Electron microscopic study of intestinal fat absorption in vitro from mixed micelles containing linolenic acid, monoolein, and bile salt

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ABSTRACT Sacs and segments of everted hamster gut were incubated in vitro in solutions of mixed micelles (containing taurodeoxycholate, monoolein, and linolenic acid) for various periods and at different temperatures. The appearance of electron micrographs of the tissues was consistent with the hypothesis that the lipids were taken up in small particles (of micellar or molecular dimensions) by means of diffusion. There was no stimulation of pinocytosis or alteration in pinocytotic vesicles in the terminal web during uptake.

In the deep cytoplasm the lipids accumulated to form large osmiophilic droplets of two kinds: (*a*) large, solitary droplets contained only by the matrix of the cytoplasm, without confining membranes; (*b*) small droplets occurring in clusters, without evidence of coalescence, within the granular and agranular endoplasmic reticulum. There was a progressive accumulation of the small droplets with time. Similar droplets appeared in extracellular spaces, including the lacteals, after prolonged incubations at 37°C. The droplets in the endoplasmic reticulum appear to be the end product of lipid absorption and to be closely related to chylomicrons.

These processes resemble those occurring during absorption of triglycerides in vivo and support the idea that this occurs predominantly through the formation of mixed micelles of the type employed in this study.

KEY WORDS electron microscopy · intestine · hamster · fat absorption · mixed micelles · bile salts · monoolein · linolenic acid · time · temperature · uptake · droplet formation · triglyceride synthesis · cytoplasmic matrix · cytoplasmic organelles

A MAJOR FRACTION of digested fat exists in the lumen of the intestine as a clear aqueous solution of mixed bile salt micelles, according to Hofmann and Borgström (1, 2). The micelles, measuring about 50 A, contain conjugated bile salts, long-chain fatty acids, and monoglycerides (3). Johnston and Borgström have recently demonstrated that the isolated intestine of the hamster rapidly forms triglyceride from such micellar lipids (4).

The present work is a morphological investigation of lipid absorption from solutions of mixed bile salt micelles. Experiments were performed in vitro in order to insure that the absorptive cells were exposed to a chemically defined lipid solution. The uptake of fatty acids and monoglycerides into the cell was associated with no detectable morphological change. Once within the cell, these lipids were converted into dense osmiophilic droplets, clearly visible within the endoplasmic reticulum and in the matrix of the cytoplasm. The possible significance of these findings will be discussed.

MATERIALS AND METHODS

Male golden hamsters weighing 100 g were fasted for 24 hr and killed by a blow on the head. The peritoneal cavity was opened, and the contents of the small intestine were washed out in situ with 25 ml of 0.9% NaCl. The jejunum and ileum were stripped from the mesentery and placed in a Petri dish containing 0.9% NaCl. The jejunum (proximal half of the intestine) was everted with a stainless steel rod and washed for 1 min in each of three dishes containing 200 ml of 0.9% NaCl. The washed intestine was placed in a Petri dish containing 50 ml of Krebs-Ringer phosphate buffer (7) minus calcium and magnesium at pH 6.3. Intestinal segments (or rings) were prepared by the method of Agar, Hird, and Sidhu (5), and everted sacs by the method of Wilson and Wiseman (6). Sacs were about 4 cm in length and contained

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0.5–1.0 ml of phosphate–saline as "serosal fluid." In all experiments, control and experimental tissue preparations were from adjacent intestinal segments.

Tissues were blotted and placed in flasks containing appropriate solutions. Six slices weighing 180–220 mg were placed in a 10 ml Erlenmeyer flask containing 1 ml of appropriate solution. An everted sac was added to a 25 ml Erlenmeyer flask containing 5 ml of incubation medium. The containers were gassed for 1 min with oxygen at a flow rate of 2 liters/min. The flasks were tightly stoppered and incubated in Dubnoff shakers. Incubations were performed for 30 sec, 2, 5, 15, 30, and 60 min. Incubation for "seconds" was performed by briefly swirling tissue in the medium without further oxygenation. When experiments were performed at low temperatures, both the tissue and the medium were chilled separately to the desired temperature (such as 0°C) before incubation.

"Postincubation" experiments were performed as follows. Intestinal slices were incubated together for 20 min in a flask containing micellar solutions at near 0°C. Then the slices were individually washed for 30 sec at near 0°C in each of three 50-ml volumes of phosphate–saline, or (giving similar results) in each of two 1.0-ml volumes of chilled phosphate–saline containing bile salt. The slices were blotted. Some of the segments were fixed. Others were rapidly placed in flasks containing 1.0 ml of phosphate–saline at 37 or at 0°C, gassed, postincubated at these temperatures for 7 and 15 min, and then fixed. Additional controls consisted of segments which were incubated and postincubated in saline alone, and also slices incubated with micelles at 0°C for 35 min.

Control preparations were incubated in Solution A, (Krebs-Ringer phosphate minus calcium and magnesium ions at pH 6.3 plus 2 mg/ml of glucose). This solution was gassed with oxygen. "Bile salt controls" were incubated in Solution B (2.4 μmoles of sodium taurodeoxycholate per ml of Solution A). The medium used for experimental preparations, Solution C, contained 2.4 μmoles of sodium taurodeoxycholate, 0.6 μmole of linolenic acid (Applied Science Laboratories, Inc., State College, Pa.) and 0.3 μmole of monoolein (Hormel Institute, Austin, Minn.) per ml of Solution A. The monoolein was a 9:1 mixture of the isomers 1- and 2-monoolein respectively. The conjugated bile salt was kindly supplied by Dr. John M. Johnston of the University of Texas, Southwestern Medical School, Dallas, Tex. Electrolytes were of analytical grade, water was distilled in glass, and the lipids were highly purified, failing to show spots of other compounds after thin-layer chromatography.

Mixed micelles were prepared by a modification of the method of Johnston and Borgström (4). For each 5 ml of micellar solution, 3.0 μmoles of linolenic acid and 1.5 μmoles of monoolein in reagent grade diethyl ether (Merck) were added to the same container. The ether was removed by evaporation with dry nitrogen and then under nitrogen in a vacuum chamber. After removal of solvent, 0.3 ml of 0.04 M taurodeoxycholate in Solution A was carefully added so as to come into contact with all of the lipid mixture. The container was gently shaken for about 2 min, the contents becoming a water-clear solution of bile salt micelles. This solution was diluted with 4.7 ml of Solution A, to arrive at the composition of Solution C.

Tissue was usually not washed before fixation. The specimen was fixed after incubation by placing an entire slice or a segment of an emptied sac into fixative and cutting the specimen into small blocks with a clean razor blade. Fixatives were usually chilled to 0–3°C, but some specimens were fixed at room temperature. The fixatives were usually osmium tetroxide buffered with veronal–acetate (8) plus sucrose (9) or in *s*-collidine (10). Tissue was also prepared with other fixatives, including Millonig's (11), Dalton's (12), osmium vapors, and glutaraldehyde (13). In some experiments, 0.2 ml of undiluted glutaraldehyde was added directly (at 37°C) to control and experimental flasks containing segments which had been incubated with 5 ml of media. This permitted a final concentration of 4% glutaraldehyde and only a slightly reduced concentration of the micelles in experimental media. These additional methods of fixation provided no additional information.

After fixation for 1–2 hr, tissues were dehydrated rapidly with methanol. The specimens were then infiltrated and embedded in Epon (14). This was accomplished by soaking them in a mixture containing equal volumes of propylene oxide and complete epoxy resin for 3 hr at room temperature. Subsequently the tissues were placed in gelatin capsules with epoxy resin which was cured overnight in an oven at 60°C. The Huxley Cambridge ultra-microtome was used for sectioning. "Thick" sections were examined by phase contrast light microscopy. The distal end of a villus was located on a block face, and only this was trimmed and sectioned for electron microscopy. Sections were stained with lead citrate (15) or "double-stained" with a saturated aqueous solution of uranyl acetate followed by lead citrate. Micrographs were obtained by means of an RCA model EMU-3F electron microscope.

RESULTS

Previous work from this laboratory demonstrated that the fine structure of isolated hamster intestine is maintained for as long as 4 hr under physiological conditions in vitro (16). The present work, performed under different in vitro conditions, confirms and extends those

findings. Electron micrographs of control tissues resemble intestine of fasted adult hamster in vivo and in vitro at 37°C (16), and in rat (17), mouse (18), and human intestine (19) in vivo.

The mucosal surface of hamster intestine is composed predominantly of finger-like projections or villi, which are covered by a simple columnar epithelium (Fig. 1). This consists mostly of absorptive cells and a few mucus-secreting goblet cells. The epithelium and its basement membrane rest on a loose connective tissue which comprises the villus core. The borders of an absorptive cell are formed by an apparently continuous plasma membrane. The apical part of the plasmalemma covers numerous cylindrical microvilli which are closely associated with a wispy material or "fuzz" (20). There are fine granules among the wisps of the "fuzz" and in the underlying cytoplasm (Fig. 1, inset). The plasma membrane is thicker at the cell apex than at its lateral and basal surfaces.

The lateral surfaces of adjacent absorptive cells are in close apposition except toward the base, where they are frequently separated by a sizeable intercellular space. The lateral membranes fuse near the luminal surfaces of the cells, obliterating the intercellular space (53). In other types of epithelium these fused or "tight" junctions appear to function as a diffusion barrier against materials entering between the cells from the lumen, at least against substances such as concentrated protein solutions. The basal part of the plasma membrane is flat and rests on a continuous basement membrane of amorphous material.

The cytoplasm of absorptive cells is distinguished by a narrow ectoplasmic zone which is rich in fibrillar material and an endoplasm containing numerous organelles. The ectoplasm has its greatest development in the apex of the cell, subjacent to the microvilli, where the agglomerated fibrils constitute a prominent band-like structure, the "terminal web." Filaments from the web extend into the cores of the overlying microvilli. The terminal web is generally devoid of membranous structures except for invaginations of the apical plasmalemma and vesicular profiles. The invaginations rarely penetrate through the full thickness of the web. The "pinocytotic vesicles" may vary in frequency from cell to cell, and they appear to have different densities (Fig. 1, inset). These differences in density seem attributable to variations in their contents, when transverse sections of the vesicles are seen at higher magnifications. It is unusual to find membranous connections between these structures and the endoplasmic reticulum, even though profiles of the latter may occur in the deep parts of the web.

