Electron microscopic study of the role of lipid micelles in intestinal fat absorption

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ABSTRACT In vitro micellar solutions of oleic acid, monoolein, and sodium taurocholate were studied electron microscopically. They contained osmiophilic particles 30–200A in diameter. Osmium staining alone was sufficient to demonstrate the particles; lead staining had little effect on their appearance.

The intestinal intraluminal contents from rats during the absorption of unsaturated fat also contained osmiophilic particles, 40–200A, and numerous similar particles were found between the microvilli and engaged in the fine filamentous coating of microvilli. In the lumen only, larger emulsion-type droplets were also seen. The small particles were demonstrable in osmium-fixed material, with or without lead stains, and staining with lead only increased contrast of the particles.

Spherules measuring about 1000A in diameter with walls about 100A thick were observed in the terminal web during fat absorption, at which time they were slightly larger and more numerous than in fasting rats.

It is proposed that during fat absorption micellar particles are engaged in the filamentous material covering the microvilli and then enter the absorptive cell either by molecular diffusion across the plasma membrane or by being incorporated into the walls of thick-coated spherules which then pass into the subapical cytoplasm.

KEY WORDS lipid absorption · electron microscopy · micelles · in vitro · in vivo · rat · small intestine · lumen · microvilli · filamentous coating · pinocytosis · vesicles · thick-coated · thin-coated

FINE STRUCTURAL STUDIES have provided some insight into the complex process of intestinal absorption of fat, but the products of fat digestion and their incorporation into intestinal absorptive epithelial cells have not yet been successfully demonstrated. In their now classic experiments, Palay and Karlin (1, 2) studied rat intestine during lipid absorption with the electron microscope and noted occasional lipid droplets of about 650A diameter between the microvilli. They also described lipid droplets of 500–2000A diameter in vesicles in the apical cytoplasm of the absorptive intestinal cells. They suggested that lipid may be absorbed by pinocytosis, but it was concluded that the quantitative significance of this means of uptake could not be ascertained. Osmiophilic droplets of 650A diameter are sparse between the microvilli (2–4), and the inclusion of lipid droplets in pinocytotic vesicles formed in the pockets between the microvilli is infrequently observed even during active fat absorption. Moreover, lipid particles of any size are infrequently present in the vesicles located in the terminal web, and lipid droplets are never seen free in the apical cytoplasm of the absorptive cells.

Borgström (5) and Hofmann and Borgström (6) suggested that monoglycerides and fatty acids liberated by pancreatic lipolysis combine with bile acids to form mixed micelles which are then absorbed by the intestinal epithelial cell. They have estimated these micelles to be spherical particles of 30–100A diameter, consisting of a center of fluid hydrocarbon with the polar groups of the lipid micelles oriented at the surface.

In an electron microscopic study of the intestinal absorption of fatty acid (7), we described fine osmiophilic particles of about 100A in the intestinal lumen and between the microvilli. It was suggested that these might represent lipid micelles.

Rostgaard and Barrnett (8, 9) demonstrated, in lead-stained preparations of rat intestine, electron dense particles of 40–50A diameter located between the microvilli, adherent to the plasma membrane, and in the cytoplasm of the microvilli. In one of their reports (8) dense particles were described free in the cytoplasm of the terminal web, but in the other study they were unable to find particles in this location. The particles they described, however, were only slightly or not at all stained with

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osmium tetroxide and were demonstrable only in lead-stained preparations. They suggested that these particles were micelles which diffused through the plasma membrane into the cytoplasm of the microvilli and then migrated through the terminal web to enter the vesicles or tubules in the apical cytoplasm, where resynthesis to neutral fat occurred. These authors also studied micellar solutions in vitro by preparing a film of the solution on Formvar, using a negative staining technique. Particles of 150A diameter with a 50A negative-stained core were demonstrated by this procedure.

Lead salts employed as electron dense stains might introduce a problem of differentiation between fine lead aggregates and minute osmiophilic lipid particles. On the other hand, droplets of unsaturated fatty acid are readily demonstrable electron microscopically with osmium staining (7). The present study is presented as an effort to define further the morphological appearance of micellar particles in vivo and in vitro, with the use of osmium tetroxide alone as well as with lead staining, and to examine further the fine structural aspects of the incorporation of micelles into intestinal epithelial cells during fat absorption.

METHODS

In Vitro Lipid Preparations

1-Monoolein and oleic acid were mixed in the ratio 1:2 meq. An emulsion was obtained by adding 104 mg of this lipid mixture to 7 ml of phosphate buffer at pH 6.3 (prepared by dissolving 1.62 g of NaH_2PO_4 and 0.48 g of Na_2HPO_4 in 100 ml of distilled water) at 37°C, and sonicating the mixture.

