FINE STRUCTURAL LOCALIZATION OF ACYLTRANSFERASES

The Monoglyceride and α-Glycerophosphate Pathways in Intestinal Absorptive Cells

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ABSTRACT

A study of the fine structural localization of the acyltransferases of the monoglyceride and α-glycerophosphate pathways for triglyceride synthesis in the intestinal absorptive cell is reported. Glutaraldehyde-fixed tissue was found to synthesize diglyceride and triglyceride from monopalmitin and palmityl CoA, and parallel morphological studies showed the appearance of lipid droplets in the smooth endoplasmic reticulum of the absorptive cell. Glutaraldehyde-fixed tissue also synthesized triglyceride from α-glycerophosphate, although this enzyme system was more susceptible to fixation than the monoglyceride pathway acyltransferases. Cytochemical methods for the localization of free CoA were based (*a*) on the formation of the insoluble lanthanium mercaptide of CoA and (*b*) on the reduction of ferricyanide by CoA to yield ferrocyanide which forms an insoluble precipitate with manganous ions. By these methods the monoglyceride pathway acyltransferases were found to be located mainly on the inner surface of the smooth endoplasmic reticulum. The α-glycerophosphate pathway acyltransferases were localized mainly on the rough endoplasmic reticulum. Activity limited to the outer cisternae of the Golgi membranes occurred with both pathways. The possible organization of triglyceride absorption and chylomicron synthesis is discussed in view of these results.

During lipid absorption from the intestine, intraluminal hydrolysis of the triglyceride by pancreatic lipase produces mainly monoglyceride and free fatty acids which are absorbed across the striated border of the absorptive cell (17, 20, 36). Inside the cell the free fatty acids are activated by synthesis of the CoA derivatives and triglyceride and phospholipids are resynthesized and packaged with cholesterol, cholesterol esters, and a small amount of protein to yield chylomicrons which are released at the lateral borders of the cell (6, 17, 20, 29, 36, 41). The existence in intestinal mucosa of two pathways for the synthesis of triglyceride from fatty acid CoA derivatives has been established. One pathway uses the absorbed monoglycerides as an acceptor for the fatty acid to yield diglyceride and then triglyceride (7, 22, 23, 38), while the second uses α-glycerophosphate as an acceptor to yield lysophosphatidic acid, phosphatidic acid, and diglycerides as intermediates (7, 10, 11, 18, 19, 21, 37). Diglycerides are also intermediates in the synthesis of phospholipids. During lipid absorption the monoglyceride pathway is preferentially used for synthesis of triglyceride and the α-glycerophosphate pathway is probably involved in the synthesis of phospholipids (26). The two pathways, although they share a common intermediate in the diglyceride, appear to be biochemically distinct

such that the diglyceride produced by one pathway is not utilized by the enzymes of the alternative pathway (23). This separation may be due to morphological sequestering of the two enzyme systems, or may be due to binding of the intermediates so that the diglyceride is not released from the enzyme during lipid synthesis. As yet there have been no reports of direct evidence indicating which of these possibilities is correct.

We have recently developed methods for the fine structural localization of acyltransferases, based on the precipitation, in an electron-opaque form, of the CoA released during transfer of fatty acid to an acceptor (15). These methods, therefore, present a new approach to the investigation of the problems indicated above, and allow the direct localization of the acyltransferases involved in triglyceride and phospholipid synthesis, without the disruption of the cell necessary for enzyme localization by cell fractionation techniques. Such methods should yield information on the way in which the biochemical events in lipid metabolism are structurally organized. The present paper is a report of the results of these studies on intestinal absorptive cells. A preliminary report has been made previously (14).

MATERIALS AND METHODS

Purified glutaraldehyde was purchased from Ladd Industries, Ltd., Burlington, Vt. Palmityl CoA, monopalmitin, and DL-α-glycerophosphate were purchased from Sigma Chemical Co., St. Louis, Mo.

Monopalmitin was prepared in solution, at a concentration of 2.5 mg/ml, by sonication in cacodylate buffer pH 7.0, 0.025 M. This yielded a turbid suspension of monopalmitin which was used as a stock solution for biochemical and cytochemical studies.

S-palmityl-1-^{14}C Coenzyme A was purchased from Tracerlab (Tracerlab Division, Laboratory for Electronics, Inc., Richmond, Calif.), and its purity was checked by paper chromatography using pyridine:isopropanol:water 1:1:1 (v:v:v) as a solvent.

L-α-glycero-phosphate-^{14}C was purchased from International Chemical and Nuclear Corporation, Burbank, Calif.

Tissues

Rat intestine from the duodenum and the upper part of the jejunum was used for both biochemical and cytochemical studies. In most cases animals were fasted for 48 hr before sacrifice in order to deplete lipid droplets from the absorptive cells. The tissue was removed from ether-anesthetized rats and was used immediately, or was fixed by lavage of the lumen of the small intestine with 1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4 containing 4.5% dextrose. Rings of fixed intestine were sliced and immersed for 15–30 min in the same fixative. Fixed tissues were rinsed in cacodylate buffer and washed in the same buffer overnight.

Biochemical Studies

Rings of fixed or unfixed intestine were incubated at 37°C for a variety of times, as indicated in the text, in a medium containing palmityl-^{14}C CoA or L-α-glycerophosphate-^{14}C in 0.025 M cacodylate buffer pH 7.0 with or without monoglyceride, unlabeled α-glycerophosphate, unlabeled palmityl CoA, or capture reagents for CoA (see below). The individual conditions for each incubation are indicated in the text. Streptomycin and penicillin were added to the medium to avoid metabolism of the labeled substrates by bacteria. After incubation, the tissue was removed, rinsed several times with the buffer of the incubation, and the lipid was extracted with chloroform:methanol 2:1 (13). Portions of the total lipid extract were taken for counting to determine the incorporation of the labeled compound into tissue lipids. The rest of the lipid extract was separated into phospholipid, monoglycerides, diglycerides, triglycerides, free fatty acids, and cholesterol on thin layers of silica gel using 40% isopropyl ether in heptane containing 2 parts per 100 of glacial acetic acid as a solvent system (16). Fractions were scraped directly into counting vials and counted in a Packard Tri-Carb scintillation counter. Recovery of counts always exceeded 80% of the counts applied to thin layers. In all experiments, controls consisting of boiled tissue and media without tissue were also performed. Other controls were as indicated in the text.

Intestinal rings were used in these studies, so that parallel morphological investigations could be made. This necessitated the use of slightly variable amounts of tissue in each incubation. In order to express the incorporation of the labeled substrate in terms of some unit of tissue present, the chloroform:methanol insoluble residue was collected by centrifugation and weighed after extraction of the lipid. Milligrams of lipid-free, dry weight were used as a unit of tissue present in each incubation; this approximates to protein.

Cytochemical Studies

PRECIPITATION OF COA: The formation of an electron-opaque precipitate of CoA for its cytochemical localization has been reported elsewhere (15). There are two general methods for this precipitation, one based on the formation of the mer-

captide of CoA with a heavy metal, and the second based on the ability of the CoA to reduce ferricyanide to ferrocyanide, which precipitates with a second metal ion. The methods reported previously have been modified for the present study, but are still based on the same general principle.

MERCAPTIDE FORMATION: Lanthanum nitrate at a final concentration of 1 mg/ml (3 mM) in 0.025 M cacodylate buffer pH 7.0 was used as a capture reagent for CoA.

REDUCTION OF FERRICYANIDE: Manganous ions form a white precipitate with ferrocyanide in concentrations with which no precipitate is formed with ferricyanide. Manganous chloride at a concentration of 0.5 mg/ml (2.5 mM) and potassium ferricyanide at a concentration of 0.15 mg/ml (0.45 mM) in 0.025 M cacodylate buffer pH 7.0 were used as capture system. Neither lanthanum nitrate, nor potassium ferricyanide and manganous chloride, at the concentrations indicated above formed a precipitate with palmityl CoA, but both formed white precipitates instantly on addition of free CoA. After removal of the precipitate by centrifugation, there was no trace of free CoA in the solution as measured by dithionitrobenzoic acid (DTNB) (12) indicating removal of all free CoA added, either as the mercaptide or as the oxidized form in the case of the ferricyanide manganous chloride method.

PREPARATION OF TISSUE FOR ELECTRON MICROSCOPY: Blocks of fixed intestine or rings of fresh intestine were incubated in the same media that were used in the biochemical studies so that the morphological changes taking place in the tissue could be followed in parallel with the biochemical events. After incubation, except in experiments where lanthanum nitrate was added as a capture reagent, the tissue was removed from the incubation medium, rinsed several times in buffer, fixed in 3% glutaraldehyde in 0.025 M cacodylate buffer pH 7.0 containing 4.5% dextrose (15–30 min), and refixed in 1% osmium tetroxide in 0.025 M cacodylate buffer pH 7.0 containing 4.5% dextrose (30–60 min). The rings of tissue which had not been fixed before incubation were cut into small blocks at this point. All tissues were dehydrated through a graded series of ethyl alcohol (50–100%) and embedded in Epon. Tissues incubated in the presence of lanthanum nitrate as a capture reagent were transferred directly from the incubation medium to a fixative containing 1% osmium tetroxide in a mixture of 50% ethyl alcohol and 50% 0.025 M cacodylate buffer pH 7.0 containing 4.5% dextrose (15–30 min). The blocks were dehydrated with absolute alcohol alone and embedded in Epon. This procedure was used in order to preserve the lanthanum CoA precipitate which is reversible in aqueous media in the absence of an excess of lanthanum nitrate. Sections of embedded material were made on an LKB ultramicrotome, and were stained for 30 sec in lead nitrate to enhance contrast when it had been found that such staining affected only membranous elements and did not interfere with the visualization of final product of the cytochemical experiments. Sections were viewed in a Hitachi 11B electron microscope.