The cytoplasm beneath the web consists of a matrix of appreciable density containing a variety of organelles including mitochondria, the endoplasmic reticulum, and elements of the supranuclear Golgi complex. The endoplasmic reticulum is richly distributed in the apical cytoplasm. This organelle is seen in thin sections as profiles of cisternae, vesicles, and tubules. Many of these forms are believed to be interconnected, constituting a single labyrinthine organelle (16, 17, 21). The reticulum consists of both granular and agranular membranes, a distinguishing feature of the former being a close association with ribonucleoprotein-enriched granules.

These typical features are consistently present too when intestinal sacs and slices are incubated with mixed micelles. Moreover, in all experimental tissue, there is no morphological evidence of entering lipid at the apical border of absorptive cells. Surprisingly, particles representing micelles are not discernible between the microvilli or in their adherent fuzzy coat, despite the expected association of these structures with micelles during incubation. Furthermore, entering lipid is not identified within the microvilli or in the superficial terminal web. Nor are there consistent changes in apical plasmalemmal invaginations or pinocytotic vesicles in the terminal web as compared to the variations which have been encountered for these in controls. The prime difference from controls has been the presence in experimental tissue of uniformly dense, osmiophilic, rounded droplets (Figs. 2, 3, 9, 10). Because of this difference, these droplets are believed to represent absorbed fat. The droplets are abundantly present in the apical cytoplasm below the terminal web, although a few may be found in the deep part of the web. The lipid depositions are located within the endoplasmic reticulum and in the matrix of the cytoplasm. Different experimental conditions during incubation are associated with variations in these droplets, as described in the following paragraphs.

When preparations are incubated with mixed micelles for "seconds," there are no droplets of absorbed lipid in the tissues.

After incubations with micelles at 37°C for 5 min (Fig. 2) droplets occur in the cytoplasm but not in intercellular spaces. Many of these droplets are present within profiles of the agranular and granular endoplasmic reticulum (Fig. 2, inset), separated by a lucent zone from the confining membranes. Several droplets may be crowded within a single profile. The droplets are about 250 mμ in diameter. A few droplets occur in Golgi vesicles. Frequently, large droplets (*ML*, Fig. 2) are also present in the cytoplasmic matrix, not confined within membranous organelles.

When the preparations are incubated for 1 hr at 37°C (Fig. 3), absorbed fat occurs as droplets and groups of droplets which mark a trail extending completely across the absorptive epithelium, except for the notable absence of lipid among microvilli and in the underlying terminal web. By this time, most cytoplasmic droplets

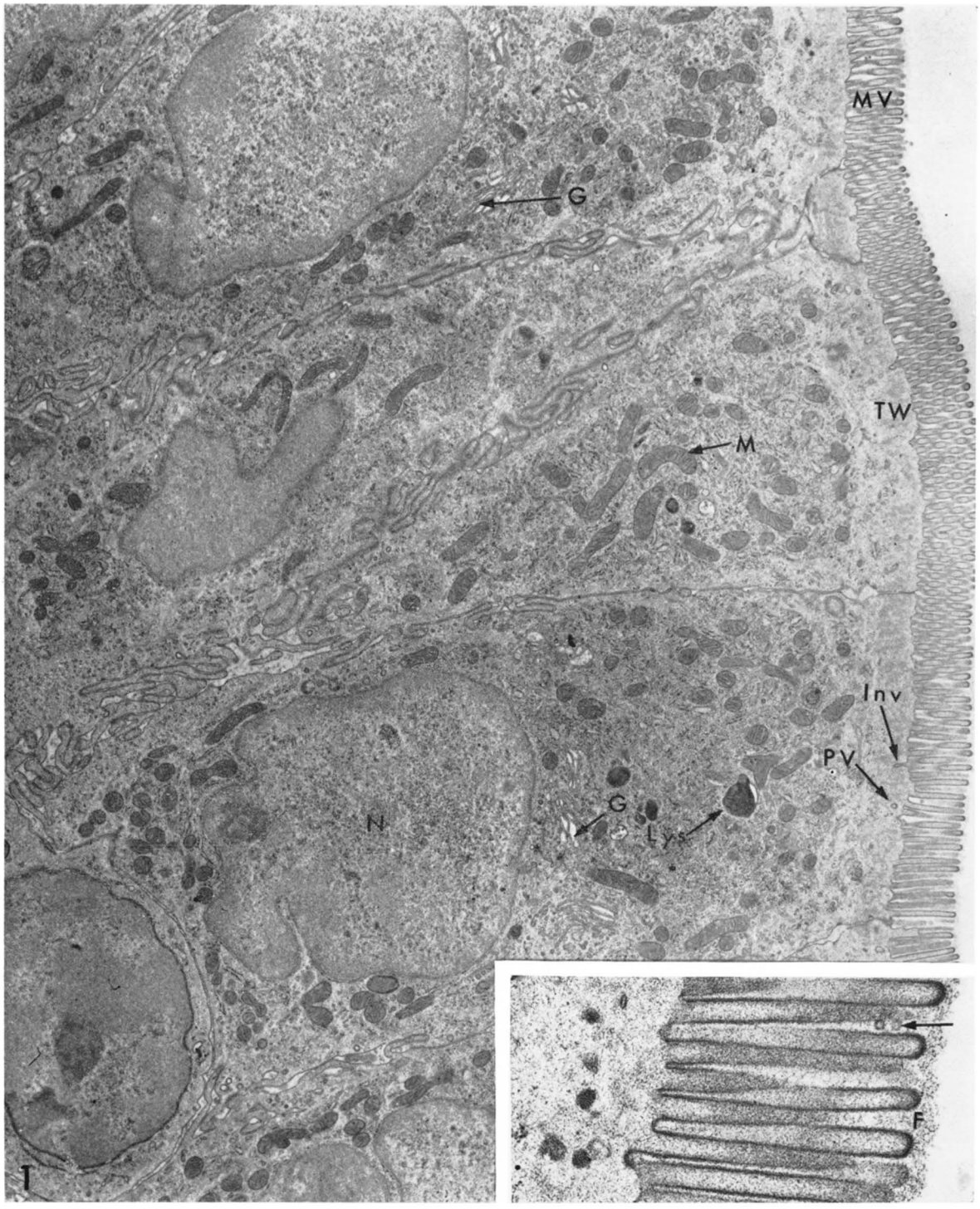

FIG. 1. Electron micrograph of epithelial absorptive cells from a control intestinal segment, incubated for 5 min at 37 °C in phosphate-buffered saline without added lipid. Obliquity of sectioning caused the apparent stratification in this simple epithelium. *MV*, microvilli; *TW*, terminal web; *M*, mitochondria. The web contains pinocytotic vesicles (*PV*) and invaginations of the apical plasmalemma (*Inv*). The endoplasmic reticulum is not easily seen at this magnification. The dense, rounded objects with a heterogeneous content are lysosomes (*Lys*). Lipid droplets are not present. Immediately above the nuclei (*N*), there are stacked profiles of flat, saccular structures and vesicles belonging to the Golgi complex (*G*). × 7,800.

The inset displays part of the free border of a control at greater magnification. This is seen to consist of "fuzz" (*F*), microvilli, and the terminal web. There are many finely granular profiles and an abundance of fibrillar material in these zones. The particles in the "fuzz" are as large as 80 A and their opacity to the electron beam is apparently variable. This normal stippling may obscure the presence of entering lipids. Vesicular objects (arrow) are occasionally seen in the spaces between microvilli. Similar profiles occur in experimental preparations (Figs. 3 and 10). Vesicles like these have been seen in vivo and may represent degenerated microvilli (52). The underlying terminal web contains seven pinocytotic vesicles which have either lightly or darkly stained contents. × 31,800.