A micellar solution was obtained by adding dropwise with agitation 104 mg of the lipid mixture of oleic acid and monoolein to 7 ml of 40 mM sodium taurocholate in the phosphate buffer at 37°C.

A third preparation (mixed emulsion and micellar solution) was obtained by adding 156 mg of the oleic acid–monoolein mixture to 7 ml of 40 mM sodium taurocholate in phosphate buffer at 37°C.

These lipid solutions were absorbed into electrophoresis paper (Beckman Paper Strips No. 320046: for use with Beckman Model R paper electrophoresis system) which was then placed in a Veronal buffered solution of osmium tetroxide [Palade's fixative (10)], passed through graded alcohols, and embedded in an epon resin, Maraglas (11), for electron microscopy. In the procedure of osmium fixation 1 mm^3 segments of the paper soaked with the mixture or solution were gently placed into the osmium solution; subsequent agitation of the tubes was avoided.

Intestinal Intraluminal Contents and Fat Absorption

Holtzman rats weighing about 250 g were given 2 ml of oral Lipomul[1] or 2 ml of a fine aqueous suspension of corn oil (50%) by stomach tube, after a 48 hr fast during which they were allowed water ad lib. These rats were sacrificed from 15 min to 2.5 hr after feeding. The intraluminal contents from different levels of the small intestine were absorbed into electrophoresis paper and fixed in osmium tetroxide for electron microscopy.

Samples of small intestinal mucosa were obtained for electron microscopy by first opening the bowel, retaining as much of the contents as possible, and then gently dropping osmium tetroxide solution onto the mucosal surface. After the mucosa and the adherent film of intestinal contents had become brown, 1 mm^3 blocks were placed in the osmium solution and prepared as usual for Maraglas embedment.

Some figures represent intestinal mucosa which was stained, where indicated, with lead by Millonig's method (12). The only advantages of lead staining were that it was easier to achieve focused electron micrographs and the contrast was improved. In vitro lipid preparations and intestinal lumen contents were stained with osmium tetroxide only; lead staining gave identical results in these cases.

All electron microscopic preparations were studied with an RCA EMU G microscope. Electron micrographs were obtained at initial magnifications from 8000 to 30,000, subsequently enlarged photographically 2.5–6 times.

RESULTS

In Vitro Preparations

The in vitro micellar solutions were optically clear (Fig. 1) and stable at room temperature for several weeks. The micellar solution became slightly opalescent as an excess of the oleic acid–monoglyceride mixture was added (Fig. 1), but the preparation was stable for several weeks.

Concentration of lipid in the emulsion and in the clear micellar solution was 1500 mg/100 ml, and in the mixed emulsion–micellar solution 2250 mg/100 ml.

Control electron micrographs of electrophoresis paper, stained and soaked in buffer, are shown in Fig. 2. The fibers did not stain with osmium and were not inherently electron dense. No osmiophilic particles were encountered.

Paper soaked with the lipid emulsion revealed smoothly rounded, homogeneous, osmiophilic droplets 1000–20,000A in diameter. No particles in the size range of micelles were observed. Paper containing mixed emulsion

[1] Oral Lipomul, Upjohn Co., Kalamazoo, Mich. Each 15 ml contains 10 g of corn oil in a vehicle containing *d*-γ-tocopheryl acetate and other antioxidants, polysorbate 80 and other emulsifying agents, and sodium cyclamate.

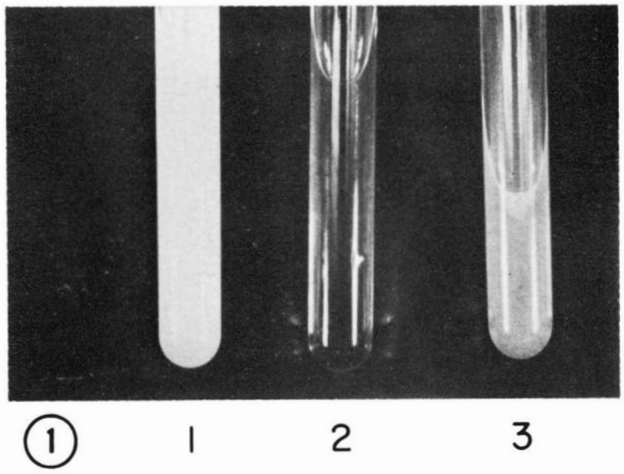

FIG. 1. Appearance of the different lipid preparations. *1*, emulsion containing 1500 mg of oleic acid per 100 ml of phosphate buffer. *2*, optically clear micellar solution containing 1500 mg of oleic acid per 100 ml of 40 mM taurocholate in buffer. *3*, emulsion–micellar mixture prepared by adding an additional 750 mg/100 ml of oleic acid to a micellar solution similar to that in tube 2.