RESULTS

Monoglyceride Pathway for Triglyceride Synthesis

METABOLISM OF PALMITYL-^{14}C COA BY GLUTARALDEHYDE-FIXED INTESTINE: The metabolism of palmityl-^{14}C CoA by slices of intestine fixed in 1% glutaraldehyde is shown in Fig. 1. In the absence of acylacceptor, palmitate-^{14}C appeared to the greatest extent in the free fatty acid fraction of the lipids, suggesting an active palmityl CoA hydrolase in the fixed tissue. The label also appeared to a small extent in the triglyceride, diglyceride, and phospholipid. The reason for the appearance of the label in esterified lipids, without addition of an acylacceptor, is not clear. As this did not occur in boiled tissue incubated under exactly the same conditions, it is presumably due to an enzyme-catalyzed reaction. This result could be due to endogenous acylacceptors remaining in the tissue or to an enzyme-catalyzed exchange between the labeled palmitate of palmityl CoA and the unlabeled fatty acids of the tissue lipids. On the basis of these results, it is not possible to distinguish between these alternatives; however, the appearance of the label in esterified lipids is small compared with that described below.

On addition of monopalmitin to the incubation medium, there was an increased incorporation of labeled palmitate into diglyceride and triglyceride, the greatest amount being into the diglyceride (Fig. 1). This was somewhat variable, however, and in some experiments the label appeared equally in triglyceride and diglyceride. The whole triglyceride synthetase system therefore survives glutaraldehyde fixation to some extent. The high level of activity in diglyceride, however, indicates a partial block in the synthesis of triglyceride. This suggestion is supported by comparison of the results in Fig. 1 with those of the metabolism of palmityl-^{14}C CoA by unfixed tissue in Fig. 2. In the latter case the label appeared predominantly in the triglyceride, without accumulation in the diglyceride. The enzyme system in unfixed tissue is therefore capable of acylating both free positions

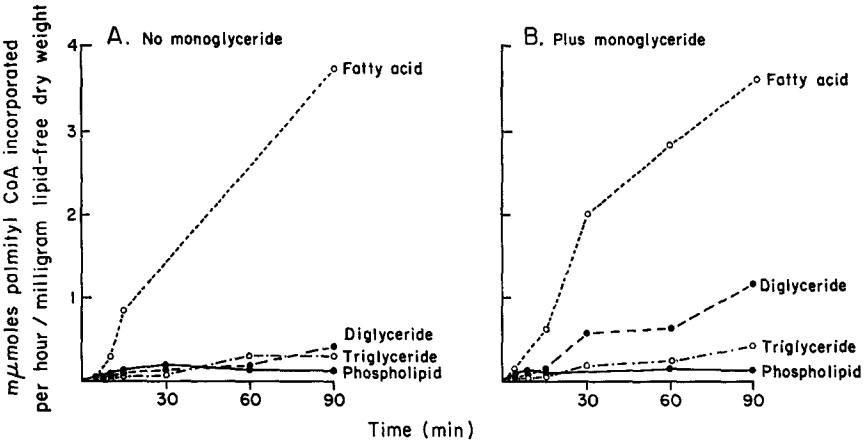


FIGURE 1 Slices of glutaraldehyde-fixed intestine were incubated at 37°C in media containing palmityl-^{14}C CoA (0.5 mM) without (A) and with (B) monopalmitate (0.75 mM) in cacodylate buffer (0.025 M) pH 7.0 containing 4.5% dextrose. Samples were taken at a variety of times, rinsed with cacodylate buffer, and extracted with chloroform:methanol 2:1. The lipids were separated as indicated in the text. Results are expressed as mμmoles of palmitate incorporated into lipid fractions per milligram of lipid-free, dry weight (see text), corrected for the small amount of label appearing in the lipids of boiled tissue.

of the monoglyceride used. The palmityl CoA diglyceride acyltransferase is presumably more susceptible to glutaraldehyde fixation than is the palmityl CoA monoglyceride acyltransferase.

Because the distribution of label in esterified lipids was different in fixed and unfixed tissue, it is difficult to determine the extent to which the triglyceride synthetase system survives fixation. The quantity of free fatty acid released in fixed and unfixed tissue was similar over a range of experiments, indicating that the palmityl CoA hydrolase survives fixation to an extent approaching 100%. This is also true of the triglyceride synthetase system if the total incorporation of labeled palmitate into esterified lipids (di- and triglycerides) is compared in fixed and unfixed tissue. There is, therefore, a high survival of the acyltransferase enzymes of the monoglyceride pathway.

LOSS OF NEWLY SYNTHESIZED LIPID FROM TISSUE ON PROCESSING FOR ELECTRON MICROSCOPY: The extent to which the newly synthesized lipids survive processing for electron microscopy was determined in order to understand the morphological changes which take place during incubation in the presence of palmityl CoA and monopalmitin. The results of this study are indicated in Table I. During dehydration with alcohol and propylene oxide by conventional techniques for preparation of tissues for electron microscopy, over 96% of the newly synthesized lipid was lost from the tissue fixed in glutaraldehyde alone. When the tissue was refixed in osmium tetroxide before processing, 35.0% of the labeled lipid was retained. Of the labeled lipid retained, the greatest amount was in the triglyceride, significant amounts were in free fatty acids, but very little was in diglyceride. The tissue examined in the electron microscope might, therefore, be expected to show morphological changes consistent with the synthesis of triglyceride and also with the accumulation of free fatty acids.

MORPHOLOGICAL CHANGES IN FIXED TISSUE INCUBATED IN THE PRESENCE OF PALMITYL COA AND MONOPALMITIN: The appearance of intestinal absorptive cells (from the upper part of the jejunum of a rat starved for 48 hr before sacrifice) fixed in 1% glutaraldehyde and incubated for 30 min in buffer is shown in Fig. 3. There are few, if any, lipid droplets in this tissue, and these are restricted to the smooth endoplasmic reticulum in the apical region of the cell. In tissue from the same region incubated in a medium containing palmityl CoA and monopalmitin, there is a large number of lipid droplets (Fig. 4). These droplets are similar in distribution to those found in animals which had been fed fat before sacrifice (6, 29, 41), but they tend to be smaller and less electron-opaque than those normally found in fat-fed animals. The Golgi region of incubated

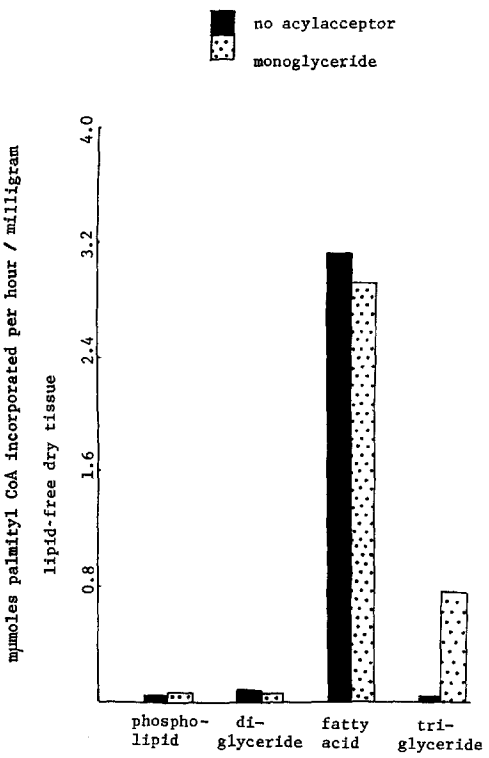


FIGURE 2 Unfixed intestinal slices were incubated in media containing palmityl CoA (0.5 mM) with and without monopalmitin (0.75 mM) in cacodylate buffer (0.025 M) pH 7.0 containing 4.5% dextrose. The tissue was removed after 1 hr of incubation, rinsed with buffer, and the lipids were extracted with chloroform:methanol 2:1. These lipids were separated on thin layers of silica gel as indicated in the text. Results are expressed as mμmoles of palmityl CoA incorporated per hour/milligram lipid-free, dry weight, corrected for label appearing in boiled tissue lipid.

tissue does not show lipid droplets, although they are found in the smooth endoplasmic reticulum close to this region (Fig. 5). From purely morphological studies, it is not possible to determine the nature of the droplets in the tissue. However, as the presence of monopalmitin and palmityl CoA is the only variable, the metabolism of these substrates must be responsible for the differences observed between the tissues in Fig. 3 and Fig. 4. Thus, from the results in Table I it would appear that the droplets are either triglyceride or free fatty acid. When tissue is incubated in a medium containing palmityl CoA without monopalmitin (Fig. 6), release of fatty acid occurs; however, there is no accumulation of lipid droplets. The droplets seen in Fig. 4 are therefore triglyceride and, possibly, some diglyceride. This is consistent with the morphological studies of chylomicrons or lipid droplets reported elsewhere (25, 34, 35).

In glutaraldehyde-fixed tissue it is unlikely that there is movement of lipid droplets, as the usual interchange and flow of membrane components is undoubtedly abolished by fixation. The location of the lipid droplets, therefore, probably marks the ultrastructural compartment of their synthesis. These observations suggest that the enzymes responsible for the triglyceride synthesized from monoglyceride and palmityl CoA are located in the smooth endoplasmic reticulum, mainly in the apical region of the absorptive cell, and not in the Golgi elements.

EFFECT OF CYTOCHEMICAL CAPTURE REAGENTS ON THE METABOLISM OF PALMITYL-^{14}C COA AND MONOPALMITIN BY FIXED TISSUE: In parallel biochemical and morphological experiments, the effects of the capture reagents on the incorporation of labeled palmitate into complex lipids in fixed intestine were studied and are shown in Table II. Potassium ferricyanide and manganous chloride had no significant effect on the metabolism of palmityl-^{14}C CoA. Lanthanum nitrate caused a reduction in the incorporation of labeled lipid into the total lipids; however, some metabolism still persisted.