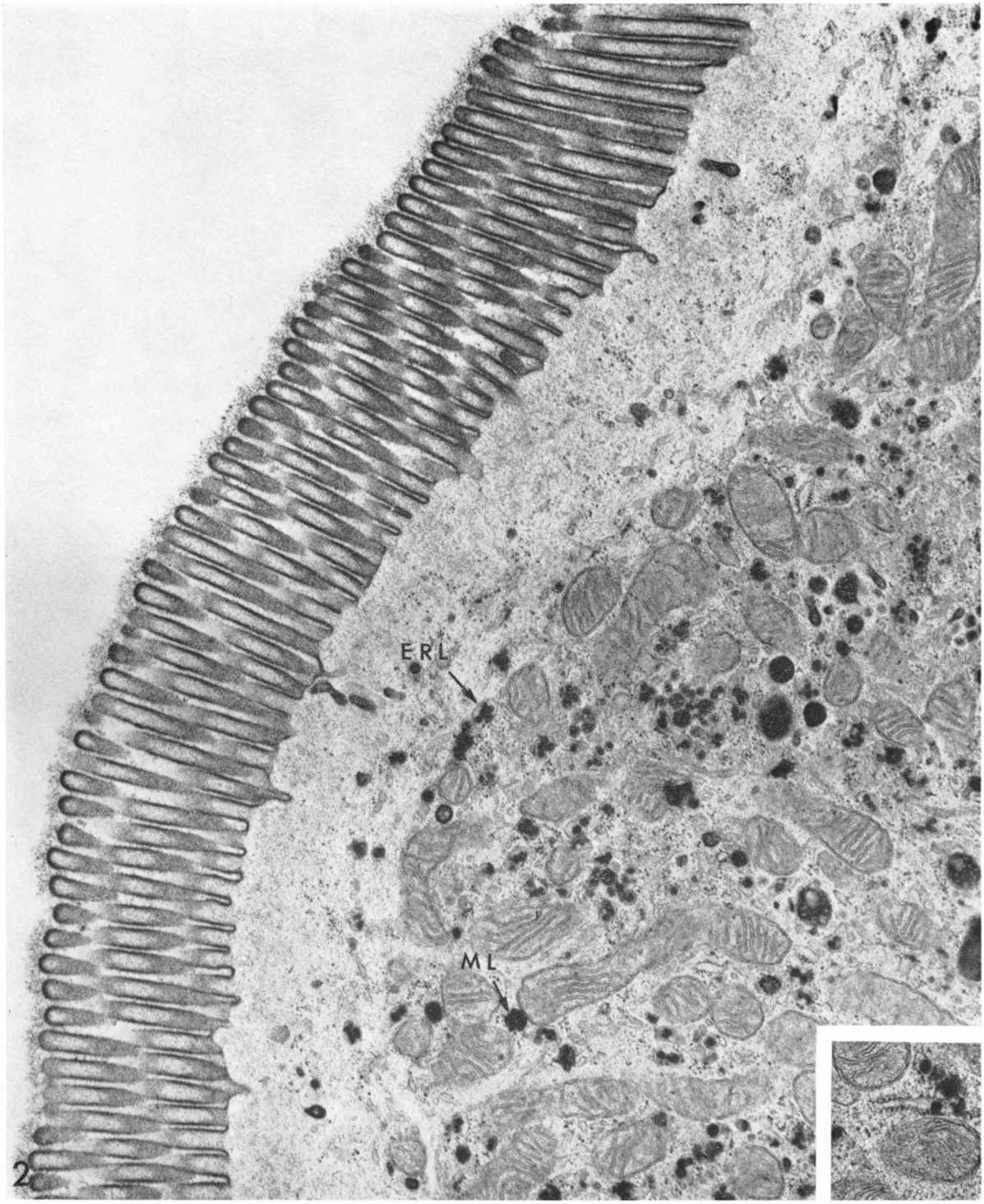

FIG. 2. Apical cytoplasm of absorptive cells after incubation with mixed micelles containing taurodeoxycholate, linolenic acid, and monoolein for 5 min at 37 °C. Fat absorption is revealed by the presence of numerous dense droplets in the deeper apical cytoplasm. However, uptake of lipids into the cytoplasm is not distinguishable in the superficial zones containing the "fuzz," the microvilli, or the terminal web. These regions contain an abundance of finely granular material which is similar to that in controls, as seen in Fig. 1, inset. There are no lipid droplets among microvilli nor is there any apparent stimulation of pinocytosis. Most of the lipid droplets in the apical cytoplasm are enclosed by membranes of the agranular endoplasmic reticulum. Clusters of these droplets (*ERL*) frequently occur in a single profile. Other droplets, usually solitary and larger (*ML*), are contained only by the matrix of the cytoplasm. × 26,800. The inset shows lipid droplets within the granular endoplasmic reticulum. The external surfaces of this rough-surfaced organelle are normally studded with numerous attached ribosomal granules. × 38,000.

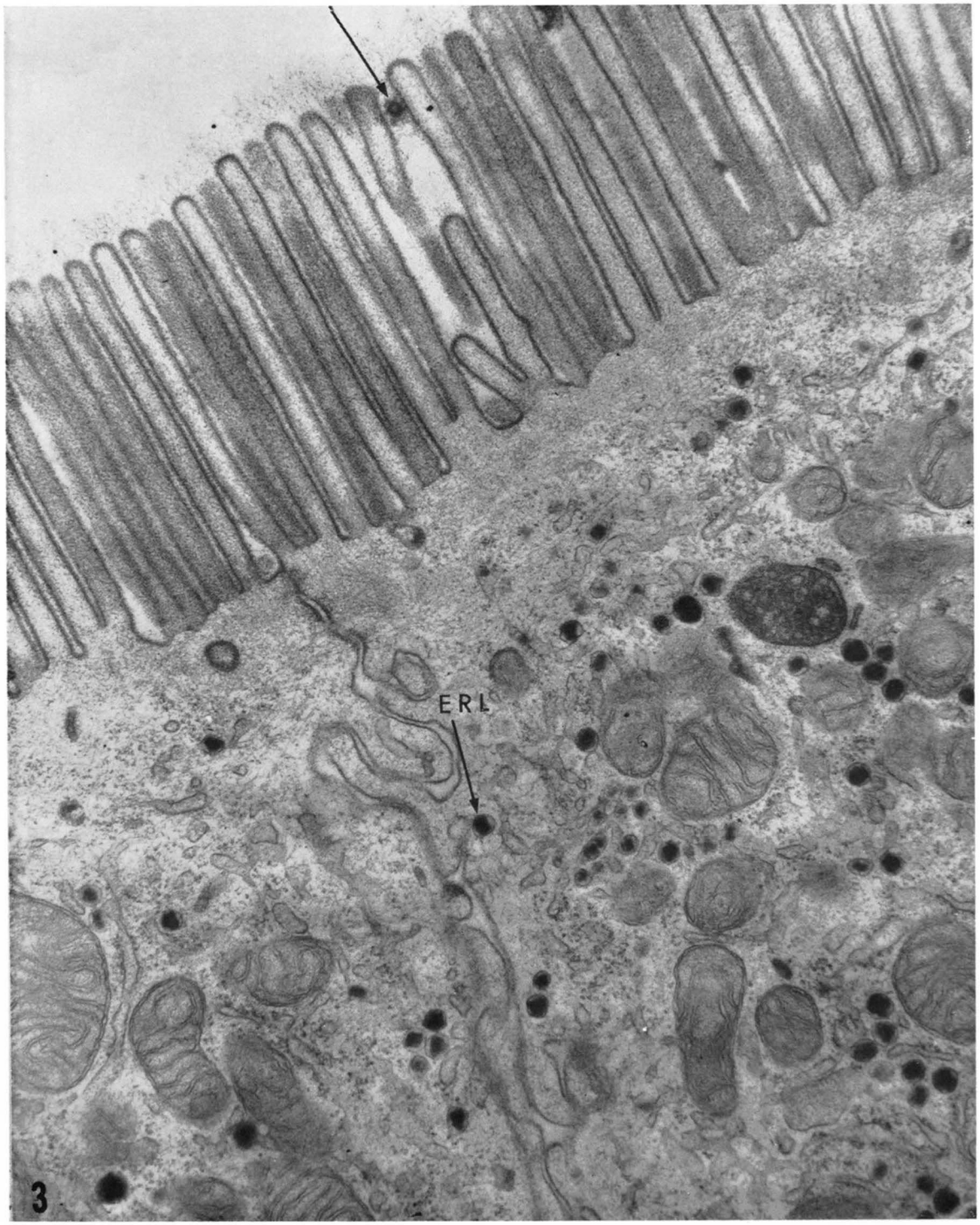

FIG. 3. Apical cytoplasm after incubation with mixed micelles for 60 min at 37°C. "Fuzz," microvilli, and terminal web are similar to controls and to preparations incubated with micelles for shorter times. Vesicular profiles (arrow) in spaces between microvilli may represent degenerate microvilli. There are numerous fat droplets (*ERL*) within vesicular and anastomosing tubular profiles of agranular reticulum. Lipid droplets have not entered the intercellular space between the upper parts of these cells. × 42,000. The next three micrographs are from deeper parts of the same villus.

are clearly located within profiles of the endoplasmic reticulum. These deposits are 300–330 mμ in diameter. However, a few large droplets may be seen without confining membranes in the cytoplasmic matrix. Osmiophilic deposits are also present within Golgi vesicles. At 60 min, a large amount of absorbed lipid also occurs in extracellular tissue spaces. Many droplets may be seen within the prominent spaces between adjacent absorptive cells (Fig. 4). These depositions are similar in size and appearance to droplets in the endoplasmic reticulum of the adjacent cells. Droplets tend to be packed in local regions of these spaces such as in the narrow parts between cytoplasmic folds, and between epithelial foot processes and the basement membrane. Droplets have arrived, too, in the underlying spaces between connective tissue cells (Fig. 5) and as chylomicrons in lacteals (Fig. 6) in the villus core. Droplets at these last two sites are similar in size and appearance to droplets within the epithelial intercellular spaces and in the endoplasmic reticulum. These droplets may be closely packed in aggregates. However, the droplets have nearly equal diameters, suggesting some protection from coalescence. Hence, the occurrence of droplets of a single type is seen to extend from the endoplasmic reticulum of absorptive cells to the lumens of lacteals.

Low-Temperature Incubations

Little, if any, absorbed fat is recognizable in experimental tissue after incubations with micelles for as long as 30 min at near 0°C (Fig. 7). Lipid droplets are not seen in the cytoplasmic matrix or within the endoplasmic reticulum of absorptive cells, or in the intercellular spaces. Furthermore, there are no morphological changes to suggest that there was uptake by these cells, such as osmiophilic accumulations among the microvilli or in the terminal web, or alteration in the apparent density of the apical plasmalemma. Micrographs are similar to those of controls, incubated in phosphate–saline at 37° or at 0°C. Nevertheless, there is considerable uptake of micellar lipid by intestine at 0°C, even though the compounds are not morphologically detectable. This has been demonstrated by "postincubating" the tissue in saline without added lipid at 37°C. The results may be seen readily, by light as well as by electron microscopy. Cytoplasmic droplets were visible in the phase contrast microscope at 7 min, and these were larger and more numerous after 15 min of supplementary incubation. In electron micrographs (Fig. 8), many small droplets are seen within granular and agranular profiles of the endoplasmic reticulum. There are also large irregular droplets which have no confining membranes, in the cytoplasmic matrix. However, very few if any fat droplets are seen in experimental tissue which was fixed immediately after incubation with micelles at 0–3°C, or in segments which were "postincubated" in saline at 0°C, or in the saline controls for this experiment.

