REGULAR STRUCTURES IN MEMBRANES

I. Membranes in the Endocytic Complex
of Ileal Epithelial Cells

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ABSTRACT
An "apical endocytic complex" in the ileal lining cells of suckling rats is described. The complex consists of a continuous network of membrane-limited tubules which originate as invaginations of the apical plasma membrane at the base of the microvilli, some associated vesicles, and a giant vacuole. The luminal surface of this tubular network of membranes and associated vesicles is covered with a regular repeating particulate structure. The repeating unit is an ~7.5-nm diameter particle which has a distinct subunit structure composed of possibly nine smaller particles each ~3 nm in diameter. The ~7.5-nm diameter particles are joined together with a center-to-center separation of ~15 nm to form long rows. These linear aggregates, when arranged laterally, give rise to several square and oblique two-dimensional lattice arrangements of the particles which cover the surface of the membrane. Whether a square or oblique lattice is generated depends on the center-to-center separation of the rows and on the relative displacement of the particles in adjacent rows.

Four membrane faces are revealed by fracturing frozen membranes of the apical tubules and vesicles: two complementary inner membrane faces exposed by the fracturing process and the luminal and cytoplasmic membrane surfaces revealed by etching. The outer membrane face reveals a distinct array of membrane particles. This array also sometimes can be seen on the outer (B) fracture face and is sometimes faintly visible on the inner (A) fracture face. Combined data from sectioned, negatively stained, and freeze-etched preparations indicate that this regular particulate structure is a specialization that is primarily localized in the outer half of the membrane mainly in the outer leaflet.

INTRODUCTION
A specialization of certain membranes in the ileal absorptive cells of suckling rats was first described by Porter et al. (17). Those authors showed that the outer leaflet of this unit membrane (21) was covered by closely packed parallel ridges. A more detailed electron microscope study by Wissig and Graney (29) showed that this membrane was part of an endocytic complex which they believed to
participate in sequestering intact proteins presumably other than antibodies (23) from the intestinal lumen into a giant intracellular vacuole and that the closely packed parallel ridges described by Porter et al. (17) were, in fact, rows of particles with a center-to-center separation of ~120 Å. Furthermore, they showed that the arrangement of the rows gave rise to a two-dimensional square lattice with a side spacing of ~120 Å. Related specializations in various other unit membranes have also been described (2, 5, 7, 8, 10, 11, 18–20, 22, 24, 25, 27).

We have first verified the findings of Wissig and Graney (29) and then further characterized the particulate array using negative-staining and freeze-fracture techniques.

MATERIALS AND METHODS

10-14-day old rats of the Sprague-Dawley strain were used throughout this study. The rats were anesthetized with ether, and short segments of the ileum (3-5 cm) adjacent to the ileocecal junction were removed and placed in mammalian Ringer solution. Small pieces (1-2 mm) of the tissue were used for thin-sectioning and freeze-fracture experiments. An epithelial scraping from the ileum was used for negative staining.

For thin sections small pieces of the tissue were fixed either in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, partially polymerized glutaraldehyde (M. K. Reedy, unpublished results), or in a hydroxyalkylperoxide fixative (15) for 1 h, postfixed 2 h in 2% OsO₄, and block stained in 0.5% uranyl acetate in Veronal-acetate buffer pH 4.6 for 1 h. In some experiments, 1% lanthanum nitrate was added to the initial aldehyde fixative according to the method of Revel and Karnovsky (19). Specimens were dehydrated through a graded series of alcohols and finally embedded in Epon. Sections were cut with diamond knives on a Reichert or LKB ultramicrotome, and micrographs were taken on a Philips EM 300 or AEI 801 electron microscope.

Fragments of membrane for negative staining were isolated by scraping the ileum with a wooden spatula to remove the epithelium. The epithelial scraping was suspended in 0.02 M Tris buffer pH 7.5 and fragmented either by mild sonication with a Branson Sonifier Cell Disruptor or by homogenization in a Dounce Teflon homogenizer. A drop of the sonicate or homogenate was dried down onto a carbon film-coated grid and negatively stained with 1% phosphotungstic acid (PTA) buffered to pH 7.0. Optical transforms and optically filtered images from electron micrographs of negatively stained material were obtained according to the method of Klug and DeRosier (9).