MORPHOLOGICAL CHANGES IN TISSUE INCUBATED FOR CYTOCHEMICAL STUDIES: Tissue incubated in the presence of potassium ferricyanide and manganous chloride, as capture reagents, and palmityl CoA without an acylacceptor is shown in Fig. 6. There was no deposition of reaction product in the smooth endoplasmic reticulum of the absorptive cells which showed lipid droplets in the previous experiments. However, occasional deposits of reaction product occurred on mitochondrial granules and between the inner and outer mitochondrial membranes. When lanthanum nitrate was used as a capture reagent, such deposits also occurred in the intracristal space. Palmityl CoA hydrolase is active in fixed intestinal slices. This enzyme has been reported in liver (39), and a mitochondrial location is consistent with its possible role. Although this reaction was not prominent in mucosal cells, it is possible that additional palmityl CoA hydrolase is located in muscle and connective tissue cells present in the tissue slices used, and that these sites may have contributed to the high level of

TABLE I

Loss of Newly Incorporated Lipid on Processing of Tissue for Electron Microscopy

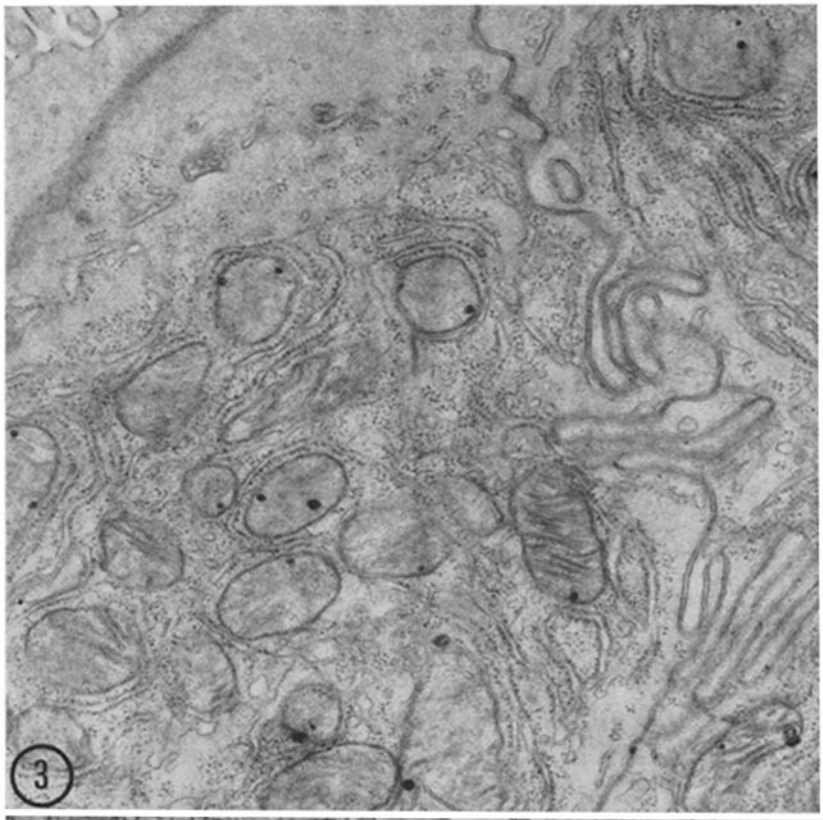
		No treatment	Glutaraldehyde and dehydration	Osmium tetroxide fixation and dehydration
mμmoles of palmityl CoA incorporated per milligram of lipid-free, dry weight	Exp. 1	0.452 ± 0.168 (4)		0.156 ± 0.036 (4)
	Exp. 2	1.252 ± 0.212 (4)	0.049 ± 0.02 (4)	
% of retention of label			3.9%	34.5%
% of distribution of label retained	Exp. 2			
	Diglyceride	9.01		2.5
	Triglyceride	11.9		36.6
	Fatty acid	64.9		18.0

Slices of glutaraldehyde-fixed intestine were incubated in the medium indicated in Fig. 1 containing monopalmitin at 37°C for 30 min to allow incorporation of the label into tissue lipids. In experiment 1, the tissue slices were divided into two groups. One group was extracted with chloroform:methanol immediately, and the second was refixed in 3% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.0, containing 4.5% dextrose for 30 min. The tissue was rinsed in buffer, dehydrated in a series of alcohol, extracted with propylene oxide, and finally the lipid was extracted with chloroform:methanol. In experiment 2, the tissue slices were divided into two groups. One group was extracted with chloroform:methanol immediately, and the second group was refixed in osmium tetroxide 1% in cacodylate buffer pH 7.0, 0.025 M, containing 4.5% dextrose. This tissue was washed in buffer, dehydrated in alcohol and propylene oxide, and the lipid was extracted with chloroform:methanol. All lipid extracts were separated as indicated in the text. Results are expressed as mμmoles of palmitate-^{14}C incorporated into lipids per milligram of tissue lipid-free, dry weight: average ± SD (No. of observations).

palmityl CoA hydrolase activity found in biochemical studies.

When monopalmitin was added to the medium used above, reaction product occurred in or on the smooth endoplasmic reticulum located mainly just below the terminal web (Figs. 7, 8, 9). In tissues from animals which had been starved for 48 hr before sacrifice, reaction product was associated both with membranes surrounding small lipid droplets and with membranes having no lipid droplets (Fig. 8). When tissue from a fat-fed animal was used, reaction product sometimes filled the space between the lipid droplet and its surrounding membrane, and in other instances it appeared only associated with the membranes of the smooth endoplasmic reticulum and the surface of the lipid droplets (Fig. 7). In tissue from fasted animals, reaction product was associated to a small extent with that part of the rough endoplasmic reticulum close to smooth membrane profiles (Fig. 8). Final product also occurred in the Golgi elements: it was located often within the membranes of small vesicles near the cisternae and occasionally in the outermost cisterna of the concave surface (1) of the complex (Fig. 12).

When lanthanum nitrate was used as capture reagent, dense deposits adhered to the surface of the striated border and were found between cells (Fig. 14). This occurred in the presence or absence of palmityl CoA or acylacceptor. Such binding of lanthanum has been reported previously (28). In the presence of monopalmitin and palmityl CoA, lipid droplets were synthesized which were more electron-opaque (Fig. 14) than those in the controls that had no capture reagent. Since it is possible that the lanthanum CoA precipitate dissolves in the lipid droplets, tissue was incubated and prepared in the same way except that postfixation in osmium tetroxide was omitted (Fig. 15). In this case, density is due to lanthanum and to the native density of the tissue. Deposits of lanthanum in the same distribution as the lipid droplets seen in Fig. 14 were found (Fig. 15). As newly synthesized lipid was lost from this tissue during proc-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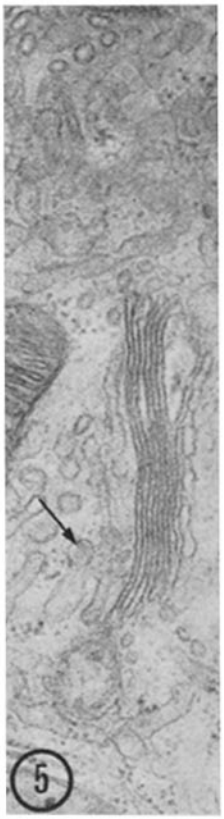
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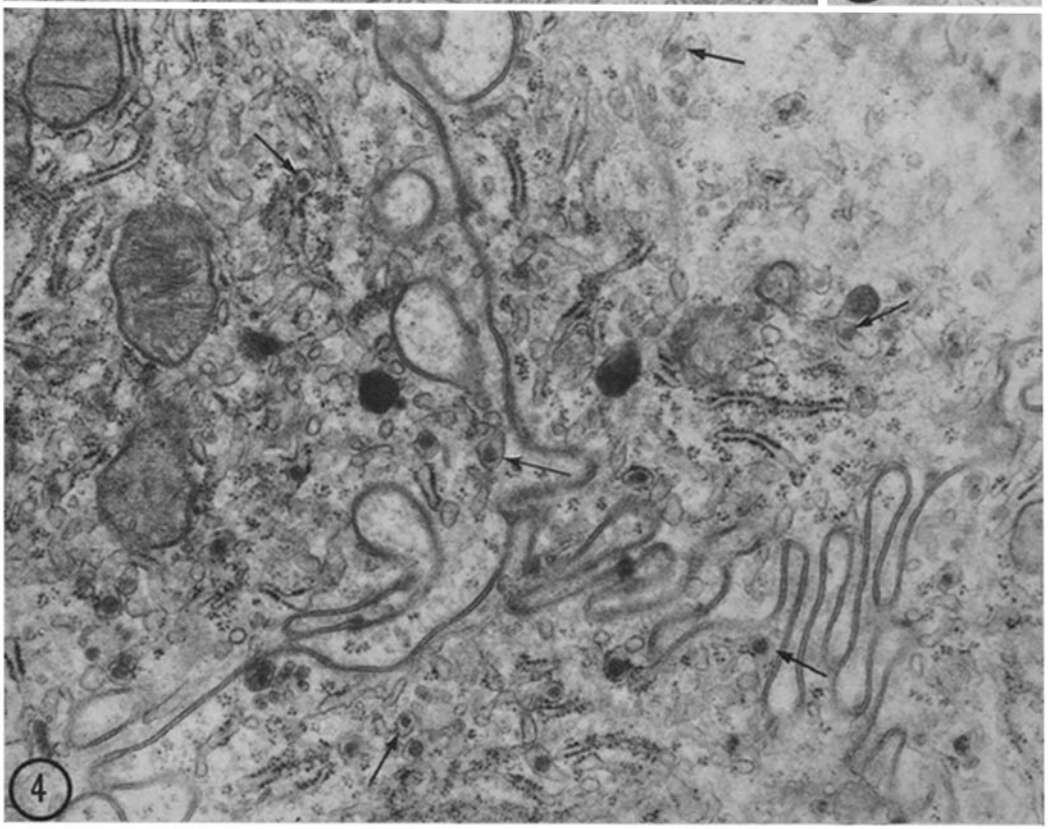
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TABLE II