A striking effect on the distribution of lipid droplets is noted during fat absorption at 10°C. After incubation with micelles for 5 min (Fig. 9), extremely minute droplets are seen in the endoplasmic reticulum but there are prominent accumulations in the cytoplasmic matrix. The two droplet types differ with respect to location, droplet size and perhaps osmiophilia. Droplets in the reticulum are frequently less than 100 mμ in diameter. Droplets are not seen in the lateral intercellular spaces or in the lamina propria mucosae. It may be seen that there are 91 droplets within the reticulum and 41 droplets in the cytoplasmic matrix (Fig. 9). Similar numbers of each class of droplet are present in micrographs of other absorptive cells. Droplets in the reticulum are so small that their combined amount can be but a small fraction of the lipid in the cytoplasmic matrix. Therefore it would seem that the rate of lipid deposition is far greater in the cytoplasmic matrix than in the endoplasmic reticulum during the initial 5 min of incubation at 10°C.

In addition to these effects on cytoplasmic distribution, temperature also affects the rate at which droplets accumulate in cells. As noted previously, very few droplets are seen even after prolonged incubations at near 0°C. A 5 min incubation at 10°C will clearly stimulate the formation of many more droplets, and an even greater deposition of lipid is seen after incubations at 37°C for 5 min. A comparison of droplet accumulation at 10 and 37°C, as seen in Figs. 9 and 2, indicates that there is a greater deposition of lipid in the endoplasmic reticulum at the higher incubation temperature.

Two populations of lipid droplets are seen in absorptive cells after incubation for 60 min at 10°C (Fig. 10). The characteristics of each population are the same as after 5 min. However, there is a greater total deposition after the longer incubation. Most of this increased lipid occurs within the endoplasmic reticulum. Individual droplets in the reticulum have increased in diameter from less than 100 mμ at 5 min to 230 mμ at 60 min at 10°C. If the droplets were spherical, there was at least an 8-fold increase in their volume. This suggests a great increase in the amount of lipid within the reticulum. The total number of droplets in this organelle is not appreciably different from that after a 5 min incubation. There seems to be relatively little change in the volume of droplets lying freely in the cytoplasmic matrix. Therefore, these considerations suggest that lipid accumulated more rapidly within the endoplasmic reticulum than in the cytoplasmic matrix at late times during a 1-hr incubation (at 10°C). It may be noticed that droplets in the reticulum are invariably much

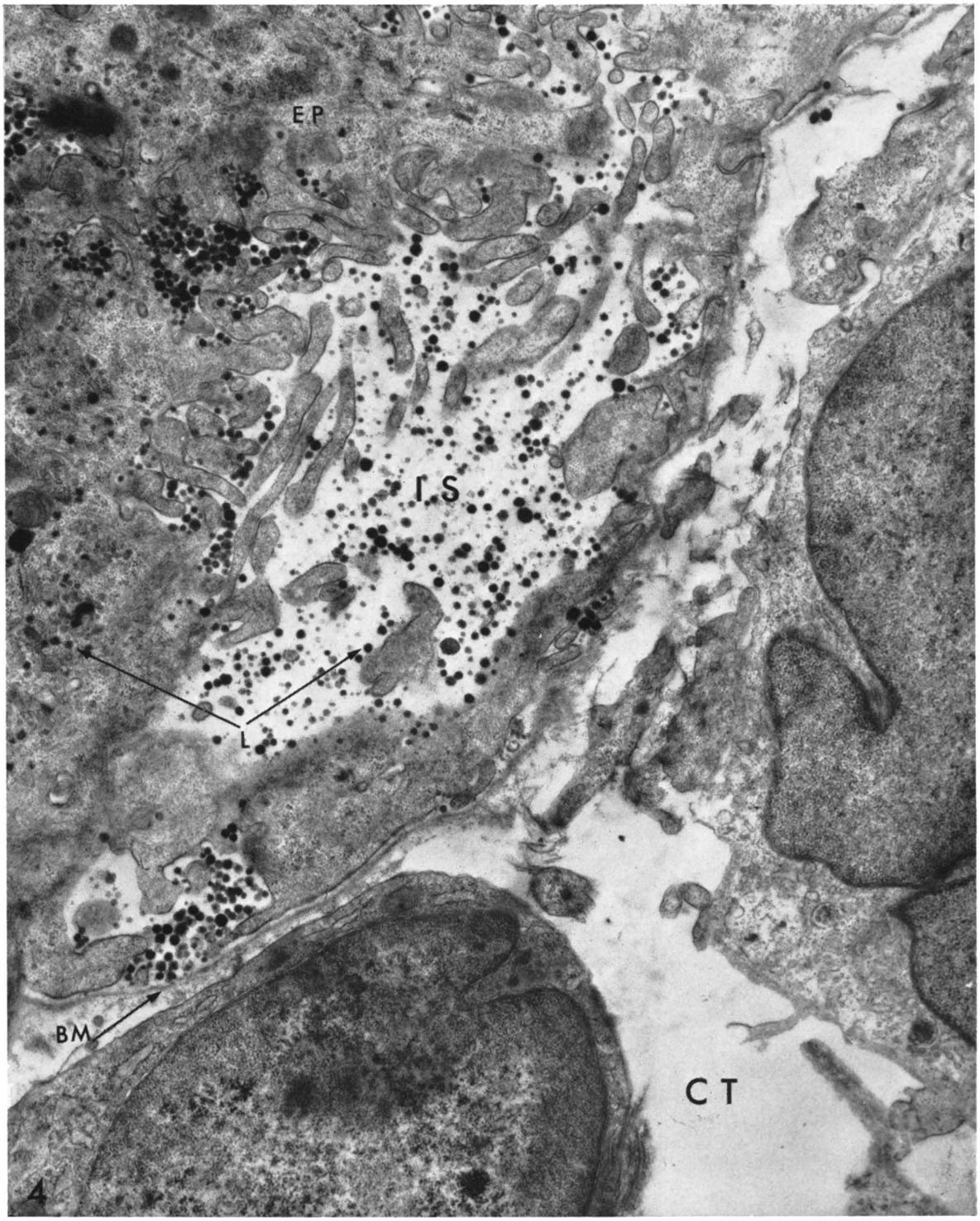

FIG. 4. Basal aspect of epithelium (*EP*) after incubation with mixed micelles for 60 min at 37 °C. A large intercellular space (*IS*) is visible. The absorptive cells rest upon a delicate basement membrane (*BM*), and below this is loosely cellular connective tissue of the lamina propria (*CT*). The epithelium contains numerous intra- and intercellular droplets of absorbed fat (*L*). Higher magnifications would show that many of the intracellular droplets are within the endoplasmic reticulum. Similar droplets are present in the intercellular space. Many of these are aggregated in narrow parts of the space between cytoplasmic folds and in local regions above the basement membrane, without apparent coalescence. Very few droplets have entered the lamina propria at this site. × 19,400.

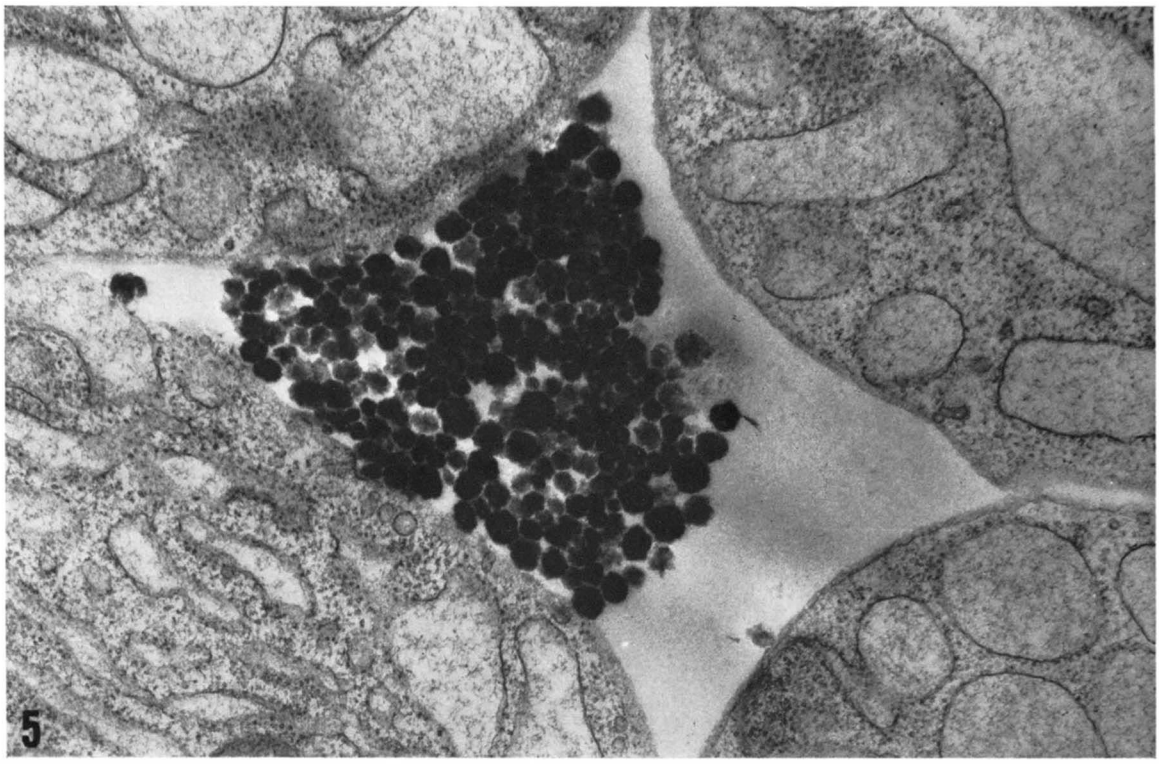

FIG. 5. Parts of four plasma cells in the core of an intestinal villus. Experimental preparation incubated with mixed micelles for 60 min at 37 °C. The intercellular space contains a cluster of lipid droplets. × 37,000.