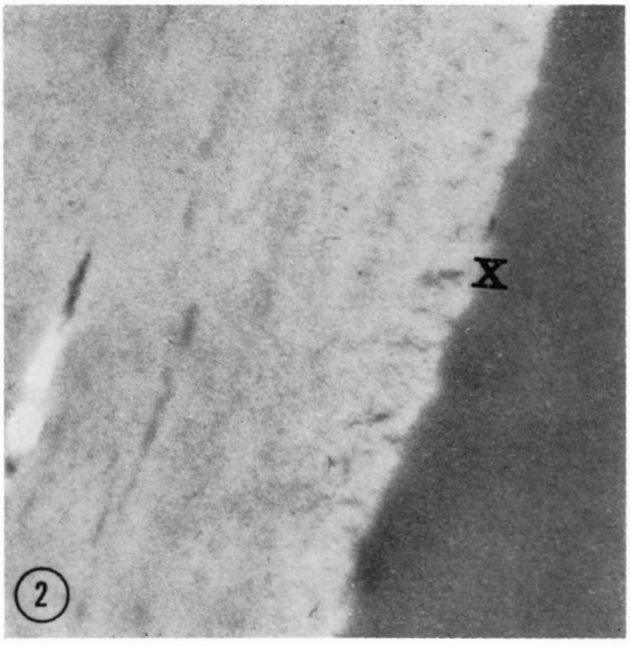

FIG. 2. Electron micrograph of a thin section of electrophoresis paper such as was used in Figs. 3–5. No lipid was employed in this preparation. The edge of a paper fiber is shown at *X*. The darker gray area represents the film of epon in which the paper was embedded. No osmiophilic elements are apparent. ×40,000.

and micellar solution contained osmiophilic particles up to 5000A in diameter as well as small droplets 30–200 A.

Paper soaked with optically clear micellar solutions contained osmiophilic particles 30–200A in diameter (Figs. 3 and 4). The smaller particles were homogeneously densely stained, and their perimeters were smooth and regular, but the larger ones contained several separate, fine granular subunits about 30A across (Fig. 4).

Intestinal Contents and Mucosa During Fat Absorption

Intestinal contents obtained during fat absorption and trapped in the paper contained a concentrated population of osmiophilic droplets and particles (Fig. 5). These ranged from 40A to 5000A in diameter.

Sections of intact intestinal mucosa made during the various stages of fat absorption revealed osmiophilic droplets and particles in intestinal lumen contents which ranged from 40A to 5000A. The better demarcated of the smaller osmiophilic droplets measured about 100–150A. Only rarely were droplets 500–1000A in diameter located between microvilli, but fine 50–150A particles were often profusely present over the tips of and between individual microvilli (Figs. 6 and 7). These particles frequently appeared to be engaged within the filamentous coating of the microvilli. The particles could be easily seen in preparations stained only with osmium tetroxide. In lead-stained preparations the particle density was somewhat increased (Fig. 8), but their shape and finer particulate subdivisions were essentially similar to that in tissue not stained with lead. The fine particles were sometimes found in contact with the cell membrane of the microvilli, and this appeared with similar frequency in all portions (tip to base) of the microvilli. In none of the tissue preparations that were stained only with

FIG. 3. Paper soaked with lipid micellar solution (tube 2 of Fig. 1). Most of the micellar particles, 40–200A in diameter, are visible along the edge of the paper (*X*). Minute osmiophilic particles are also present in the matrix of the paper. ×20,000.

FIG. 4. Higher magnification of paper containing lipid micelles. A few larger particles up to 200A diameter (*X*), apparently containing small subunits, are present, but most of the fine osmiophilic particles in the mesh of the paper measure 40–80A. ×120,000.

osmium were the fine particles seen either in the matrix of the microvilli or in the terminal web. In lead-stained sections, however, fine dense particles believed to be artifactual lead deposits of about the same size (50A) were occasionally encountered in these sites.

In the intestinal epithelium sampled during fat absorption, numerous pockets of the plasma membrane between microvilli were observed protruding into the apical portion of the cell (Fig. 9). These were sometimes continuous with hollow and occasional solid-appearing spherules of osmiophilic material in the terminal web. Both the protrusions and the hollow spherules were lined (Fig. 10) by a membrane comparable in thickness to that of the plasma membrane of the microvilli (about 100A). The membrane lining the larger vesicles that contain large osmiophilic droplets and lie deeper in the cell, however, was only about 75A thick. The spherules occasionally contained fine osmiophilic particles 50–100A in diameter, which were either free in the lumen of the spherule or adhered to the inner surface of its limiting membrane.

Long, narrow, tubular structures connecting separate spherules of the terminal web to each other and to more deeply placed droplet-containing vesicles were frequently observed in the zone immediately subjacent to the terminal web. The long tubular structures, which extended well below the terminal web, were sometimes continuous with intermicrovillous pockets, which suggests that they originate from this source.