Effect of Capture Reagents on the Incorporation of Palmityl-^{14}C CoA by Slices of Rat Intestine Fixed in 1% Glutaraldehyde

Reagents	mμmoles of palmityl-^{14}C CoA incorporated per hour/milligram lipid-free tissue		
	Diglyceride	Triglyceride	Fatty acid
(*a*) None	0.1078 ± 0.0196 (4)	0.057 ± 0.0208 (4)	0.389 ± 0.036 (4)
(*b*) Lanthanum nitrate	0.024 ± 0.0068 (4)	0.0228 ± 0.0156 (4)	0.0915 ± 0.02 (4)
(*c*) Potassium ferricyanide Manganous chloride	0.12 ± 0.0308 (4)	0.066 ± 0.0308 (4)	0.256 ± 0.072 (4)

Slices of fixed intestine were incubated in media containing palmityl-^{14}C CoA (0.5 mM) and monopalmitin (0.75 mM) in cacodylate buffer (0.025 M), pH 7.0, containing 4.5% dextrose for 30 min at 37°C with the following additions: (*a*) no addition, (*b*) lanthanum nitrate (3 mM), or (*c*) potassium ferricyanide (0.45 mM) and manganous chloride (2.5 mM). Components of each medium were added in the order indicated above to give final concentrations as shown. The solution was mixed after each addition. At the end of the incubation the slices were removed from the medium, washed in buffer, and the lipid was extracted with chloroform:methanol. The lipids were separated as indicated in the text. Results are expressed as mμmoles of palmityl CoA incorporated into lipid fractions: average ± SD (No. of observations).

essing (Table I), the deposits of lanthanum probably represent the sites of these lipid droplets.

Use of lanthanum nitrate as a capture reagent for CoA thus confirms the results of the experiments in which potassium ferricyanide and manganous chloride were used. However, tissue incubated in lanthanum nitrate is not well preserved, probably because postfixation of the tissue in alcoholic osmium tetroxide was necessary. With the use of potassium ferricyanide and manganous chloride, the deposits of reaction product are discrete and delicate, and require a reasonable preservation of morphology for identification. Thus, the use of lanthanum is not completely satisfactory for this study.

α-Glycerophosphate Pathway

INCORPORATION OF α-GLYCEROPHOSPHATE-^{14}C INTO COMPLEX LIPIDS BY UNFIXED AND FIXED INTESTINE: EFFECTS OF CAPTURE REAGENTS: When α-glycerophosphate replaced monopalmitin in the medium used in Fig. 1, the incorporation of the palmityl-^{14}C CoA into esterified lipid was only slightly greater than that occurring in the absence of acylacceptor. Experiments were therefore performed in which α-glycerophosphate-^{14}C was used with unlabeled palmityl CoA. Direct comparisons of unfixed and fixed intestine from the same animal were made and are given in Table III. Incorporation of α-glycerophosphate into triglyceride and phospholipid fractions occurred, and a small amount of label was incorporated into diglyceride. Fixation in 1% glutaraldehyde decreased the incorporation of label by approximately 80% into all lipids. Addition of potassium ferricyanide and manganous chloride to the incubation medium had no effect on the incorporation of the labeled lipids by unfixed or fixed tissue.

MORPHOLOGICAL CHANGES IN TISSUE INCUBATED FOR CYTOCHEMICAL STUDIES: Tissue incubated in the presence of α-glycero-

FIGURE 3 Apical region of intestinal absorptive cell from a fasted rat incubated in cacodylate buffer for 60 min at 37°C. Note absence of lipid droplets. × 30,000.

FIGURE 4 Apical region of intestinal absorptive cell from fasted rat incubated in a medium containing palmityl CoA and monopalmitin in cacodylate buffer (see text for details) for 60 min at 37°C. Note lipid droplets (arrows) within the smooth endoplasmic reticulum. × 37,000.

FIGURE 5 Golgi region of same tissue shown in Fig. 4. Suggestion of lipid droplet (arrow) occurs in membranous profile close to but not within the Golgi cisternae. × 37,000.

phosphate and palmityl CoA with potassium ferricyanide and manganous chloride, as capture reagents, is shown in Figs. 10 and 11. Deposits of reaction product occurred mainly on the membrane (or its inner surface) limiting the rough endoplasmic reticulum. Deposition also occurred occasionally on the membranes of smooth vesicles, especially in regions where these elements were close to or continuous with rough-surfaced forms. The Golgi membranes of the cell also showed delicate deposition of reaction product mainly on the dilated outermost cisterna of the concave surface of the complex (Fig. 13).

Because fixation in glutaraldehyde inhibited the incorporation of α-glycerophosphate into complex lipids by 80%, experiments were performed on unfixed tissue. Morphological preservation in these experiments was poor; however, reaction product was located primarily on the rough endoplasmic reticulum, there being no significant deposition elsewhere.

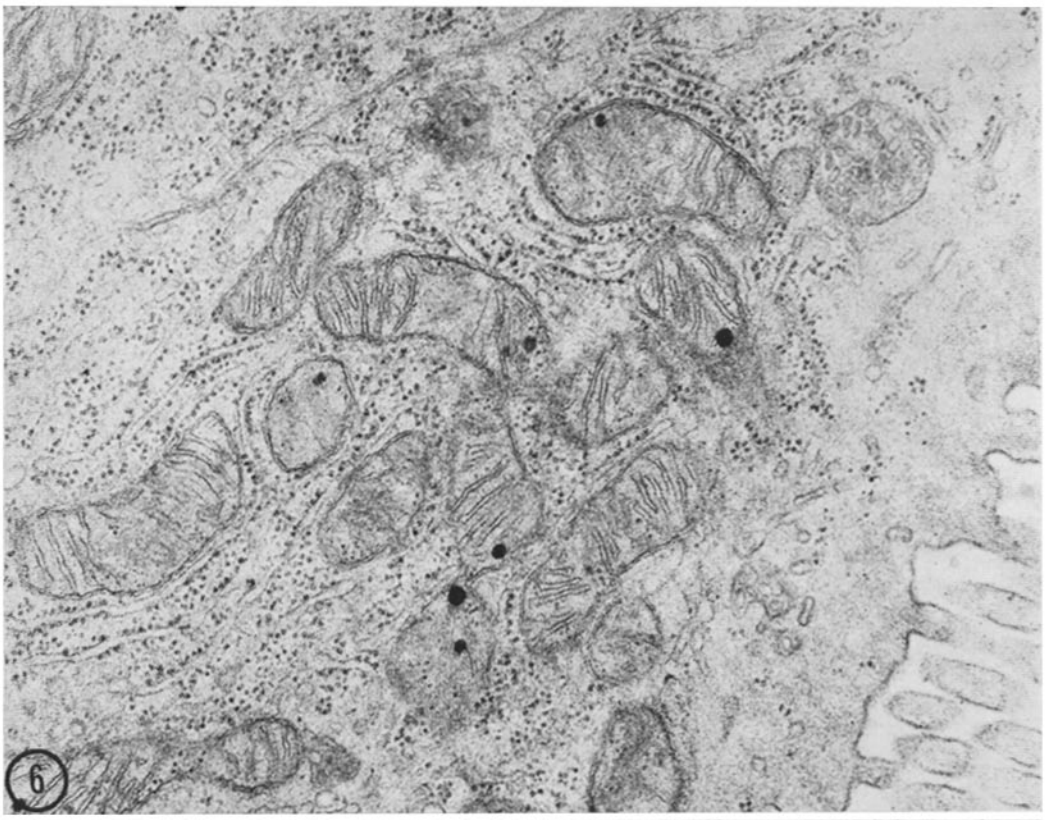
DISCUSSION

The application of cytochemistry to the study of the fine structural and functional interrelationships of the cell suffers from a number of possible sources of artifact, and some of these are worthy of consideration here. Final reaction product occurred within the intestinal absorptive cell, indicating penetration of the capture reagents. Parallel biochemical studies were performed on the same tissue; as metabolism took place penetration of the substrates must have occurred. From these observations, it is reasonable to conclude that penetration of the reactants into the cells was not significantly impeded. The rate of metabolism of palmityl CoA was sufficiently slow to necessitate incubation times of up to 2 hr in order to obtain sufficient reaction product. In addition, the concentrations of capture reagents used were far in excess of those needed to precipitate free CoA produced in these experiments. These precautions should avoid artifacts due to diffusion of the products of enzyme activity before precipitation; such diffusion, if present, usually results in a characteristic gradient of deposition of final product in the cytoplasm, having its highest concentration at the site of the enzyme and then decreasing as it spreads from this site. A third possible source of artifact, selective inhibition by glutaraldehyde of enzymes at different morphological sites, is the most serious source of error. The monoglyceride pathway acyltransferases were virtually unaffected by fixation; acyl CoA diglyceride acyltransferase was the most susceptible to fixation, and this was variable.However, it has been indicated by Rao and Johnston that the triglyceride synthetase system is a complex, comparable with the fatty acid synthetase enzyme system (30). If this is true, localization of one of its components will localize the whole enzyme system. In the case of the α-glycerophosphate pathway, glutaraldehyde caused a significant loss of activity approaching 80%. This could represent loss of activity of the enzyme at one or more morphological sites. However, experiments in which unfixed tissue was used showed essentially the same localization as experiments with fixed tissue. Thus, loss of enzyme activity due to fixation is probably random rather than selective.