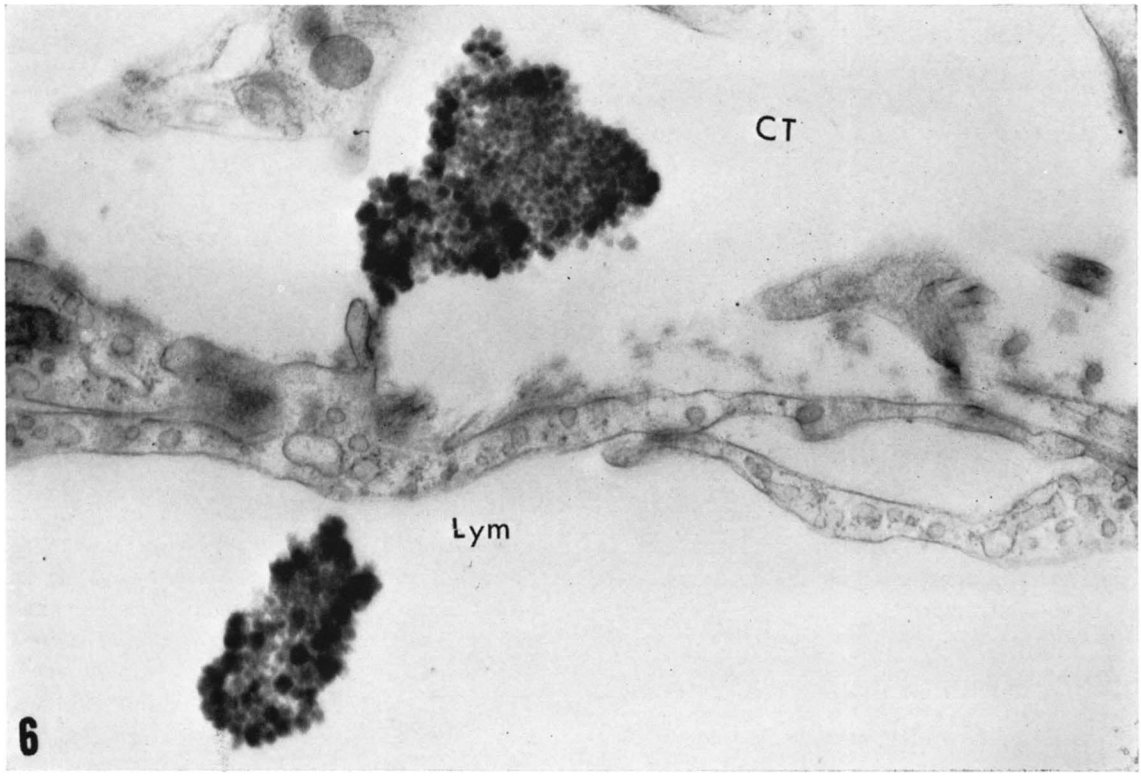

FIG. 6. Droplets of absorbed fat in the lamina propria (*CT*) and in the lumen of a lacteal (*Lym*), from an experimental preparation which was incubated for 60 min at 37 °C. The droplets are closely clustered but there is little evidence of coalescence, judging by similar droplet sizes. Droplets in the lacteal are believed to represent chylomicrons. × 21,800.

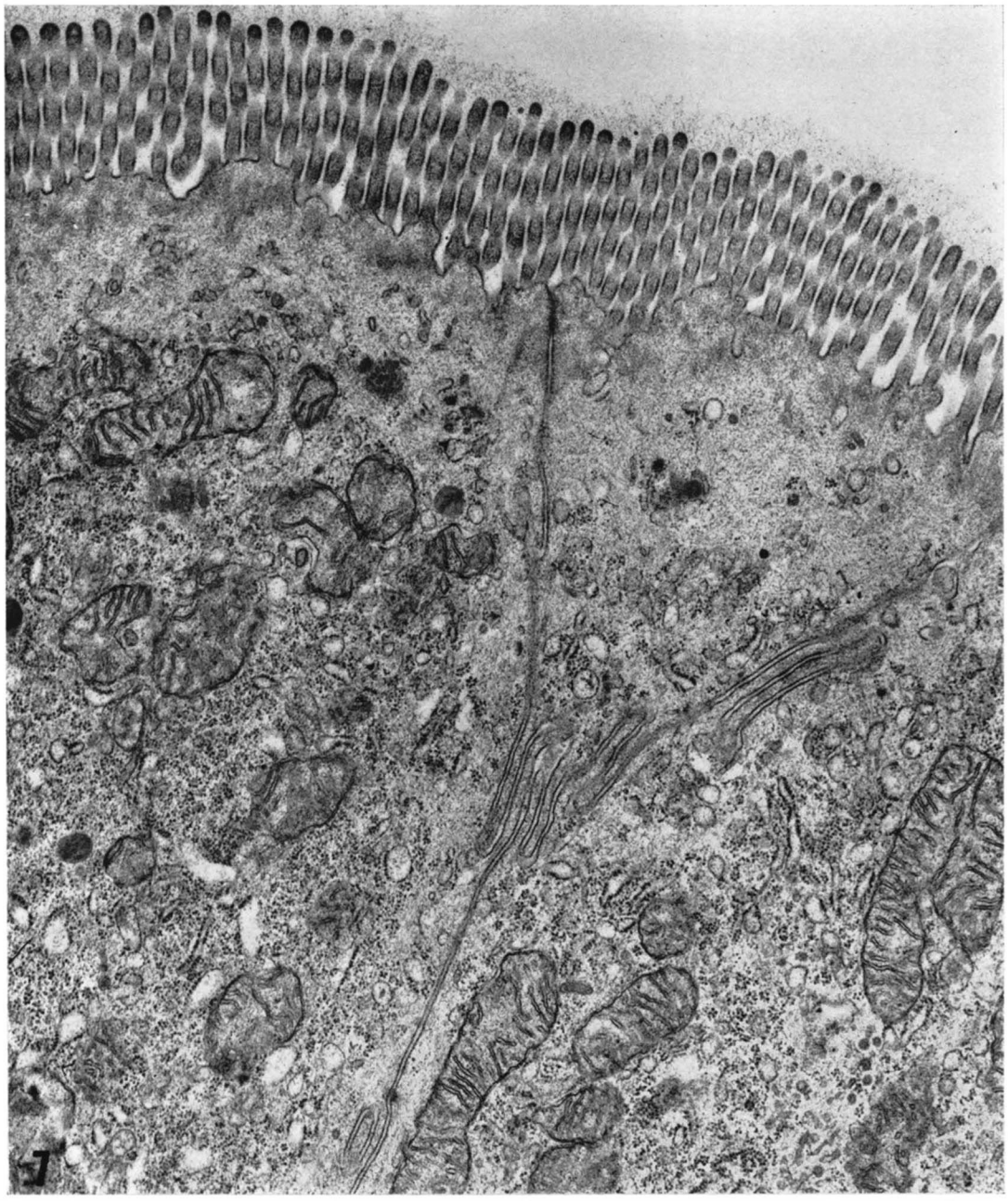

FIG. 7. Parts of the apical cytoplasm of three absorptive cells, after incubation with mixed micelles for 30 min at 0–3 °C. Accumulation of lipid is not morphologically apparent. × 23,800.

smaller than those in the matrix, even when there are several in a single profile. Finally, droplets were not seen in Golgi vesicles or in intercellular spaces, despite prolonged incubations at 10°C.

DISCUSSION

Morphologists have known for many years that droplets of lipid accumulate in intestinal tissues during the absorption of triglycerides of long-chain fatty acids. The occurrence of these depositions in cells has been the main morphological evidence for fat absorption. Palay and Karlin (17, 22) and Palay (23) were the first to show, by means of electron microscopy, the existence of a mechanism by which lipid droplets like these entered and traversed intestinal absorptive cells in the adult rat in vivo. The transport of lipid droplets was considered to be similar to pinocytosis. These investigators observed that lipid droplets, measuring about 500 A, were distributed in spaces between microvilli and in pinocytotic vesicles in the terminal web. Larger and more numerous droplets occurred in the endoplasmic reticulum. Even-

tually droplets appeared at extracellular sites, including the lacteals. They hypothesized that droplets of oil from a luminal emulsion (24) may enter and traverse the cytoplasm of absorptive cells by means of a pathway which is entirely within the confines of membranous organelles. Their data were confirmed by others in rat (25, 26), in different species (16, 19, 27) and after administering different lipids (29, 30) or chemically inert substances (31, 32). Pinocytosis of lipid droplets was observed to a striking degree in hydra, an organism which is not known to contain an extracellular lipase (28).

The concept that the endoplasmic reticulum may be a route for intracellular transport of lipid droplets has been generally accepted (33). This agrees with morphological and chemical work concerning the role of