Unfixed tissue for freeze etching was either immersed in mammalian Ringer solution or infiltrated over a 1-2-h period with 20–30% glycerol also in Ringer solution. The epithelial homogenate was also used in some freeze-etch experiments. Freeze etching was carried out in a Balzer's 360 freeze-etch machine used according to the method of Moor and Mühlethaler (14). Small circular pieces of ileum with the villi facing upwards were mounted on gold-alloy specimen holders and frozen in liquid Freon 22 at -150°C. Specimens were fractured at -100°C in vacuo and etched for up to 2 min also at -100°C before replication with platinum and carbon. Tissue was digested first in concentrated sulfuric acid followed by Chlorox, and the replicas finally were washed in distilled water. This digestion procedure was sometimes reversed, with similar results. Replicas were placed on uncoated copper grids and examined in a Philips EM 300 or AEI 801 electron microscope.

The encircled arrows at the lower corner of freeze-etch micrographs indicate the direction of shadowing.

RESULTS

The endocytic complex in ileal cells of suckling rats consists of a network of membrane-limited tubules, some associated vesicles, and a giant intracellular vacuole (Figs. 1 and 2). The network of tubules originates as invaginations of the plasma membrane into the apical cytoplasmic matrix. In thin-section preparations the apical network of tubules appears as many rounded and elongated membrane profiles (Fig. 1) but the tubular nature of the system is more apparent after lanthanum hydroxide has been instilled into the lumen of the ileum. This results in the filling of most of the tubular system with colloidal lanthanum, demonstrating the continuity of most of its components (Fig. 3). Some of the large associated vesicles and particularly the components just beneath the apical ends of the cells as well as the giant vacuole did not fill with lanthanum in these experiments. The tubular nature of the system is also apparent in freeze-fracture preparations where oblique fractures through the tubules and fractures along considerable lengths of the tubular network often occur (Figs. 10 and 11). The limiting membrane of the apical system of tubules and associated vesicles features a regular periodic structure when observed in thin sections (Figs. 4 and 5), negatively stained preparations (Figs. 6-9), or by freeze etching (Figs. 10-17).

The appearance of this regular particulate structure in thin sections has previously been described by Wissig and Graney (29), and our results confirm their observations. When thin sections are cut normal to the plane of the membrane, the sections usually feature a conventional ~8-nm thick unit...
membrane structure on the external surface of which is located an ~8-nm thick specialization. This specialization can take on one of two different appearances depending on the orientation of the membrane with regard to the plane of section. It sometimes appears as an ~8-nm thick layer of fairly uniform density somewhat less electron opaque than the outer dense leaflet of the unit membrane structure with which it merges. Alternatively, it may appear as a row of regularly
spaced ~8-nm diameter particles with a center-to-center separation of ~15 nm (Fig. 4). A third appearance, in which the outer dense leaflet appears interrupted or hyphenated at regular intervals along its length with one particle overlying each hyphen, was described by Wissig and Graney (29). We have also observed this appearance as in the inset to Fig. 4. The outer leaflet sometimes appears partially interrupted here but more often it does not. In such regions it is usually present as a faint continuous dense stratum. The effect appears to us to be mainly one of accentuation of the density of the outer dense stratum where the particles are attached. We agree with the suggestion of Wissig and Graney (29) that this hyphenated appearance with apparent absence of the outer dense stratum is probably an artifact. Nevertheless, the differences in density of the outer leaflet leading to the hyphenated appearance probably indicate focal chemical differentiations of the outer leaflet of the attachment regions. When the membrane is sectioned tangentially it has the appearance of particles arranged in a two-dimensional repeating pattern. This pattern can either be square when the particles in adjacent rows are in register (29) or oblique when the particles are not in register (Fig. 5).

In thin transverse sections one sometimes sees the particles as fairly uniform, roughly spherical densities. Of course, in such sections which arc several tens of nanometers thick one is seeing several overlapping particles, and the outlines are hence quite blurred. In negatively stained preparations, however, the appearance of the particles is much more detailed. Here in frontal views of sheets of membrane each particle is seen as a figure resembling a capital letter H (Fig. 6). Optically filtered images have been reconstructed from light diffraction patterns of the arrays. These images show that, in face view, the particles are composed of two cigar-shaped components each ~7.5 nm long and ~3 nm wide. Such images show the two vertical bars of the H but the cross member is lost, and so far these images have added no new information. We can only say that the two vertical oblong components and the connecting crosspiece always stay together in register and appear to be identical halves of a single particle. Therefore, we refer to them together with the crosspiece as a particle.