Three groups of enzymic activities were localized in these studies. That activity indicated in the controls, presumably palmityl CoA hydrolase, was active in fixed tissue. The present studies indicate that this enzyme has a mitochondrial localization in the absorptive cell. This localization was variable; reaction product occurred between inner and outer membranes, between the cristae, and in association with mitochondrial granules. Further studies of this enzyme system were not made except as controls for the experi-

FIGURE 6 Apical region of intestinal absorptive cell from fasted rat incubated in a medium containing palmityl CoA, potassium ferricyanide and manganous chloride but no acylacceptor in cacodylate buffer for 2 hr at 37°C (see text for details). Larger than usual electron-opaque granules occur in the mitochondria. No lipid droplets are visible in any elements of the reticulum. × 40,000.

FIGURE 7 Apical region of intestinal absorptive cell from fat-fed rat incubated in a medium containing palmityl CoA, monopalmitin, potassium ferricyanide, and manganous chloride in cacodylate buffer at 37°C for 2 hr (see text for details). Note accumulation of reaction product between lipid droplets and membrane (arrows) as well as on the limiting smooth membrane and the surface of the droplets. × 70,000.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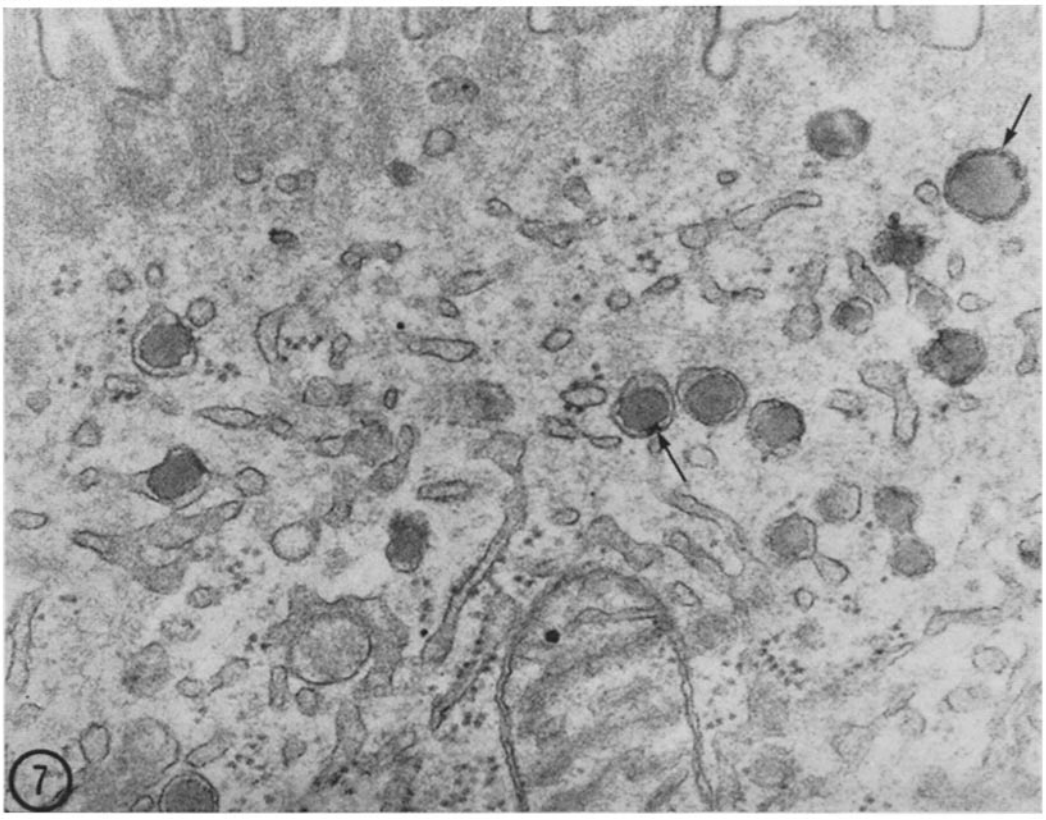
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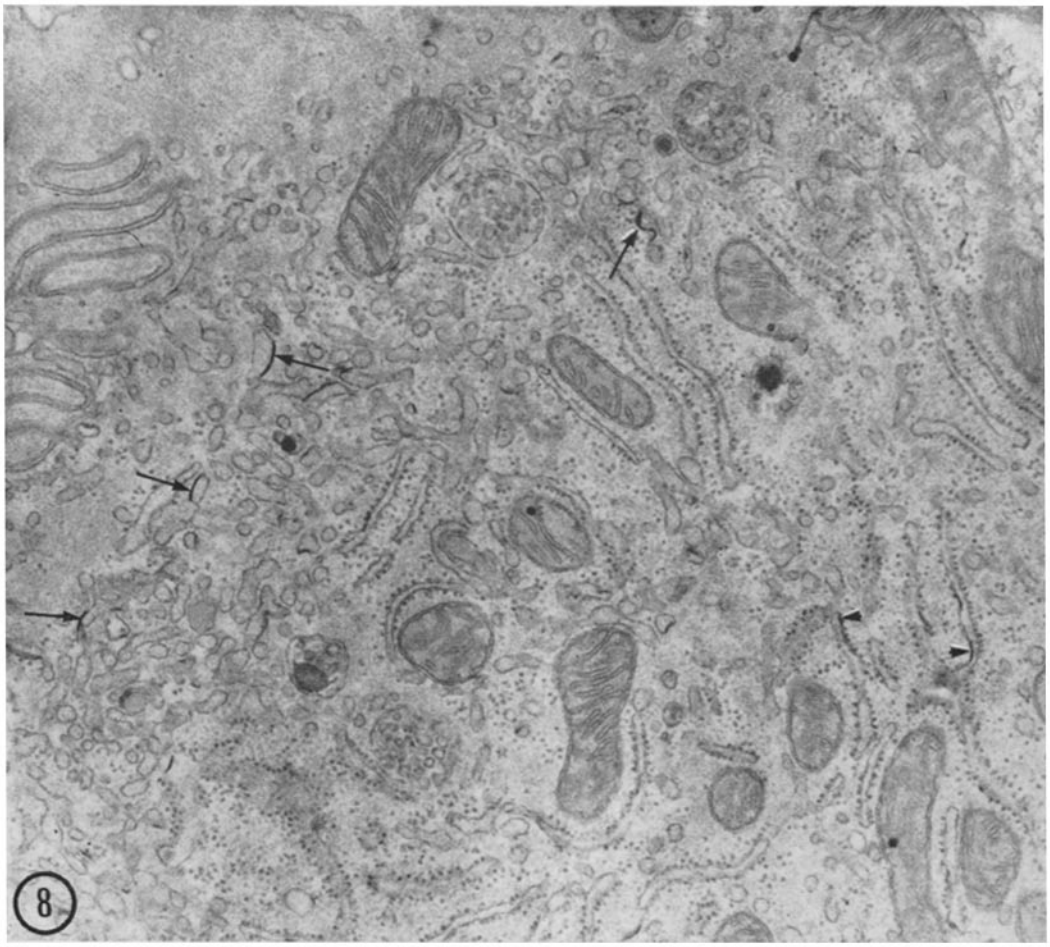

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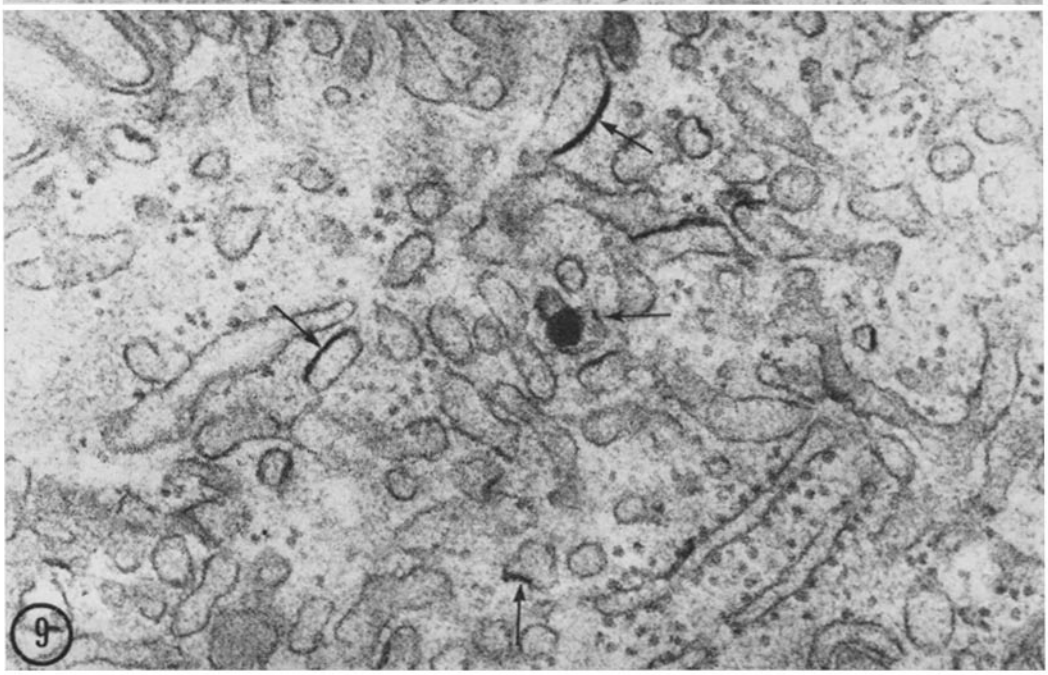