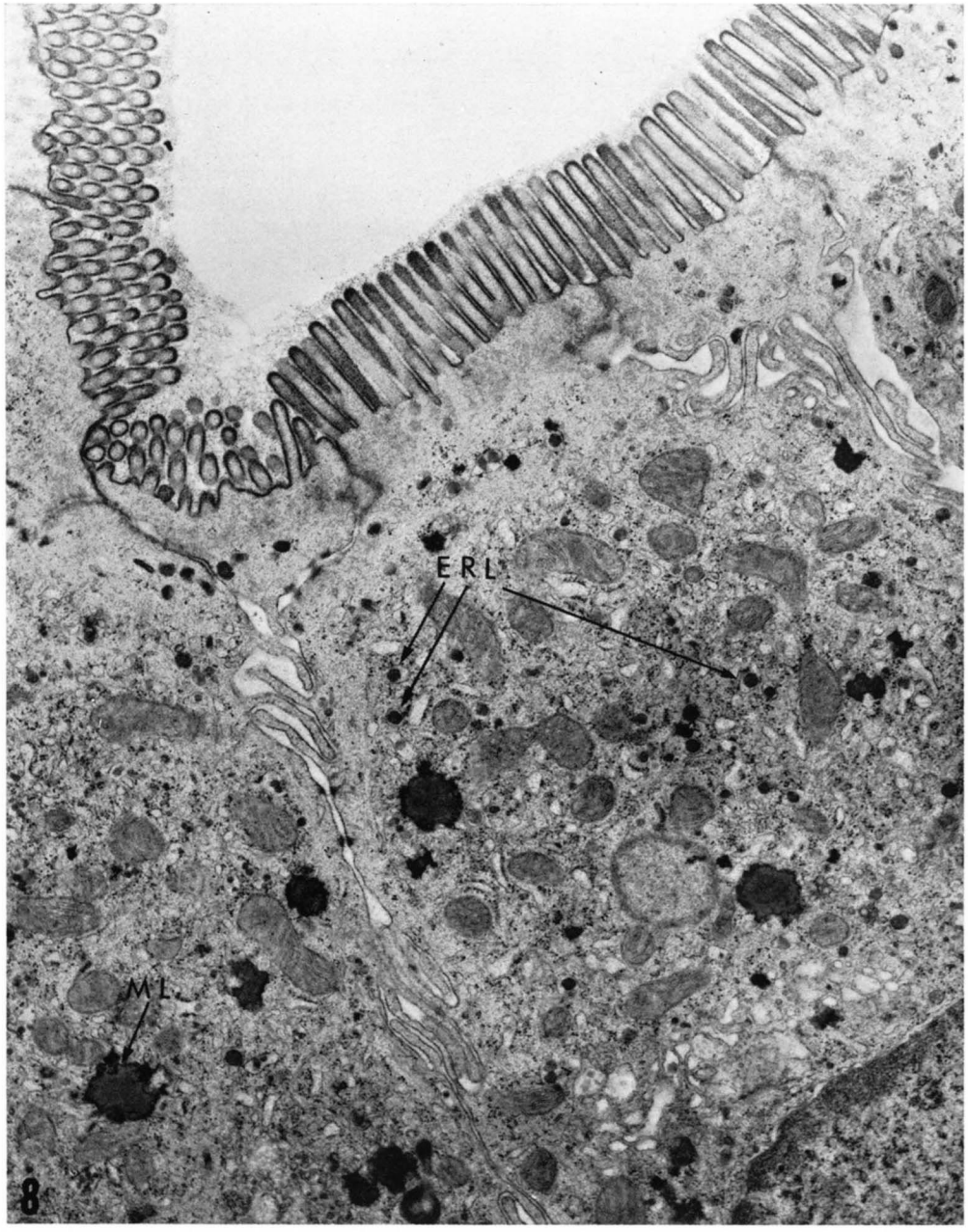

FIG. 8. Apical and supranuclear cytoplasm of absorptive cells which were incubated with mixed micelles for 20 min at near 0 °C. The specimen was afterwards incubated in saline at 37 °C for 15 min. There are many droplets of cytoplasmic lipid below the terminal web. Large droplets (*ML*) are contained only by the matrix of the cytoplasm. Sharply defined membranes of the endoplasmic reticulum confine the smaller droplets (*ERL*). There is a clear space between the droplet and the membrane. × 18,600.

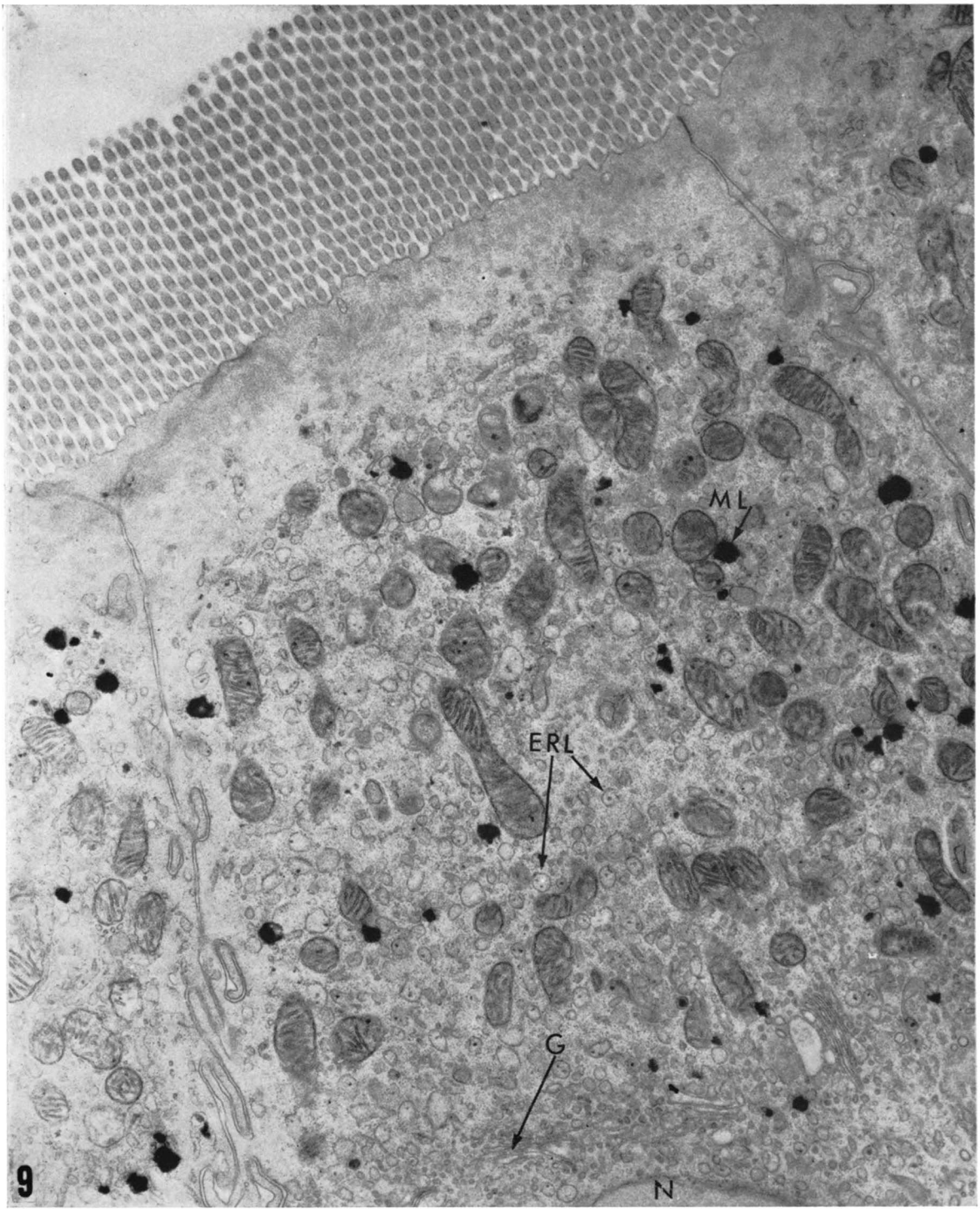

FIG. 9. Experimental preparation, incubated at 10 °C for 5 min. There are striking differences between the two types of lipid droplets under these experimental conditions. Extremely small droplets (*ERL*) are seen throughout the apical cytoplasm as dense punctate objects within vesicular profiles of the endoplasmic reticulum. At this early time, considerably larger, solitary droplets (*ML*) are found in the cytoplasmic matrix. *G*, Golgi complex; *N*, nucleus. × 21,800.

this organelle in secretory processes in other cell types (34, 51). However, the hypothesis concerning uptake of particulate lipid by means of pinocytosis has been criticized on both morphological and chemical grounds. With regard to the former, the number of droplets entering cells is too small to account for very much of the absorbed fat (16). In some in vivo studies, neither particulate uptake (35, 36) nor pinocytosis (36) was

observed. The possible quantitative insufficiency of pinocytosis was pointed out originally by Palay and Karlin (22). More recently Palay and Revel showed that pinocytosis may be intense in rat after feeding linolenic acid containing a small amount of tristearin (29), but this has not been demonstrated after triglyceride feeding.

Additional criticism of pinocytosis depends on advances in knowledge of the digestion of fats. Physicochem-