Sometimes at the edges of sheets of membrane fragments displaying the square lattice as at the lower left in Fig. 8, we see rows of particles which might represent lateral views of the ~7.5-nm particles. These sometimes show suggestions of substructure leading us to believe that each lateral component consists of four subunits each ~3 nm in diameter. This will be dealt with in a separate paper.

We have noted that the particles when separated from the membrane tend to stay together in rows as in Fig. 9. Furthermore, in face views of the arrays in negatively stained preparations of reasonably intact membranes, the end-to-end relationships of the particles remain constant at a spacing of ~15 nm (Figs. 6 and 18 a). The side-to-side relationships of rows of particles, however, are quite variable. Thus adjacent rows may be shifted out of register with one another to give an oblique lattice (Figs. 7 and 18 b). The adjacent rows may also be closer than ~15 nm, sometimes being spaced at ~11 nm. From these observations we conclude that the particles are bound end-to-end in some way even though we see no definite structural element clearly mediating this. We indicate this feature of the particles in Fig. 18 by dotted lines. Similarly, as mentioned, the lateral components of the particle are believed to

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**FIGURE 3** Electron micrograph of the apical region of an ileal absorptive cell of a suckling rat. Instillation of lanthanum into the lumen of the ileum during fixation resulted in the almost complete filling of the tubular membrane system (T) of the endocytic complex demonstrating its continuity. Some apical vesicles (V) and the giant vacuole (GV) did not fill with lanthanum. Microvilli (MV). x 180,000.

**FIGURE 4** Part of the tubular network of the membranes of the endocytic complex. The limiting membranes of the tubules when seen in normal sections feature a conventional ~7.5-nm thick specialization. This specialization appears either as an ~7.5-nm thick layer of fairly uniform electron density (arrow) or as a row of regularly spaced ~7.5-nm diameter particles (arrowhead) with a center-to-center separation of ~14.0 nm. x 110,000. Inset, x 280,000.

**FIGURE 5** Tangential section through membranes of the endocytic complex. The ~7.5-nm diameter particles are seen arranged in a two-dimensional oblique lattice at the top. x 120,000.
FIGURE 6  Fragment of membrane isolated from the endocytic complex and negatively stained with 1% PTA. The surface of the membrane is covered with a regular repeating structure. Individual ~7.5-nm diameter particles each having the appearance of a capital letter H are joined together to form long rows. Lateral arrangement of the rows gives rise to a regular square lattice arrangement of the particles with a side spacing of ~15 nm. × 186,000.
be bound together by some kind of cross member. In some regions in Figs. 6 and 7 we believe we can clearly see the cross member joining the two components. We have not been able to decide about the size or shape of this component as yet. It is shown as a spherical particle between the lateral components in Fig. 18 merely for convenience of illustration. We do not know its size or shape at present but feel confident of its existence.

A square lattice with a side spacing of ~15 nm is observed when the particles are in register and the center-to-center separation of the rows is also ~15 nm (Figs. 6 and 18 a). A second type of rhomboid lattice occurs when the adjacent rows of particles are shifted relative to one another but remain ~15 nm apart. A third type of square lattice is observed when the rows of particles are closely packed with a center-to-center separation of ~7.5 nm and the particles in adjacent rows are displaced one-half repeat period (i.e., ~7.5 nm) relative to particles in the next row. In this case a square lattice with a side spacing of ~11 nm oriented 45° to the rows of particles is observed. Fig. 8 shows such an oblique lattice in which the center-to-center separation of the rows is ~11 nm. These three principal types of lattice arrangements of the particles are illustrated diagrammatically in Fig. 18 a–c.