The spherules in the terminal web ranged from 500 to 1500A diameter. Some of them appeared as solid osmiophilic bodies, although the central portion was usually less electron dense than the outer rim. Many of the spherules contained a clearly visible, relatively thick outer rim of 100A thickness, and the central paler zone was usually occupied by finely fibrillar or granular material of moderate electron density. An average of 20 such spherules were seen in the transverse dimension of the terminal web per absorptive epithelial cell. If we assume an average thickness of 5μ for each cell, an average diameter of 1000A for each spherule, and an average thickness of 500A for each ultrathin section in which the cells were counted we get an estimate of 1000 spherules in the terminal web per cell at a given moment during fat absorption. In nonabsorbing intestinal epithelium these spherules were

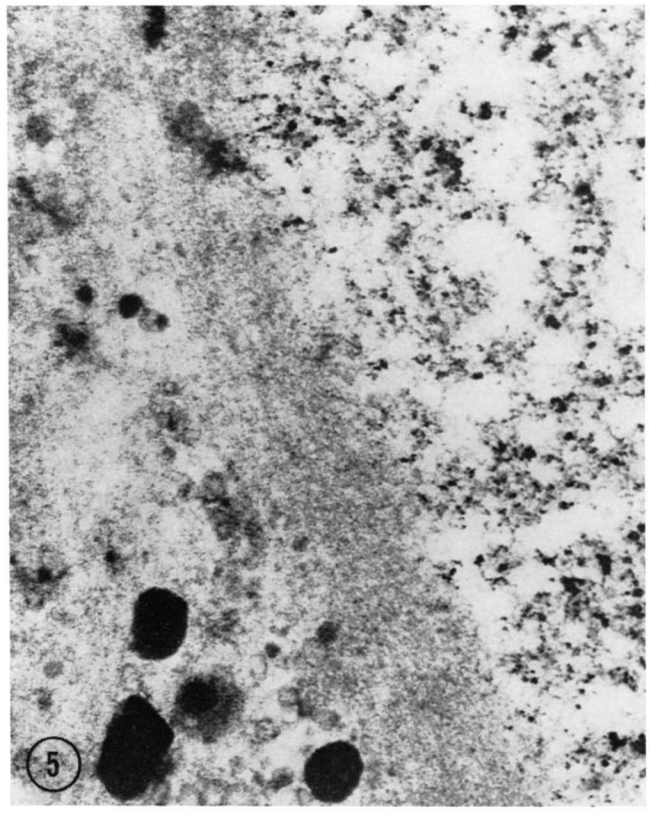

FIG. 5. Intestinal contents of jejunum of fat-absorbing animal. Emulsion-type droplets are present, but numerous osmiophilic particles of micellar size, 40–200A, are also visible. ×50,000.

only slightly less numerous (Fig. 11) but were smaller than those in animals absorbing fat.

DISCUSSION

The role of lipid micelles in intestinal absorption of fat has recently been emphasized from the biochemical standpoint (5, 6, 13, 14), but electron microscopic visualization and identification of micelles has been only briefly and rarely undertaken (7, 8). It is believed that the morphologic identification of micellar particles would contribute evidence pertaining to intestinal epithelial uptake of fat. Rostgaard and Barrnett (8, 9) illustrated particles of appropriate size for micelles in both in vivo and in vitro preparations. While the particles they illustrated may be micelles, their demonstration was complicated by the fact that the particles shown were not osmiophilic; the use of lead salts as stains introduced a possible artifact of lead precipitates (15, 16); and their in vitro demonstration was dependent upon a negative staining technique. Strauss (17) has recently reported an in vitro study on the absorption of micellar lipid by sacs and segments of hamster intestine. While he did not actually visualize micelles and considered that they were not large enough to be differentiated from other material around microvilli, he did describe the formation of the larger lipid droplets in the cytoplasmic vesicles beneath the terminal web. He interpreted the results of his study to favor diffusion of micelles across the cell membrane into the apical cytoplasm of the absorptive cells.

In our efforts to demonstrate micelles in the intestinal lumen contents and in micellar solutions prepared in vitro, material with and without lead staining was used, and osmiophilia was employed as a characteristic of unsaturated fats. Recently, the dependence of the process of osmiophilia upon unsaturated bonds in long carbon chains has been reaffirmed (18, 19), and it seems appropriate to conclude that typical dense osmiophilia is an