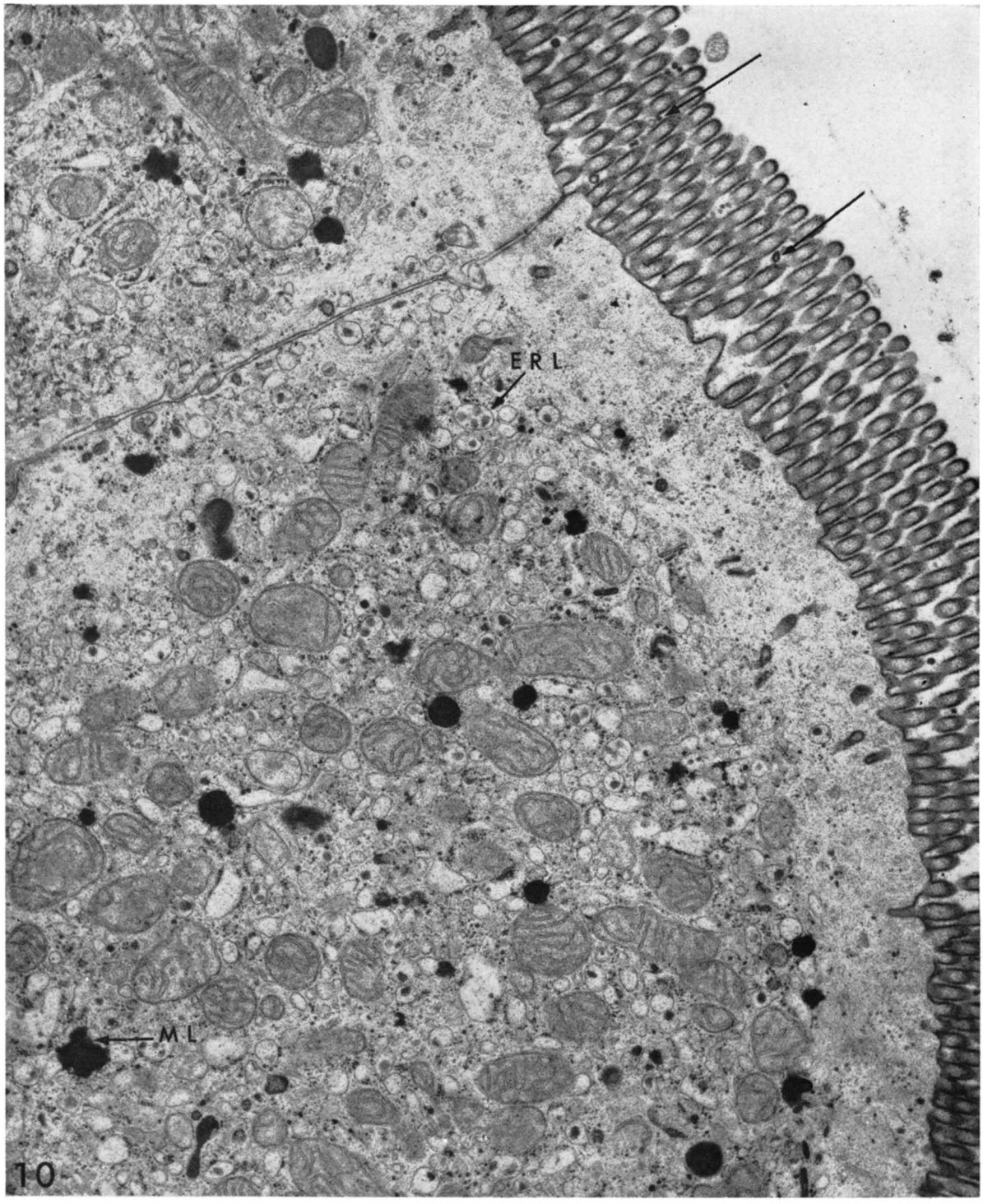

FIG. 10. Experimental preparation, incubated with mixed micelles at 10°C for 60 min. There are many lipid droplets (*ERL*) within the endoplasmic reticulum, typically separated from the confining membranes by an electron-lucent space. Droplets (*ML*) lying freely in the matrix, are larger and apparently more osmiophilic than the depositions in the reticulum. Arrows without label point to vesicular objects which are believed to represent degenerated microvilli. × 17,000.

ical studies of digestion have demonstrated that a large fraction of triglyceride is hydrolyzed in the intestinal lumen in vivo into fatty acids and monoglycerides, and that these products are solubilized by micelles of conjugated bile acids (1, 2). Therefore, the final digestive products appear to exist in a diffusible form. It is difficult to explain the uptake of these soluble end products by means of a mechanism such as pinocytosis, which appears better suited for the bulk uptake of compounds. Indeed, there is abundant evidence that lipid-soluble substances such as fatty acids and monoglycerides may easily diffuse into intestinal absorptive cells (37, 38). Recent experiments on rat in vivo have suggested that digested fat may enter the microvilli as minute osmiophilic particles (39) or as very small non-osmiophilic forms (40) which were believed to be lipid micelles. However, as far as is known, neither monoglycerides, fatty acids, nor mixed micelles, resulting from digestion of triglycerides in vivo, have been identified in electron micrographs during intestinal uptake. For this reason, it has not been possible to evaluate the role of these major products of fat digestion on the basis of morphological data.

The present work has attempted to approach this problem by incubating the intestine in vitro with synthetic solutions of mixed micelles (41). Fat absorbed from these solutions must be derived solely from the monoglyceride and fatty acid added to the media. It was expected that absorption from these synthetic solutions would be morphologically similar to triglyceride absorption in vivo, which is derived from the micellar phase of chyme. The experimental results have confirmed these expectations by showing that fat was absorbed from micellar solutions in vitro in a manner strikingly similar to that of triglyceride absorption in vivo. However, droplets of lipid did not occur among the microvilli nor was there the stimulation of pinocytosis in vitro that Palay and Karlin observed at early absorptive times in vivo (22). Therefore, the mechanism of uptake from micellar solutions clearly does not involve particulate uptake or pinocytosis. Instead, it has been shown in the present work that the presence of lipid droplets in cells is not directly related to uptake, but rather to a later synthetic step in fat absorption. This has been inferred from an experimental manipulation in which uptake and droplet-formation occurred independently. There was uptake from micellar solutions but no droplet formation at 0°C. However, cytoplasmic fat droplets appeared when the tissues were not taking up lipids during a "postincubation" in saline at 37°C. These and other observations in the current study are consistent with the hypothesis that uptake of micellar lipids occurs by means of diffusion. After uptake, fatty acid and monoglyceride are transformed into fat droplets within the cytoplasm of the absorptive cell.

Morphology of Lipid Uptake

In both sacs and segments of everted gut at 10 and 37°C, after incubations for "seconds" to an hour, the uptake of micellar monoolein and linolenic acid was demonstrable only indirectly, by the presence of osmiophilic droplets in the deep apical cytoplasm. Despite the use of several different fixative procedures, as described under Methods, the lipids were not detected during uptake among the microvilli, in the terminal web, or in the deeper cytoplasm. Nevertheless, they must have been present in the superficial cytoplasm at early absorptive times when there were high concentrations of mixed micelles in the media and when droplets were forming in the underlying cytoplasm. This suggests that fatty acid and monoglyceride were present but not morphologically evident during uptake in the ectoplasm and in the deeper cytoplasm, up to the sites where droplets were formed. However, these observations do not permit an identification of the specific ectoplasmic structures, such as the microvilli, which may participate in the uptake of lipid.

An hypothesis for the apparent absence of particles representing mixed micelles, in or about the fuzzy coat of microvilli, depends on their particle size of about only 50 A (3). There may be great difficulty in distinguishing these particles in electron micrographs from similar images ranging up to 80 A in size, which are normally and abundantly present in the "fuzz," as seen in Fig. 1, inset. Furthermore, the fact that newly taken up lipids are not detected in the superficial cytoplasm may be due to their small particle size within the cell too, their dimensions possibly approaching those for micelles or even molecules. Only the images of *triglyceride* droplets are evident in the cytoplasm after uptake. Their large size must make them easily visible.

Results of incubations at 0°C support these inferences. Micellar lipids were taken up at this temperature, but micrographs show very little evidence of the transferred compounds within or about the absorptive cells. One cannot attribute the apparent absence of fatty acid and monoglyceride to their rapid conversion to triglyceride droplets in this case, since droplet formation is inhibited at 0°C. Furthermore, the amount of lipids taken up at 0°C was relatively large, as revealed by the numerous fat droplets which appeared in the absorptive cells after "postincubation" in saline at 37°C. This large amount of lipid would have been easily seen in electron micrographs if it had been concentrated at a specific site during uptake at 0°C, such as at the apical plasma membrane. The fact that there was almost no demonstrable localization at 0°C suggests that the lipid

existed diffusely throughout the cytoplasm in the form of morphologically indistinguishable micelles or even molecules, as in a solution.[1]

Mechanism of Uptake

Biochemical data by Johnston and Borgström demonstrated that the radioactive micellar lipids, 1-monoolein and oleic acid, were firmly bound by hamster gut in vitro at 0 and 37°C (4). A large part of the bound lipids was converted to triglyceride at the higher temperature only. They concluded that uptake occurred by means of a physical process and that synthetic mechanisms were the rate-limiting step for triglyceride formation in vitro.

The present work used taurodeoxycholate micelles which contained linolenic acid and a mixture of 1- and 2-monoolein. Uptake of micellar lipids has been shown to occur at 0°C or at higher temperatures. This confirms the conclusion by Johnston and Borgström that uptake depends on a temperature-insensitive physical process. Furthermore, the fact that uptake occurred at near 0°C without morphological change suggests, as previously discussed, that micelles or individual molecules of the newly taken-up lipids entered the interior of the cell. These lipids became visible only after their chemical transformation into triglyceride droplets during "postincubations" in saline at 37°C. Uptake and droplet formation occurred continuously when a temperature of 37 or 10°C was used throughout incubation. At these temperatures, the lipids were not distinguishable during uptake in the "fuzz," among the microvilli, the terminal web, or in the deeper cytoplasm. They were easily seen, however, after their transformation into osmiophilic droplets below the terminal web. These apparent distributions of lipids at 0, 10, and 37°C are consistent with uptake by diffusion. A consideration of the fine structure of the intestinal columnar cell suggests that during absorption from micellar solutions, there is diffusion of lipid across the apical plasma membrane from the medium into the terminal web and underlying cytoplasmic matrix, and that only later may the lipid enter cytoplasmic organelles such as the endoplasmic reticulum. The precise physicochemical state of the lipids during diffusion awaits further experimental study.

Cytoplasmic Transport Mechanisms

Lipid droplets form and accumulate in the cytoplasm during transport through the cell. The formation of lipid droplets has been shown to depend on temperature, for although an abundance of lipid entered cells from the media at near 0°C, droplet formation was inhibited until "postincubation" at a higher temperature. This experiment demonstrates that droplets are not formed as a direct result of uptake. Their formation requires a further mechanism which is temperature dependent, and which appears to be the rate-limiting step in droplet formation. The unequal accumulation of droplets at 10 and 37°C is further evidence for the temperature dependence. The sensitivity of this step to temperature suggests that droplets are formed by means of chemical or enzymatic reactions. These observations of temperature dependence enhance the correspondence between droplet formation and triglyceride synthesis. The two phenomena appear to be, respectively, morphological and biochemical equivalents. Both occur after lipid uptake in hamster gut and have a similar temperature dependence (4). The absence of triglyceride synthesis in isolated brush borders, as observed by Johnston and Borgström (4), is also consistent with the electron microscopic distribution of lipid droplets in the present study.