**Membrane Faces Revealed by Freeze Etching**

Fractures through the ileal lining cells reveal the limiting membranes of the microvilli and of
FIGURE 10 Freeze-fracture replica of the apical region of an ileal absorptive cell after glycerination. The tubular network of membranes of the endocytic complex (T) originates as invaginations of the apical plasma membrane (arrows) at the base of the microvilli (MV) and honeycomb the entire cytoplasmic matrix along with the associated vesicles (V). A part of the giant vacuole (GV) is visible. A series of parallel ridges can occasionally be seen in light relief (arrowheads and inset enlargement) on the convex B fracture face of membranes of the apical tubules. The ridges in the micrograph have a center-to-center separation of ~15 nm. × 31,000. Inset, × 55,000.
the components of the endocytic complex. In each case the fracture faces observed represent split internal membrane faces whereas the true lumenal and cytoplasmic membrane faces are only revealed after etching. The tubular and vesicular membranes of the endocytic complex fracture along the core of the membrane so as to reveal two complementary fracture faces: a concave face (A) which is the split surface of the cytoplasmic half of the membrane, and a convex face (B) which is the split surface of the outer lumenal leaflet (Figs. 10 and 11). A few randomly distributed ~8.5-nm diameter membrane-associated particles are seen on the A fracture face whereas the B fracture face is virtually devoid of particles of this kind (Fig. 11). The remainder of both fracture faces usually appears smooth and typical of a fracture of a pure unperturbed lipid bilayer. Frequently a series of parallel ridges can be seen on the convex B face (Fig. 10 broad arrows and inset enlargement). Occasionally, complementary rows of ridges can be seen on the concave A face as in the inset in Fig. 11. This regular structure, which only appears in light relief on the unetched fracture faces, is often more apparent when a cryoprotective agent has not been used as in Fig. 14.

Extensive areas of the lumenal and cytoplasmic membrane can be visualized after deep etching as in Figs. 12, 13, and 15–17. Although there is some distortion of the tissue in the absence of a cryoprotective reagent the microvilli and the membranes of the endocytic complex can still be easily identified, and where deep etching has occurred a regular
particulate structure is exposed on the luminal membrane surface of the apical tubules and vesicles. The cytoplasmic surface in such etched preparations appears smooth (Fig. 14). When the shadowing angle is approximately perpendicular to the rows of particles, only the rows show up clearly. Fig. 15 shows an area of etch face in which the rows of particles have a center-to-center separation of ~15 nm. However, when the shadowing angle is more favorable, individual ~7.5-nm diameter particles arranged in a two-dimensional lattice can be seen as in Figs. 16 and 17. Fig. 16 shows a square lattice of particles with a side spacing of ~11 nm while Fig. 17 shows an area of etch face in which most of the particles are arranged in an oblique lattice. No subunit detail has yet been visualized in any of the individual particles seen by freeze-etch methods.

DISCUSSION

Fine Structure of the Regular Particulate Array

Although the functional role of the ileum particles remains unknown and a matter of speculation, a comparison with known macromolecular structures suggests that the particles are probably protein and that they may possibly have some associated enzyme activity. As shown by Miller and Crane (12, 13) digestive disaccharide hydrolases are associated with isolated intestinal brush border fractions. These authors suggested that it might be justifiable to think of the absorptive surface of the brush borders as being also a "digestive surface," at least for carbohydrates (reference 13, p. 298). It seems reasonable to suspect that the particulate arrays of the endocytic complex may serve some such function though this matter must await isolation and characterization of the particles for elucidation.

So far, our observations suggest that the particles might not exist independently since whenever we have seen them apart from the membrane they are linked together to form linear aggregates. This is not usually apparent in either thin-section or freeze-fracture preparations but is clearly seen in face views of the rows of particles seen in negatively stained material (Figs. 6-9). It is possible, therefore, that the rows of particles rather than individual particles constitute some kind of functional unit. It is also quite possible that the particles do separate individually and that we simply have not recognized them in our preparations so far.

Several square and oblique lattice arrangements of the particles have been observed in both thin-section, negatively stained, and freeze-etch preparations, indicating that these different types of lattice probably exist in vivo and are not an artifact of any one preparative technique. Wissig and Graney (29) observed in thin sections a square lattice with a side spacing of ~12 nm, and this can be correlated with the square lattice with a side spacing of ~11 nm observed in both negatively stained (Fig. 8) and freeze-etch preparations (Fig. 16). The small difference in unit cell dimensions is presumably a reflection of the different preparative techniques used. Since the function of the particles remains unknown it is not possible to say whether the different types of square and oblique lattices simply represent different ways in which the rows of particles can be arranged so as to cover the surface of the membrane or whether they are a reflection of some more dynamic process which involves movement of the rows of particles relative to one another. It also is possible that the three different patterns reflect molecular differences in the particles that are too subtle to be detected in the micrographs. Thus, the particles in the different arrays could be different and have different functions. We have treated all the particles in the three arrays as though they were the same but they might very well be quite different. Sometimes particles of similar size are seen on the membranes at the bases of the microvilli but we have not studied these sufficiently to say how they may relate to the particles of the endocytic complex.