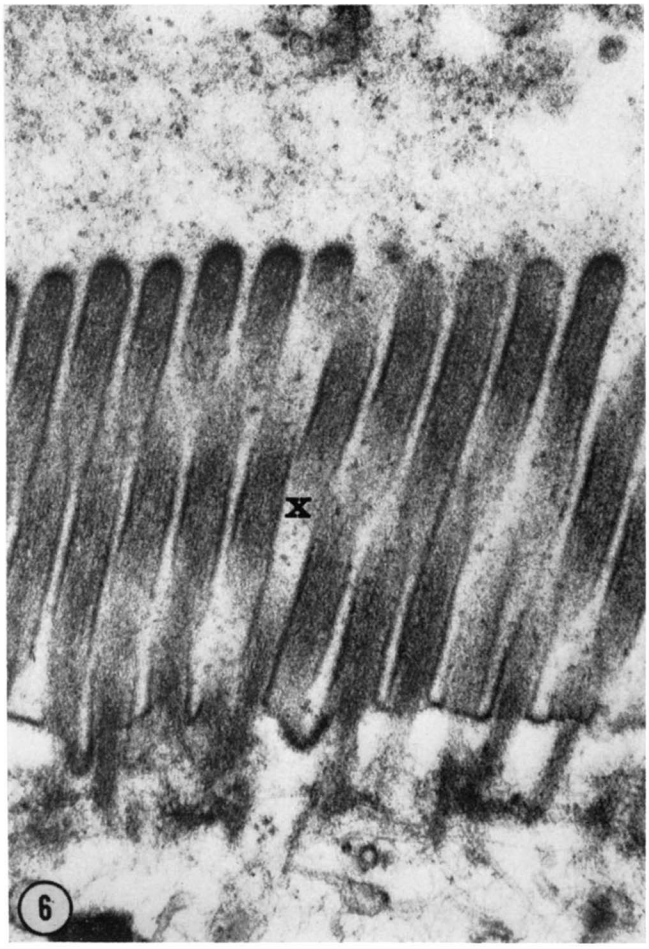

FIG. 6. Microvilli and an area of lumen contents. Moderately dense osmiophilic particles, 40–200A in size, are present in the lumen and between the microvilli (*X*). Lead staining was not employed. ×40,000.

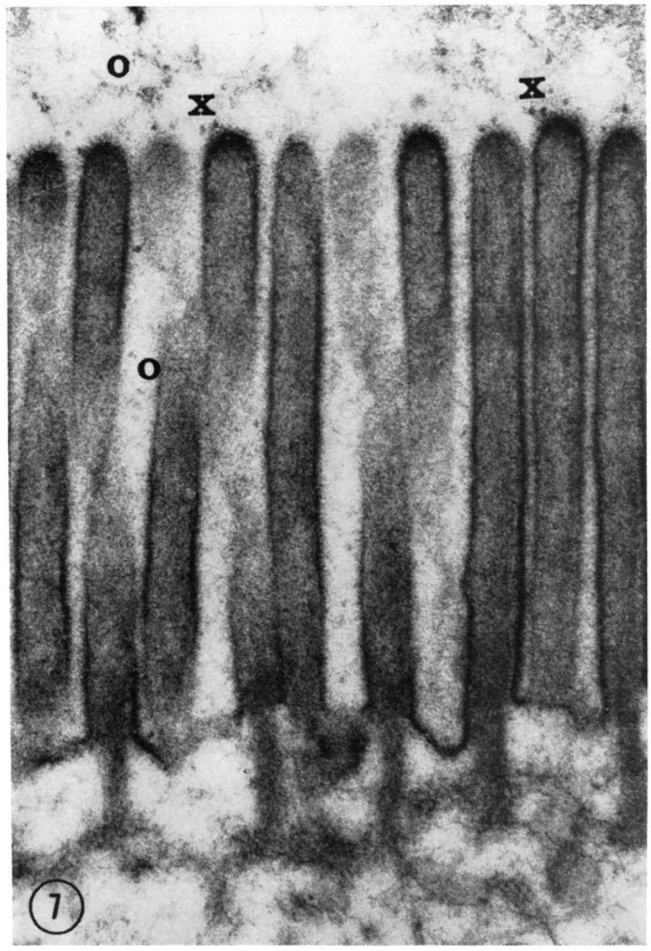

FIG. 7. Higher magnification of microvilli and adjacent lumen contents during fat absorption. Osmiophilic particles of micellar size are observed in the lumen (*O*), and a few are present between microvilli. At *X* are micellar particles apparently in contact with the fine filamentous coating about the microvilli. Tissue not lead-stained. ×65,000.