Lipid droplets occurred in both the matrix of the cytoplasm and within the endoplasmic reticulum. The absence of membranes around the matrix droplets is not artifactual, since this feature may be correlated with the size of these droplets, their tendency not to occur in clusters, and perhaps with their osmiophilia. Previous electron microscopic studies of intestinal fat absorption have emphasized that droplets occurred in the reticulum, although Palay and Karlin (22) and Palay and Revel (29) observed that some droplets were present in the matrix without confining membranes. The emphasis on the presence of droplets in the endoplasmic reticulum in earlier studies has been commensurate with the abundance of droplets in this organelle. The present work has demonstrated that matrix droplets may be prominent in intestinal cells at early absorptive periods or during incubations at 10°C, rather than at late absorptive times only, as suggested by Palay and Revel (29). Their occurrence shortly after uptake suggests that the cytoplasmic ground substance is an important part of the early absorptive pathway. This is compatible with the previously discussed idea that lipids diffuse into the cytoplasmic matrix from the medium. However, most enzymatic activities for triglyceride synthesis are present in the microsomes (42–47), a subcellular fraction derived mainly from the endoplasmic reticulum. Perhaps higher glycerides may be formed in the matrix by means of enzymes on the outer surface of the endoplasmic reticulum.

Droplets in the reticulum seem to be the main absorptive product. They appear to be closely related to chylomicrons, the end product of fat absorption in vivo (48). There was a progressive increase in the amount of lipid within the endoplasmic reticulum with time at

[1] This hypothesis is consistent with the results of recent autoradiographic experiments performed in collaboration with Dr. S. Ito, Department of Anatomy, Harvard Medical School.

both 10 and 37°C, but there was little apparent change in the total deposition in the matrix. When droplets of absorbed fat accumulated in the reticulum at 37°C, similar droplets appeared extracellularly between absorptive cells, in connective tissue, and in lacteals. Droplets in the latter site probably represented chylomicrons. These observations suggest that lipid droplets were transported through the endoplasmic reticulum as intact units, and "spilled over" into extracellular spaces. Droplets in the reticulum seem to be closely related to chylomicrons not only on the basis of size and distribution but also by their apparent protection from coalescence. This is indicated by their uniformly small size even when they occur in clusters. Conversely, matrix droplets may become quite large. These observations support the data by Palay and Karlin (22) which indicated that droplets in the reticulum may resemble chylomicrons and that the reticulum represents a secretory pathway leading to the extracellular spaces. Recent biochemical information indicates that the lipoprotein (49, 50) parts of chylomicrons may be formed in the endoplasmic reticulum.

The data of Hofmann and Borgström have revealed that mixed micelles are a quantitatively major digestive product (1, 2). Furthermore, the demonstration by Johnston and Borgström of intestinal triglyceride synthesis from micellar lipids, together with the current work, makes it plain that most fat is absorbed from the micellar fraction of intestinal chyme in vivo. The present data are incompatible with the idea that micellar lipids are taken into cells by means of pinocytosis, since uptake did not affect the appearance of pinocytotic channels and vesicles as seen in experimental and control tissues. It is also difficult to believe that uptake by pinocytosis can occur at 0°C, if this involves the active participation of cell membranes. The demonstration that lipid droplets occurred in the cytoplasmic matrix at early absorptive times also argues against the postulate that pinocytosis plays a role, since the latter mechanism requires that transport be conducted within membranous channels. However, these findings do not constitute evidence against the occurrence of pinocytosis during triglyceride absorption in vivo. Droplets may be absorbed from nonmicellar components of the fatty chyme in vivo. It seems probable that pinocytosis may occur in columnar absorptive cells of mammals but that this is of little quantitative significance as compared to mechanisms for the absorption of micellar lipids.

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References

1. Hofmann, A. F., and B. Borgström. *Federation Proc.* **21:** 43, 1962.
2. Hofmann, A. F., and B. Borgström. *J. Clin. Invest.* **43:** 247, 1964.
3. Borgström, B. In *Metabolism and Physiological Significance of Lipids*, edited by R. M. C. Dawson and D. N. Rhodes. John Wiley and Sons Limited, London, 1964, pp. 221–228.
4. Johnston, J. M., and B. Borgström. *Biochim. Biophys. Acta* **84:** 412, 1964.
5. Agar, W. T., F. J. R. Hird, and G. S. Sidhu. *Biochim. Biophys. Acta* **14:** 80, 1954.
6. Wilson, T. H., and G. Wiseman. *J. Physiol.* **123:** 116, 1954.
7. Luca, H. F. de, and P. P. Cohen. In *Manometric Techniques*, edited by W. W. Umbreit, R. H. Burris, and J. F. Stauffer. Burgess Publishing Company, Minneapolis, 1964, p. 132.
8. Palade, G. E. *J. Exptl. Med.* **95:** 285, 1952.
9. Caulfield, J. B. *J. Biophys. Biochem. Cytol.* **3:** 827, 1957.
10. Bennett, H. S., and J. Luft. *J. Biophys. Biochem. Cytol.* **6:** 113, 1959.
11. Millonig, G. *J. Appl. Physics* **32:** 1637, 1961.
12. Dalton, A. J. *Anat. Rec.* **121:** 281, 1955.
13. Sabatini, D. D., K. Bensch, and R. J. Barrnett. *J. Cell Biol.* **17:** 19, 1963.
14. Luft, J. H. *J. Biophys. Biochem. Cytol.* **9:** 409, 1961.
15. Reynolds, E. S. *J. Cell Biol.* **17:** 208, 1963.
16. Strauss, E. W. *J. Cell Biol.* **17:** 597, 1963.
17. Palay, S. L., and L. J. Karlin. *J. Biophys. Biochem. Cytol.* **5:** 363, 1959.
18. Zetterqvist, H. *The Ultra Structural Organization of the Columnar Absorbing Cells of Mouse Jejunum.* Antiebolaget Godvil, Stockholm, 1956.
19. Ladman, A. J., H. A. Padykula, and E. W. Strauss. *Am. J. Anat.* **112:** 389, 1963.
20. Ito, S. *Anat. Rec.* **148:** 294, 1964 (abstract).
21. Palade, G. E., and K. R. Porter, *J. Exptl. Med.* **100:** 641, 1954.
22. Palay, S. L., and L. J. Karlin. *J. Biophys. Biochem. Cytol.* **5:** 373, 1959.
23. Palay, S. L. *J. Biophys. Biochem. Cytol.* **7:** 391, 1960.
24. Frazer, A. C., J. H. Schulman, and H. C. Stewart. *J. Physiol.* **103:** 306, 1944–45.
25. Ashworth, C. T., V. A. Stembridge, and E. Sanders. *Am. J. Physiol.* **198:** 1326, 1960.
26. Millington, P. F., O. C. Forbes, J. B. Finean, and A. C. Frazer. *Exptl. Cell Res.* **28:** 179, 1962.
27. Napolitano, L. M., and J. Kleinerman. *J. Cell Biol.* **23:** 65A, 1964 (abstract).
28. Gauthier, G. F. *J. Exptl. Zool.* **152:** 13, 1963.
29. Palay, S. L., and J. P. Revel. In *Proceedings of An Inter-*

national Symposium on Lipid Transport, edited by H. C. Meng. Charles C Thomas, Springfield, 1964, pp. 1–11.
30. Ashworth, C. T., and J. M. Johnston. *J. Lipid Res.* **4:** 454, 1963.
31. Sanders, E., and C. T. Ashworth. *Exptl. Cell Res.* **22:** 137, 1961.
32. Barrnett, R. J. *Exptl. Cell Res. Suppl.* **7:** 65, 1959.
33. Porter, K. R. In *The Cell*, edited by J. Brachet and A. Mirsky. Academic Press, Inc., New York, 1961, Vol. 2, pp. 621–675.
34. Siekevitz, P., and G. E. Palade. *J. Biophys. Biochem. Cytol.* **7:** 619, 1960.
35. Weiss, J. M. *J. Exptl. Med.* **102:** 775, 1955.
36. Sjöstrand, F. S. In *Biochemical Problems of Lipids*, edited by A. C. Frazer. Elsevier, Amsterdam, 1963, pp. 91–115.
37. Hogben, C. A. M. *Ann. Rev. Physiol.* **22:** 381, 1960.
38. Wilson, T. H., *Intestinal Absorption*. Saunders, Philadelphia, 1962, p. 182.
39. Lacy, D., and A. B. Taylor. *Am. J. Anat.* **110:** 155, 1962.
40. Rostgaard, J., and R. J. Barrnett. *Anat. Rec.* **148:** 398, 1964 (abstract).
41. Strauss, E. W. *J. Cell. Biol.* **23:** 90A, 1964 (abstract).
42. Senior, J. R. *J. Lipid Res.* **5:** 495, 1964.
43. Senior, J. R., and K. J. Isselbacher. *Biochim. Biophys. Acta* **44:** 399, 1960.
44. Senior, J. R., and K. J. Isselbacher. *J. Biol. Chem.* **237:** 1454, 1962.
45. Hübscher, G., B. Clark, M. E. Webb, and H. S. A. Sherratt. In *Biochemical Problems of Lipids*, edited by A. C. Frazer. Elsevier, Amsterdam, 1963, pp. 201–210.
46. Johnston, J. M., and J. H. Bearden. *Biochim. Biophys. Acta* **56:** 365, 1962.
47. Ailhaud, G., D. Samuel, and P. Desnuelle. In *Biochemical Problems of Lipids*, edited by A. C. Frazer. Elsevier, Amsterdam, 1963, pp. 197–200.
48. Gage, S. H., and P. A. Fish. *Am. J. Anat.* **34:** 1, 1924.
49. Isselbacher, K. J., and D. M. Budz. *Nature* **200:** 364, 1963.
50. Hatch, F. T., L. M. Hagopian, J. J. Rubenstein, and G. P. Canellos. *Circulation* **28:** 659, 1963(abstract).
51. Fawcett, D. W. In *Modern Developments in Electron Microscopy*, edited by Benjamin M. Siegel. Academic Press, New York, 1964, pp. 257–333.
52. Millington, P. F., and J. B. Finean. *J. Cell Biol.* **14:** 125, 1962.
53. Farquhar, M. G., and G. E. Palade. *J. Cell Biol.* **17:** 375, 1963.