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TABLE III

Metabolism of α-Glycerophosphate-^{14}C by Slices of Intestine Unfixed and Fixed in 1% Glutaraldehyde: Effect of Capture Reagents

	mμmoles α-glycerophosphate incorporated per hour/milligram lipid-free tissue		
	Phospholipid	Diglyceride	Triglyceride
No capture reagents			
Unfixed tissue	0.624 ± 0.34 (4)	0.427 ± 0.05 (4)	3.84 ± 1.1 (4)
Fixed tissue	0.174 ± 0.065 (4)	0.1007 ± 0.013 (4)	0.373 ± 0.065 (4)
Plus capture reagents			
Unfixed tissue	1.15 ± 0.025 (4)	0.426 ± 0.034 (4)	4.42 ± 1.23 (4)
Fixed tissue	0.18 ± 0.07 (4)	0.103 ± 0.026 (4)	0.398 ± 0.077 (4)

Slices of fixed and unfixed intestine were incubated in media containing α-glycerophosphate-^{14}C (1.5 mM) and palmityl CoA (0.5 mM) in cacodylate buffer (0.025 M), pH 7.0, containing 4.5% dextrose at 37°C for 1 hr with and without addition of potassium ferricyanide (0.45 mM) and manganous chloride (2.5 mM). Components were added in the order indicated and the solution mixed after each addition. At the end of the incubation the slices were removed from the medium, washed with buffer, and the lipid was extracted with chloroform:methanol. The lipids were separated as indicated in the text. Results are expressed as mμmoles palmityl CoA incorporated into lipid fractions: average ± SD (No. of observations).

ments described here; however, these studies are in progress.

Several pieces of evidence are presented which indicate that the smooth endoplasmic reticulum is the major site of triglyceride synthetase activity. In the morphological studies, fixed tissue incubated in the presence of palmityl CoA and monopalmitin showed triglyceride droplets in membranous envelopes of the smooth endoplasmic reticulum. When capture reagents were present the electron-opaque deposits indicating release of CoA were formed on the membranes surrounding lipid droplets, but they also were associated with membranes having no droplets. This is probably due to loss of newly synthesized lipid during processing. When tissue from a fat-fed animal was used for cytochemical studies, reaction product was deposited between the lipid droplet and its surrounding membrane. These results indicate that triglyceride synthetase is probably located on the inner surface of the smooth endoplasmic reticulum, or is oriented on this membrane so as to release the products of its activity at the inner surface.

Using purified triglyceride synthetase from hamster intestine, Rao and Johnston have reported that CoA is not released during triglyceride synthesis from acyl CoA and monoglyceride (31). However, studies in which microsomes from rat intestine were used showed release of free CoA (33). In the present study, attempts to assay CoA release by using DTNB were unsuccessful because of the high blank value obtained with fixed tissue. However, deposition of reaction product occurred and was dependent on the metabolism of palmityl CoA and monopalmitin, indicating that release of free CoA occurred. A quantitative determination of the cytochemical results is difficult because of the extremely small amount of tissue examined as compared with that used in the biochemical measurement. However, deposition of reaction product did appear to be greater in the presence

FIGURE 8 Apical region of intestinal absorptive cell from fasted rat incubated in a medium containing palmityl CoA, monopalmitin, potassium ferricyanide, and manganous chloride in cacodylate buffer at 37°C for 2 hr (see text for details). Note reaction product associated with the smooth membranes in the apical region of the cell (arrows) and occasionally associated with rough endoplasmic reticulum membranes (arrowheads). × 30,000.

FIGURE 9 High magnification of tissue in Fig. 8. Note reaction product related to membrane of the smooth endoplasmic reticulum (arrows). × 80,000.

of α-glycerophosphate, although the incorporation of palmitate from palmityl CoA into esterified lipid was lower in this case than in the case of the monoglyceride pathway. It is possible, therefore, that some of the CoA released by triglyceride synthetase remained bound to the enzyme, but that sufficient CoA was released to form an electron-opaque deposit of manganous ferrocyanide or lanthanum CoA.

In these studies a commercial preparation of monopalmitin was used as an acylacceptor. This was used rather than monoolein, which is acylated more rapidly, in order to avoid the production of triglyceride of considerable density after osmium tetroxide fixation, which might mask the localization of the cytochemical reaction product. The product of the reaction between palmityl CoA and monopalmitin is tripalmitin, which has no double bonds and, in theory, should not react with osmium tetroxide. However, osmium tetroxide does cause retention of 35% of this lipid, indicating some form of reaction. In addition, lipid droplets persisted which, although pale, had some electron opacity. The reason for this is not clear. However, it is becoming increasingly apparent that the reactions of osmium tetroxide with lipids and other tissue constituents is complex and probably involves reactions or associations in addition to simple osmication of double bonds (27).

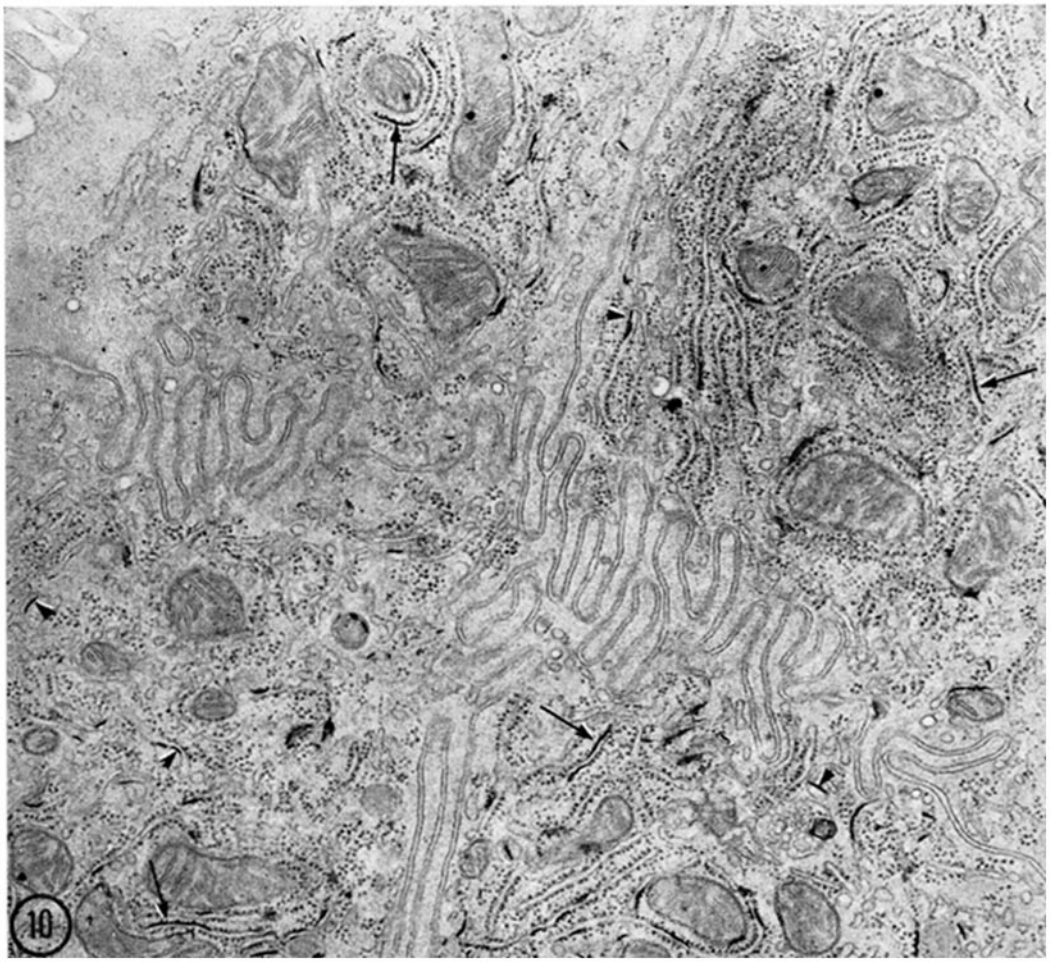
Commerical monopalmitin consists of a mixture of 1-monopalmitin and 2-monopalmitin. Some authors have reported that, with fractions of intestinal mucosa being used as a tissue source, 1-monoglycerides yield 1,3-diglycerides and 2-monoglycerides yield triglycerides as well as diglycerides (5, 8, 38). However, Kern and Borgstrom (26) and Johnston and Brown (22) have shown that slices of hamster intestine or whole mucosal scrapings synthesize triglyceride from either isomer of monoglyceride. Similarly, in the present study, when unfixed slices of intestine were used, triglyceride was the major product of the reaction and there was little accumulation of the label in diglyceride. Kern and Borgstrom (26) interpreted their observations as indicating loss of activity of acyl CoA diglyceride acyltransferase during preparation of microsomes. A similar loss of this activity occurred in the present study when tissue was fixed in 1% glutaraldehyde.

One surprising result from these studies was the observation that intestine fixed in glutaraldehyde retained the ability to incorporate α-glycerophosphate into triglyceride in the presence of palmityl CoA. This indicated that the following enzymes survived fixation: α-glycerophosphate palmityl CoA acyltransferase, lysophosphatidic acid palmityl CoA acyltransferase, phosphatidic acid phosphatase, and diglyceride palmityl CoA acyltransferase; and it also indicated that these enzymes are closely related in a structural sense, so that even in fixed tissue the complete synthetic pathway to triglyceride takes place. The distribution of label in the different lipid classes was similar in fixed and unfixed tissue, the only difference being the rate of incorporation of the label. This could be due to equal inhibition of all enzymes involved, or more likely to the fact that the rate-limiting step in the process is affected to the greatest extent. In mucosal cell fractions, the existence of a stimulated and an unstimulated pathway for triglyceride and phospholipid synthesis has been demonstrated, the former requiring addition of a soluble supernatant fraction to the mucosa microsomal fraction (4). Johnston et al. (24) have demonstrated that the stimulating factor in the supernatant is probably phosphatidic acid phosphatase. This enzyme is probably more loosely bound to the microsomal membrane than the rest of the enzymes involved in triglyceride synthesis. Although cell fractionation might promote the release of this enzyme, it is reasonable that in fixed tissue phosphatidic acid phosphatase remains bound at its site of action.