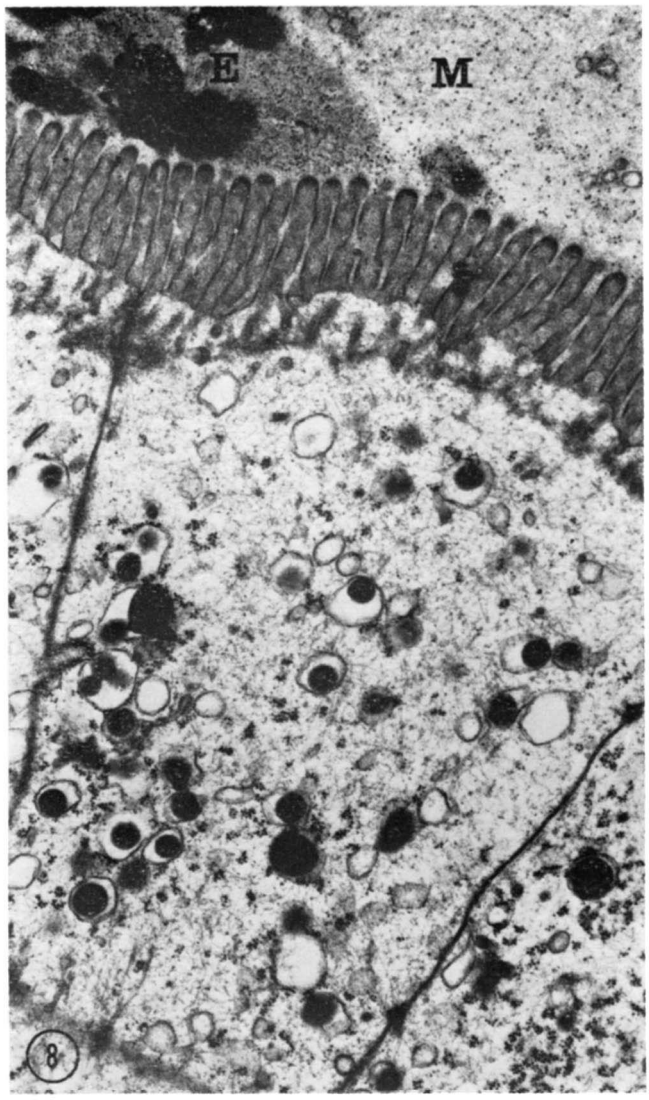

FIG. 8. Lead-stained section of intestinal epithelium during fat absorption. Osmiophilic particles up to 150A in size are readily seen in the lumen (*M*), and there are also larger emulsion-type lipid droplets (*E*). ×20,000.

indication of unsaturated lipids. Furthermore, our earlier studies of fatty acid absorption (7) revealed that dense osmiophilia was characteristic of fatty acid in the intestinal lumen. This was true both for larger droplets and fine particles, the latter of which were suggested at that time to be micelles. Thus, in our present study of in vitro micellar solutions which were absorbed in paper, treated with osmium, and prepared for thin sectioning for electron microscopy, it is believed that the fine osmiophilic particles measuring 30–200A in diameter represent micelles. Similar particles in intestinal lumen contents during fat absorption were demonstrated between microvilli and were seen in contact with the plasma membrane surface of the microvilli. These particles are believed to be lipid micelles resulting from the intraluminal digestion of fat.

Among the possible mechanisms by which micellar lipid may be incorporated into the intestinal epithelial cell are the following: (*a*) further breakdown of the micelle to smaller units, possibly single fatty acid molecules, which then pass across the cell membrane into the cytoplasm by some unspecified mechanism; (*b*) pinocytosis of intact micelles; (*c*) passage of the intact micelle across the plasma membrane into the cytoplasm; and (*d*) incorporation of the micellar lipid into the microvillous cell membrane, either as a micellar lipid–membrane complex or as newly synthesized lipoprotein membrane, which subsequently passes into the cell as thick membrane-coated vesicles (20) by a process of membrane flow (21).