Figure 12 Suckling rat ileum freeze cleaved in the absence of a cryoprotective reagent and etched for 2 min at ~100°C before replication. Fractured microvilli (MV) and the tubular membranes of the endocytic complex (T) can still be readily identified and where deep etching has occurred a regular particulate structure is revealed on the luminal surface of membranes of the tubules (arrows and inset enlargements). 

x X 36,000. Insets, x 100,000.

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FIGURE 13 Freeze-fracture replica of a fragment of membrane isolated from the endocytic complex, washed in distilled water, and etched for 2 min at 100°C before replication. A regular particulate structure consisting of parallel rows with a center-to-center separation of ~150 nm can be seen on the lumenal membrane surface exposed by deep etching. A clearly defined ridge (R) separates the A fracture face from the outer (O) lumenal etch surface. × 74,000.
FIGURE 14 Freeze-fracture replica of a fragment of membrane isolated from the endocytic complex, washed in distilled water, and etched for 2 min at −100°C before replication. A regular particulate structure consisting of parallel ridges can be seen in light relief on much of the convex B fracture face. The series of ridges indicated by the arrow have a center-to-center separation of ~150 nm. Etching has exposed an area of the inner (I) cytoplasmic membrane surface which appears smooth. A clearly defined ridge (R) separates the B fracture face from the cytoplasmic membrane surface. × 74,000.
Regular particulate structures seen on the lumenal membrane surface of membranes of the endocytic complex after deep etching. Only the rows of particles show up clearly when the shadowing direction is perpendicular to the rows (Fig. 15). The center-to-center separation of the rows is ~14 nm. Fig. 16 shows an area of etch face in which individual ~7.5-nm diameter particles are arranged in a regular square lattice with a side spacing of ~11 nm. Fig. 17 shows an area of etch face in which the particles are arranged in an oblique lattice. Fig. 15, × 175,000; Fig. 16, × 140,000; Fig. 17, × 100,000.

Diagram illustrating the three types of regular lattice that have been observed in membranes of the endocytic complex: (a) square lattice with a side spacing of ~15 nm; (b) oblique lattice; (c) square lattice with a side spacing of ~10.5 nm oriented 45° to the rows of particles.

Relationship Between the Regular Particulate Structure and the Membrane

In thin sections the particles are seen merged with and extending out from the external surface of the lumenal dense leaflet of the unit membrane structure (Fig. 4). Freeze etching allows one to look at both the hydrophobic interior of a membrane and the hydrophilic surface regions. This technique, therefore, should provide more informative data as to the relationship between the regular structure and the membrane than is possible with either thin-sectioning or negative-staining techniques. When biological membranes are fractured they tend to split preferentially along the hydrophobic interior of the membrane (1). Both the double-replica technique (28) and labeling the
external surface with specific markers (16, 26) have confirmed that true membrane surfaces generally are not exposed by the fracturing process but are only revealed after etching. The extensive smooth areas of fracture faces observed when membranes are fractured probably correspond to a fractured lipid bilayer (3, 4), while evidence is accumulating to suggest that particulate areas are related in some way to specializations of nonlipid components. The exact way in which such specializations are expressed in the hydrophobic fracture face to yield a particulate appearance in freeze-fracture preparations remains to be decided.

Two complementary fracture faces are produced when the tubular membranes and associated vesicles of the endocytic complexes are cleaved: a concave A face and a convex B face which faintly reveals the particulate array. The particles show up only faintly as ridges in the fracture faces (Figs. 10 and 11) and are seen much more clearly in the etch face (Figs. 12, 13, and 15–17). We have the impression that this effect is more pronounced if cryoprotective agents are not used even though the particulate array does also sometimes appear quite distinctly on the fracture face in glycerinated specimens as in Figs. 10 and 11.

Perhaps the rows of surface particles show up in the core of the bilayer simply because the polar surface of the bilayer tends to follow the contours of the particulate array. The variations in the lipid monolayers would be expected to be minimal, and one would expect to have it revealed only when the shadowing angle and thickness of deposited metal were exactly right. Another explanation is suggested by our observation that the particulate array in the fracture faces is accentuated if cryoprotective agents are not used even though the particulate array does also sometimes appear quite distinctly on the fracture face in glycerinated specimens as in Figs. 10 and 11.

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