}C\), lymph radioactivity and triglyceride were determined in control and protein-inhibited rats. Results are the means of six animals in each group.
Figure 3  Electron microscopy of whole lymph from a representative control, and acetoxy cycloheximide (ACH)-treated animal. Samples of whole lymph at the indicated times were directly examined after negative staining with 2% phosphotungstic acid. Control (4-1 hr), left. ACH (1-2 hr), right. × 5400.
lipid absorption was distributed over the entire 4 hr after infusion and there appeared to be no clear relationship between lymph triglyceride concentration and chylomicron size. While chylomicron size was greatest in the protein-inhibited group, the highest triglyceride concentrations occurred in the control animals during peak lipid absorption i.e. 1–1 hr).

Triglyceride-phospholipid determinations. As further verification of the interpretation of the sucrose gradient density distribution, triglyceride and phospholipid determinations were performed on washed chylomicrons from control and protein synthesis-inhibited animals at each time interval (Table II). If chylomicron size had increased during inhibition of protein synthesis, one would expect an increase in the relative amount of triglyceride to phospholipid in the chylomicrons. This would be reflected by a decreased TG/PL ratio. The TG/PL ratio would also provide a check on whether aggregation of chylomicrons had occurred and was responsible for the gradient distribution, since under these conditions there should be no change in these ratios. As is clearly shown in Table II, at each time period there was a significant increase in the amount of triglyceride to phospholipid in the inhibited group, a finding which is consistent with an increased in chylomicron size. As with the gradient distributions (Fig. 1), TG/PL ratios increased and then returned to initial values in the control group, while in the inhibited group they remain increased for the duration of the study.

Electron microscopy. Further confirmation of increased chylomicron size during inhibition of protein synthesis was provided by direct visualization of lymph using the negative staining technique (see Methods). Representative lymph samples from a control and an ACH-treated animal are shown in Fig. 3 where an increased chylomicron size in the protein-inhibited animal is readily apparent. It should be stressed that at the dose of lipid infused, the control sample shown in Fig. 3 represented the maximal chylomicron size observed in the control group. When chylomicron size was directly measured with a calibrated ocular, the greater particle size in the ACH-treated group was confirmed (Table III). These size distributions were determined on particles having a diameter greater than 2000 Å, a size well within the chylomicron range.

DISCUSSION

The use of inhibitors of protein synthesis by Sabesin and Isselbacher (9) seemed to reproduce in the rat some features comparable to the human disease, abetalipoproteinemia. In that study, the intestinal mucosa was laden with lipid and there was an absence of chylomicronemia after puromycin administration. These investigators pro-

### Table II

*Effect of Acetoxycycloheximide Treatment on Triglyceride/Phospholipid Ratios on Lymph Chylomicrons*

<table>
<thead>
<tr>
<th>Time after infusion</th>
<th>Triglyceride/phospholipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0–1 hr</td>
<td>8.5</td>
</tr>
<tr>
<td>1–2 hr</td>
<td>10.8 ±0.89</td>
</tr>
<tr>
<td>2–3 hr</td>
<td>10.2 ±0.44</td>
</tr>
<tr>
<td>3–4 hr</td>
<td>7.2 ±0.58</td>
</tr>
</tbody>
</table>

Washed lymph chylomicrons were prepared (Methods) from control and ACH-treated animals at each time interval and triglyceride and phospholipid values determined. Values are the mean ±SE of five animals in each group, except that the 0–1 hr period represents the average of only two determinations.

posed that intestinal protein synthesis, specifically β-lipoprotein synthesis, was important for the normal movement of lipid from the intestine into lymph. This hypothesis was questioned, however, by Redgrave (10), who found significant lipid absorption into lymph after cycloheximide treatment and attributed any decrease in lipid absorption to decreases in lymph flow produced by the toxic actions of the drug employed. In a further study Redgrave and Zilversmit (11), by using puromycin, again found absorption of triglyceride into lymph. Chylomicrons from puromycin-treated animals seemed to have normal protein and phospholipid content. However, in those studies intestinal protein synthesis was not inhibited but actually appeared to be stimulated by 20%.

Whereas in the present study the inhibition of intestinal protein synthesis was associated with a sig-

### Table III

*Effect of ACH Treatment on Size of Lymph Chylomicrons*

<table>
<thead>
<tr>
<th>Particle size</th>
<th>Control (1–1 hr)</th>
<th>ACH treated (1–2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000–3000 Å</td>
<td>70%</td>
<td>40%</td>
</tr>
<tr>
<td>3000–4000 Å</td>
<td>22%</td>
<td>22%</td>
</tr>
<tr>
<td>4000–5000 Å</td>
<td>6%</td>
<td>16%</td>
</tr>
<tr>
<td>&gt;5000 Å</td>
<td>2%</td>
<td>22%</td>
</tr>
</tbody>
</table>

Electron microscopy of whole lymph was carried out as in Fig. 3. The size determination of chylomicrons >2000 Å was determined with a graduated ocular from at least three unselected fields from each lymph sample. 276 particles were examined in the control and 191 in the ACH-treated groups. Two animals were studied in each group.
significant decrease in the amount of oleic acid-\(^{(13)}\)C recovered in lymph (Table I), both in this study and earlier (13) significant amounts of triglyceride did appear in lymph during inhibition of protein synthesis. However, can one conclude from these data that protein synthesis is not required for intestinal lipid absorption? It should be emphasized that because of the toxicity of the agents employed, it is not possible to completely inhibit intestinal protein synthesis in vivo. Therefore, the possibility exists that when presented with lipid mucosal lipoprotein synthesis might continue at a rate greater than that of other cellular proteins. Also, the possibility that preformed lipoprotein was being utilized for lipid transport during protein inhibition cannot be excluded. It seemed, therefore, that the appearance of triglyceride in lymph during protein inhibition was not sufficient to exclude a role for protein synthesis in lipid absorption.