The first of these possibilities, further breakdown of

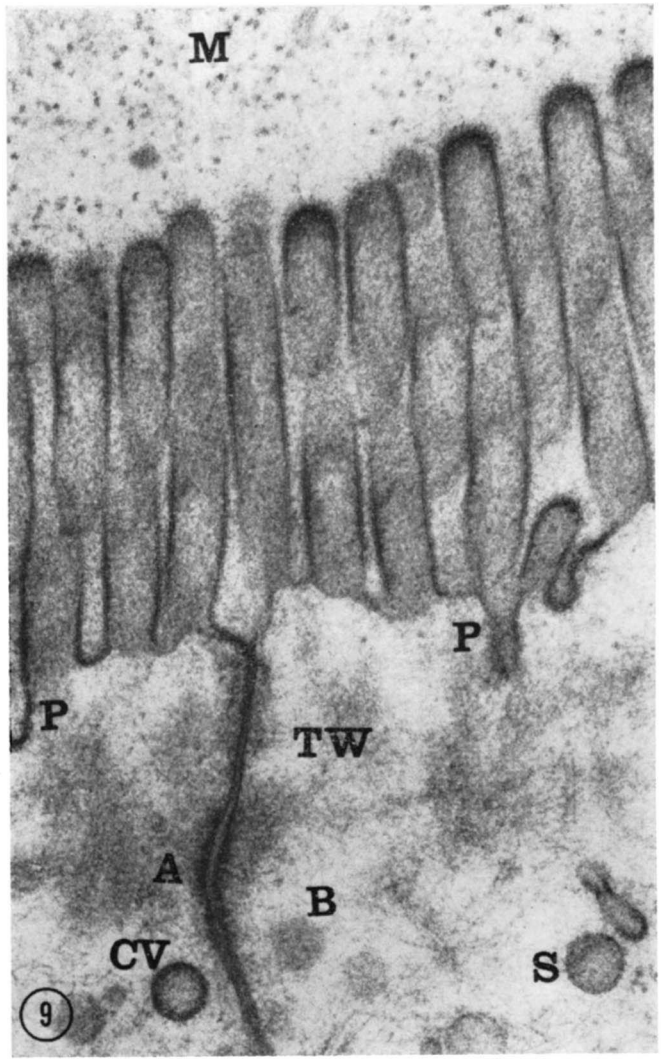

FIG. 9. Intestinal epithelium during fat absorption. A portion of lumen containing micelles (*M*), microvilli, and some of the terminal web (*TW*) of two adjacent cells (*A* and *B*) are shown. At *P* are pinocytotic membrane inclusions. Deeper in the cytoplasm are thick-coated hollow spherules (*CV*) and solid-appearing spherules (*S*) about 1000A in diameter. Lead-stained tissue. ×65,000.

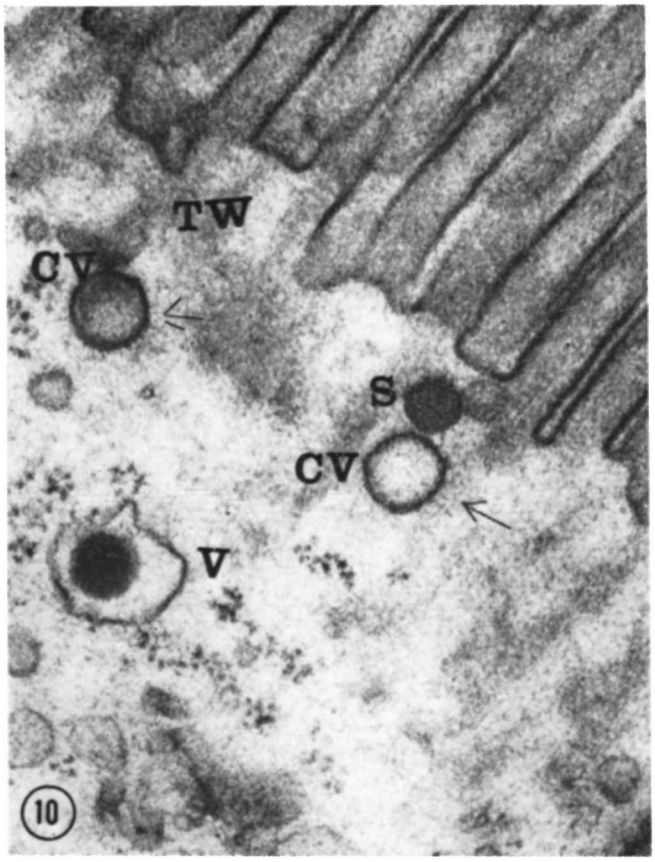

FIG. 10. Terminal web (*TW*) area and bases of microvilli in intestinal epithelium during fat absorption. A solid-appearing spherule (*S*) and two thick-coated hollow spherules (*CV*) are present in the terminal web. The membranes of the thick-coated spherules are similar in thickness, about 100A, and resemble the structure of the microvillous cell membranes. Deeper in the cytoplasm a thin-walled vesicle (*V*) containing a lipid droplet is visible. ×65,000.

micellar lipid, is a possibility which probably cannot be confirmed or excluded by standard methods of electron microscopy.

If pinocytosis of intact micelles were the primary mechanism of intracellular lipid incorporation, micellar particles should be frequently seen in the pinocytotic vesicles; but in fact they are very rarely present. Furthermore, bile salts are thought to be released to the bowel lumen from the micelles as the latter enter the absorptive cells (14), which would be difficult to explain in pinocytosis of intact micelles.

Passage of the intact micelle through the plasma membrane into the cytoplasm was suggested by Rostgaard and Barrnett (8, 9). Their preparations were lead-stained, and lead staining tends to produce electron dense, finely granular artifacts. In their electron micrograph of intestinal epithelium sectioned in the longitudinal axis of the microvilli, many microvilli are seen to be tangentially cut, and some sections pass near the surface of the microvilli. In their Fig. 1 (8) the particles are seen only where the microvilli are tangentially cut near their surface, as indicated by lack of definition of their plasma membranes. Thus, the illustrated particles actually appear to be located between and on the surface of the microvilli. Furthermore, it is difficult to explain the passage of an intact lipid micelle through the lipoprotein plasma membrane without its molecular incorporation into the membrane structure. We have not been able to demonstrate electron dense particles of the size of micelles in the ground cytoplasm of microvilli or terminal web in tissue in which lead staining was not employed.