The cytochemical methods used localize the site

FIGURE 10 Apical region of intestinal absorptive cell from fasted rat incubated in a medium containing α-glycerophosphate, palmityl CoA, potassium ferricyanide, and manganous chloride in cacodylate buffer at 37°C for 2 hr (see text for details). Note reaction product associated with membrane of the rough endoplasmic reticulum (arrows) and occasionally with smooth-surfaced membranes (arrowheads). × 30,000.

FIGURE 11 High magnification of tissue in Fig. 10. Note reaction product associated with the surface of the rough endoplasmic reticulum (arrows). × 100,000.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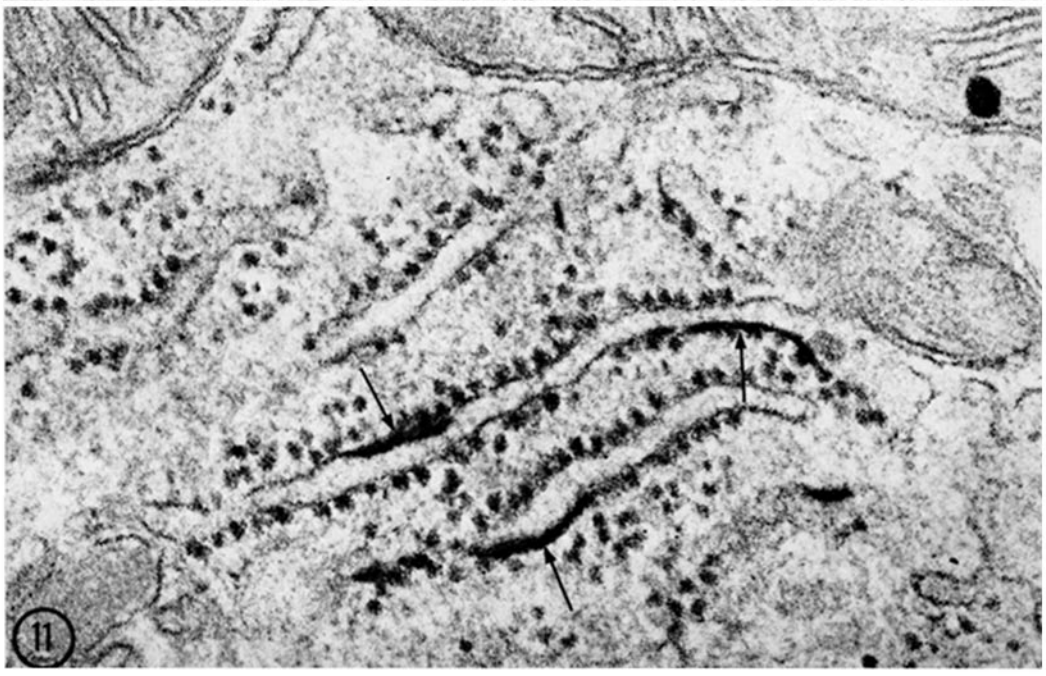
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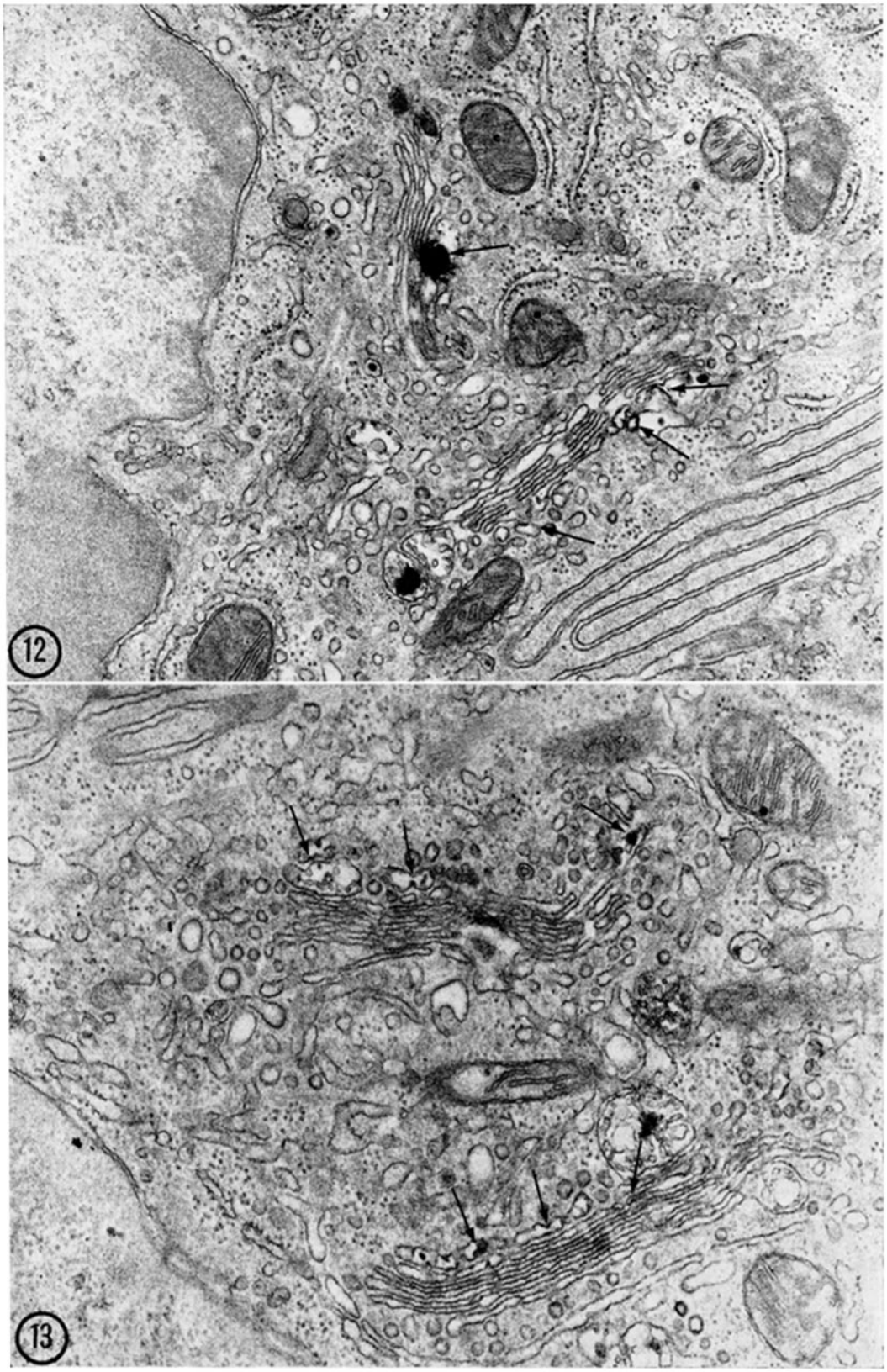


FIGURE 12 Intestinal absorptive cell incubated in medium shown in Fig. 8. Note reaction product associated with outer cisternae and vesicles of the Golgi apparatus (arrows). × 30,000.

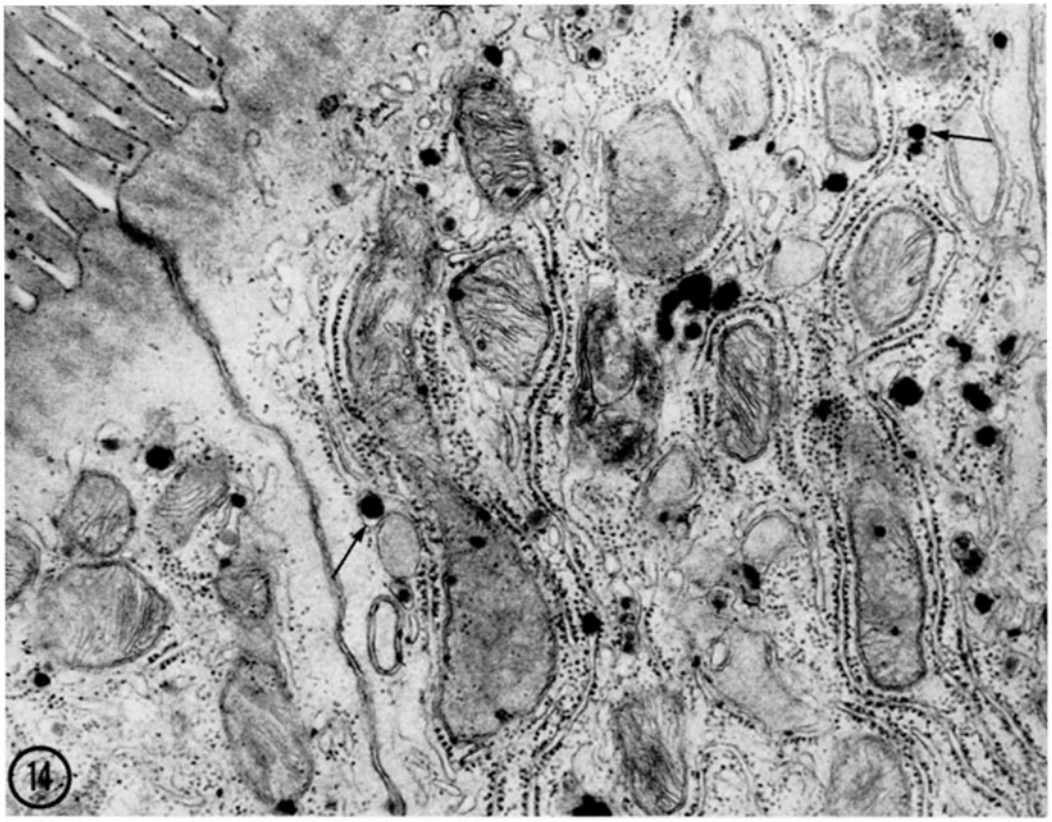
FIGURE 13 Intestinal absorptive cell incubated in the medium shown in Fig. 10. Note reaction product associated with the outer elements of the Golgi apparatus (arrows). × 30,000.

of production of free CoA. Therefore they locate the acyltransferases only, and cannot differentiate between the triglyceride- and phospholipid-synthesizing pathways when α-glycerophosphate is used as a substrate. However, biochemical evidence suggests that the α-glycerophosphate pathway system has common enzymes for the production of triglyceride or phospholipid, depending on the availability of bases and unsaturated fatty acids for the latter.