If, indeed, some decrease in the ability to synthesize lipoprotein does occur during protein inhibition, then this might be reflected in the size of chylomicrons appearing in lymph. Since the lipoprotein and phospholipid moieties of the chylomicron appear to be in a surface coat surrounding a core of triglyceride (15), the relationship of these surface components to triglyceride should follow the surface area–volume relationships of a sphere. An increase in the radius of a chylomicron will lead to a proportionally greater increase in volume than surface area. Therefore, if one or more surface components of the chylomicron become limiting with inhibition of protein synthesis, one would expect that a compensatory mechanism might lead to formation of larger chylomicrons in an effort to transport the triglycerides into lymph. Indeed, this appeared to be the case. As demonstrated by sucrose density gradient distribution (Fig. 2), triglyceride/phospholipid ratios (Table I), and by electron microscopy (Fig. 3, Table III), a larger chylomicron size occurred after actetoxycycloheximide treatment.

The present studies utilized micellar solutions of oleic acid infused directly into the duodenum and avoided the variable effects of the protein synthesis inhibitors on gastric emptying, bile flow, and lipolysis as pointed out by others (11). Also, by studying absorption directly into lymph rather than plasma, the effects of the inhibitors on hepatic lipoprotein synthesis and secretion were avoided.

While decreasing lymph flow may lead to decreased absorption of triglyceride into lymph in spite of compensatory increases in lymph triglyceride concentration (10, 11), our data demonstrate that the increased chylomicron size observed during protein inhibition is not clearly related to lymph triglyceride concentration (Table I). While chylomicron size was greatest in the protein synthesis–inhibited animals, the highest triglyceride concentrations occurred during the peak period of lipid absorption (4–1 hr) in the control animals. Also, in one animal the infusion of intraduodenal saline after protein synthesis was inhibited and chylomicron size had increased, produced an increase in lymph flow, but chylomicron size remained unchanged. Therefore, while decreased lymph flow may affect the total amount of triglyceride recovered in lymph, no evidence was obtained that this affects the size of chylomicrons in lymph.

Since the protein (i.e. apoprotein) content of chylomicrons after ACH treatment was not measured, it is not possible to determine from the present experiments whether the increase in chylomicron size was a direct result of decreased apoprotein production by the intestinal mucosa. However, the recent work of Kessler, Stein, Dannacker, and Narcessari (2) utilizing an in vitro microsomal system from rat intestine which was capable of synthesizing \(\beta\)-lipoprotein would support this interpretation. These workers showed that when puromycin was added in vitro to such a system, larger amounts of lipid were associated with the \(\beta\)-lipoprotein fraction demonstrating that with inhibition of protein synthesis adaptive changes in lipoprotein structure may occur. Furthermore, recent studies (Glickman, unpublished observations) indicate that a similar increase in lymph chylomicron size occurs during dietary protein deficiency or protein depletion, further instances of impaired protein synthesis. However, the possibility that a decrease in phospholipid synthesis occurs with inhibition of protein synthesis and that this is responsible for or contributes to the observed increase in chylomicron size cannot be excluded and studies of chylomicron apoprotein composition are currently in progress.

We have confirmed the findings of Fraser, Clif, and Courtice (19) that in the normal animal chylomicron size increases during fat absorption. Whereas the factors influencing the formation of chylomicrons remain largely unknown, it is possible that during peak period of chylomicron assembly within the intestinal cell the availability of apoprotein may be limiting and that the apoprotein subunits may combine with more lipid to form larger particles, as suggested by Windmueller, Lindgren, Lossow, and Levy (20).

The present studies were carried out at only one modest dose level of lipid. Nevertheless, the rapid and prolonged increase in chylomicron size in the inhibited animal would seem to indicate that large preformed stores of \(\beta\)-apolipoproteins do not exist within the intestinal cell. The findings of Alcendor, Infante, Soler-Argilaga, Raisounier, Polonovski, and Cardi (21) that protein synthesis inhibitors prevent the release of \(d < 1.063\)
lipoproteins by perfused rat liver suggest that large quantities of preformed \( \beta \)-apoproteins also do not exist in liver.

The present studies clearly demonstrate that the intestinal inhibition of protein synthesis is associated with an increase in the size of intestinal lymph chylomicrons and support the concept that protein synthesis is important in the formation and transport of chylomicrons from the mucosal cell into lymph.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Dr. Richard Cornell in performing the electron microscopy of lymph samples.

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