The presence of micelles in the fine filamentous coating (22) around microvilli suggests that they may be first incorporated and held within this fine filamentous layer just outside the plasma membrane. Johnston and Borgström (13) showed that lipid micelles could be incorporated by isolated brush borders of intestinal epithelium by a mechanism not requiring energy. It seems plausible that loose chemical bondings of micelles within the filamentous layer of the microvilli could explain this phenomenon. The microvillous filamentous covering may be identical with Bennett's ubiquitous cell mucoprotein coating or glycocalyx (23). Bennett discussed the

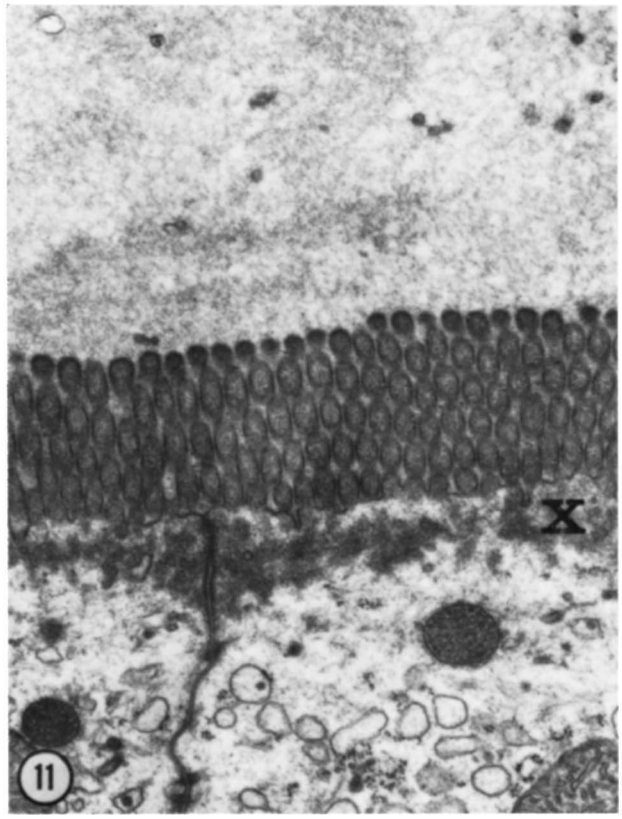

FIG. 11. Intestinal epithelium of 48 hr fasting rat. The lumen contents were scanty; electron microscopy reveals a faintly electron dense, fine filamentous material there. No particles resembling micelles were seen. A few small pinocytotic membrane inclusions and small thick-coated spherules are present in the terminal web (*X*). Lead-stained tissue. ×20,000.

possibility of loose chemical bonding between this glycocalyx and molecules or particles which engage the cell surface.

The possible role of thick-coated vesicles or pinocytotic inclusions of the cell membrane as a transport vehicle for fat during absorption has frequently been considered. Usually it has been considered that the material being transported is carried as luminal contents of the vesicle. It is also possible that the vesicle could serve as a transport vehicle simply by virtue of the composition of its walls, or the adherence of very fine particulate material to the internalized mucoprotein coating. As a detraction from the concept of vesicles as a transport system in intestinal epithelium, Sjöstrand (24) reported that the vesicles in the apical portion of the intestinal absorptive cell were lined by membranes of only 70A thickness. Since the plasma cell membrane of the microvilli is about 100A thick, he concluded that the vesicles were not derived from pinocytotic inclusions in the intermicrovillous pockets. While we also found that vesicles deeper in the cell possessed 70–75A thick membranes, we observed that the spherules within the terminal web possessed thickly coated membranes. In this area the vesicles were either hollow spherules with walls of 100A thickness or greater, or they were apparently solid. The number and size of these spherules during fat absorption both increased, but only slightly. It is impossible at this time to know whether the rate of their formation and migration in the cell increases during the absorption of fat.

If it can be assumed that lipid micelles are incorporated first and loosely bound to the mucoprotein coat of the microvilli, the subsequent means of internalization of lipid is still an elusive subject insofar as electron microscopic morphologic observations are concerned. Our findings suggest that micellar particles are neither transported as such through the microvillous or terminal web matrix, nor transported as visible particles within the lumens of pinocytotic vesicles. It appears that they either become incorporated in or attached to the cell membrane itself and then give up their lipid, which is carried deeper into the cell, possibly by membrane flow (21); or the micelles are broken down to separate electron microscopically invisible fatty acid and monoglyceride molecules which diffuse across the plasma membrane of the microvilli and are carried through the microvillous and terminal web matrix, then to be converted to larger visible intravesicular lipid droplets.

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