There have been a number of studies of intestinal mucosal cell organization with regard to lipid metabolism, by means of cell fractionation techniques. These studies have shown that both the monoglyceride and the α-glycerophosphate pathways are associated with the microsomal fraction of the mucosal cell (4, 5, 38). Brindley and Hubscher (4) have further investigated the distribution of these enzymes in subfractions of the microsomal fraction and found that both pathways are associated with the rough-surfaced microsomes and to a lesser extent with one of two smooth vesicular fractions. The association of the α-glycerophosphate pathway with the rough-surfaced microsomes is consistent with the findings reported here. However, at first examination, the appearance of the monoglyceride pathway in the rough-surfaced microsomes conflicts with our finding that this pathway occurs mainly in the smooth endoplasmic reticulum. This is explicable, however, when the structure of the endoplasmic reticulum of the intestinal mucosal cell is considered. The rough and smooth membranes are frequently continuous, and lipid droplets occur within smooth outpouchings of the rough endoplasmic reticulum during lipid absorption. Separation of rough and smooth elements by cell fractionation is thus difficult to achieve, and it is possible that the rough-surfaced vesicles isolated by Brindley and Hubscher include smooth elements, which in the intact cell represent the part of the endoplasmic reticulum where the lipid droplets form. The fraction of the smooth vesicles, which also has monoglyceride and α-glycerophosphate pathway enzymes, was not identified with any cell organelle. It is possible that these Golgi membranes, which in this study show some enzymatic activity, might appear in such a smooth membrane fraction.

In the present studies the monoglyceride palmityl CoA acyltransferases were found to be located in association with the membrane of the smooth endoplasmic reticulum in the apical region of the cell, and to a lesser extent with the rough endoplasmic reticulum which is continuous with the smooth membranes; and it was also found to be associated with elements of the concave surface of the Golgi apparatus. The α-glycerophosphate palmityl CoA acyltransferases are associated mainly with the rough endoplasmic reticulum, occasionally with smooth elements, and with elements of the Golgi region described above. The consistent finding that the monoglyceride and α-glycerophosphate pathways are mainly associated with different compartments of the endoplasmic reticulum is a curious one and deserves comment. The enzymes and structural proteins of both membrane compartments are most likely synthesized by the rough endoplasmic reticulum (9), and the present study suggests that this is also true for the phospholipid component. This is partly confirmed by recent experiments on hepatic cells (2) which indicate that a portion of the rough endoplasmic reticulum contains the enzymes involved in the α-glycerophosphate pathway. The problem arises, therefore, as to how the monoglyceride pathway is segregated mainly in the smooth membranes, although the source of its components is the rough endoplasmic reticulum. This must, in turn, be related to the synthesis of smooth from the rough endoplasmic reticulum. From the present evidence for the location of the monoglyceride pathway, unless the enzymes are produced and transported in an inactive state, the sites of activity (and presumed synthesis) in the rough endoplasmic reticulum are limited and most of them are close to the smooth elements in the apical region of the cell.

In view of our results, the following organization of the cell, with regard to lipid absorption, may be postulated. Monoglycerides and free fatty acids cross the microvilli of the intestinal cell probably by diffusion and not by pinocytosis. If pinocytosis occurred, the membranes surrounding the lipid droplets would be expected to show the same enzymic activity as the microvilli. On the contrary, most of the apical vesicles containing lipid droplets showed activity of acyltransferase and this activity was not present on the apical plasma membrane. Inside the cell, the absorbed fatty acid is activated by synthesis of its CoA derivative and triglyceride resynthesized by acylation of the absorbed monoglyceride, by an enzyme system associated with the membranes of the smooth endoplasmic reticulum. Triglyceride droplets thus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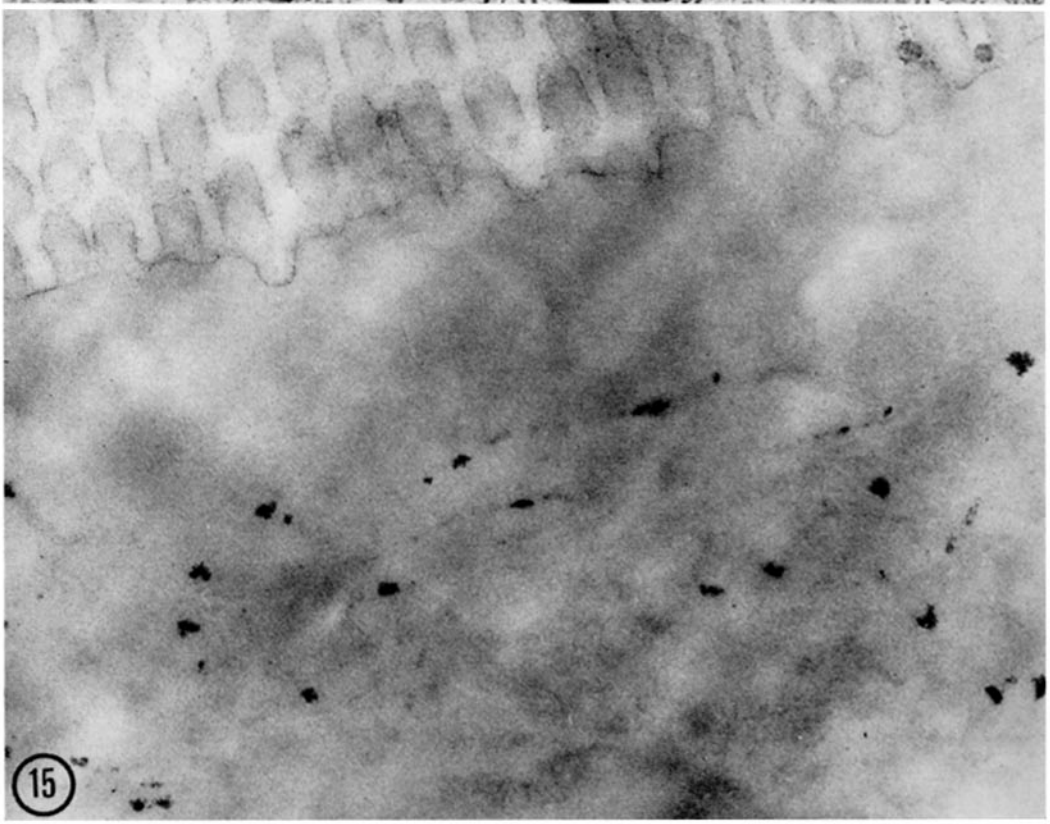
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accumulate within these membranous envelopes. The membrane-bound droplets then move to the Golgi apparatus, where they apparently fuse with the elements on the concave surface. The membranes of the smooth endoplasmic reticulum thus show a cytochemical localization for acyltransferase that is similar to that of the outermost concave elements of the Golgi membranes, and the fusion may account for the cytochemical reaction occurring in the latter. Such a pathway for absorbed triglyceride has been suggested from both morphological and radioautographic studies in vivo and in vitro (6, 29, 40, 41, 42). Phospholipids, the second major constituent of chylomicrons, are synthesized mainly by an enzyme system associated with the limiting membrane of the rough endoplasmic reticulum. The phospholipid may move from its site of synthesis to directly available triglyceride droplets in contiguous smooth surface regions of the endoplasmic reticulum. Alternatively, it may move separately within pinched-off smooth vesicles to the outer elements of the Golgi apparatus. At some point during this process, the triglyceride and phospholipid are packaged with cholesterol, cholesterol esters, and some protein to yield chylomicrons (3, 32), which have a constant composition and structure. There must, therefore, be some control of the rate of synthesis of the different components or the rate at which these are packaged. It is possible that, as the major components appear to be synthesized in different structural compartments, the control lies in this separation. The rate of movement of the lipid-containing vesicles to the Golgi elements and their fusion with these elements may represent an additional controlling factor.

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FIGURE 14 Apical region of intestinal absorptive cell from fasted rat incubated in a medium containing palmityl CoA, monopalmitin, and lanthanum nitrate in cacodylate buffer for 2 hr at 37°C (see text for details). Note very dense deposits mainly in elements of the smooth endoplasmic reticulum which have the size and form of the lipid droplets in Fig. 4 (arrows). × 37,000.

FIGURE 15 Tissue incubated as in Fig. 14, omitting the refixation in osmium tetroxide. All density is due to native density and to lanthanum. Note electron-opaque deposits in the same distribution as those in Fig. 14. × 37,000.

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