BRUSH BORDER DEVELOPMENT IN THE INTESTINAL ABSORPTIVE CELLS OF *XENOPUS* DURING METAMORPHOSIS

MARY A. BONNEVILLE and MELVYN WEINSTOCK

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. Dr. Weinstock's present address is the Department of Anatomy, McGill University, Montreal

ABSTRACT

The differentiation of the brush border which makes up the apical free surface of intestinal absorptive cells has been studied by electron microscopy. Specimens of *Xenopus* small intestine were fixed at various stages during metamorphosis, the time when a new intestinal epithelium forms. The interpretation of details described herein emphasizes the role of "surface-forming" vesicles. These vesicles are thought to provide membrane both for the initial expansion of the apical surface and for the later elongation of the microvilli. The latter are believed to be "molded" around filamentous cores that appear early in differentiation. The cores are attached to the apical membrane and extend vertically into the supranuclear cytoplasm. This interpretation rests chiefly on (*a*) the resemblance, both in morphology and in staining properties with colloidal thorium, between the membrane that limits the vesicles and that which limits the microvilli and (*b*) the distribution and time of appearance of the vesicles with respect to development of the microvilli. According to this view, the specific properties of surface membrane reside in preformed units that arise within the supranuclear cytoplasm. This morphogenetic process probably involves participation of the Golgi region as the site where the complex macromolecular architecture of the cell surface is assembled.

INTRODUCTION

Since the pioneering work of Granger and Baker (1950), the morphological complexity of the striated, or brush, border of intestinal absorptive cells has gradually received better definition, until at present its basic pattern of organization has been clearly demonstrated in small mammals by a number of workers, particularly Ito (1965), Mukherjee and Williams (1967), and Cardell et al. (1967). In all instances, microvilli, straight, finger-like projections of regular height, diameter, and frequency, are a characteristic feature. The plasma membrane which limits the microvilli is thicker than that of the lateral and basal regions of the cell (Zetterqvist, 1956; Palay and Karlin, 1959), and the asymmetry of its unit membrane structure has been noted (Sjöstrand, 1963; Millington, 1964; Revel and Ito, 1967). Enclosed in each microvillus is a central core, a bundle of fine filaments which run the length of the structure and extend into the terminal web. The latter is a fibrous cortical layer of cytoplasm from which most cytoplasmic organelles (e.g., mitochondria, endoplasmic reticulum [ER]) are excluded.

The structural feature of the microvilli most recently described in detail and one that is of particular interest here is the acid mucopolysaccharide-rich filamentous coat (Ito, 1965; Revel, 1964; Spicer, 1965; Wetzel et al., 1966; Rambourg

and Leblond, 1967) that covers their outer surface and is considered to be an integral part of the plasma membrane (Ito, 1965; Revel and Ito, 1967). The latter interpretation rests chiefly on the observations (*a*) that the enteric surface coat has distinct morphological characteristics that vary depending on the epithelial cell type and on the species examined and (*b*) that the coat can not be removed from the plasma membrane by a variety of proteolytic and mucolytic agents. Subsequently it was found that the appearance of the coat also varies when absorptive cells from the small and large intestines of the same animal are compared (Mukherjee and Williams, 1967). These facts make it seem unlikely that the coat is simply adsorbed to the surface, spreading out after its secretion by mucous cells.

Further knowledge of the macromolecular complexity of the free surface of intestinal epithelial cells has been derived from histochemical studies and analyses of isolated brush borders. For example, alkaline phosphatase appears to be closely associated with the plasma membrane limiting the microvilli (Ito, 1965; Eichholz and Crane, 1965a; Overton et al., 1965; Overton, 1965; Hugon and Borgers, 1966), and other enzymes, including several disaccharidases (see review of Crane, 1966), a dipeptidase (Forstner et al., 1966), and ATPase (Ashworth et al., 1963; Eichholz and Crane, 1965b) have been localized in the brush border region. Each feature of morphological and chemical complexity mentioned here is presumed to have significance during absorption and transport across the plasma membrane, at which time each would play a precise role.

Of particular interest is the model proposed by Crane (1966) to explain active transport of sugars across the apical plasma membrane of intestinal absorptive cells. After having considered results from a variety of experimental approaches, he has proposed a spatial ordering of the essential molecules within the plasma membrane in order to account for the specific transport properties believed to exist there. Presumably, an active transport system for amino acids (Kinter and Wilson, 1965) and a mechanism by which monoglycerides and fatty acids are separated from bile salts before entering the absorptive cells (Senior, 1964) also could be defined in terms of the macromolecular architecture of the cell surface. Thus, although its plasma membrane shares a basic trilaminar structural pattern with other cell surfaces, the apical surface of the intestinal absorptive cell is a highly specialized area which has distinct and unique properties of structure and function.

Such specialization raises the question of how this complex structure may be assembled during development. In interpreting their observations on chicks (Overton and Shoup, 1964) and mice (Overton, 1965), Overton and Shoup have suggested that the developing filamentous cores play a role in the morphogenesis of microvilli. This hypothesis does not conflict with the less detailed descriptions of immature microvilli that have been presented by Brown (1962), by Trier (1963), and by Mukherjee and Williams (1967).

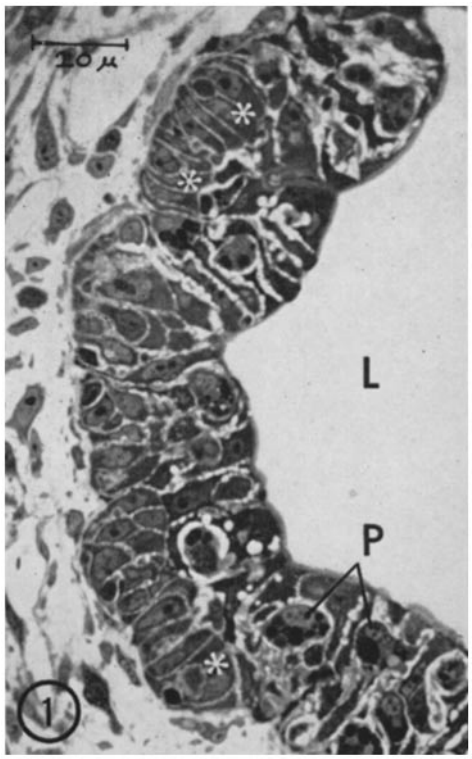


FIGURE 1 Cross-section through the intestinal epithelium of *Xenopus* at stage 61 (Nieuwkoop and Faber, 1956). This light micrograph shows "nests" of cells (*) at the base of the epithelium during metamorphosis. These will give rise to the functional adult epithelium, which replaces that of the larva. Degenerating larval tissue covers the "nests" and lines the lumen (*L*) of the gut. In some instances the formerly columnar larval absorptive cells have become pyknotic (*P*) and are seen as rounded cells lying beneath the surface of the epithelium. × 670.

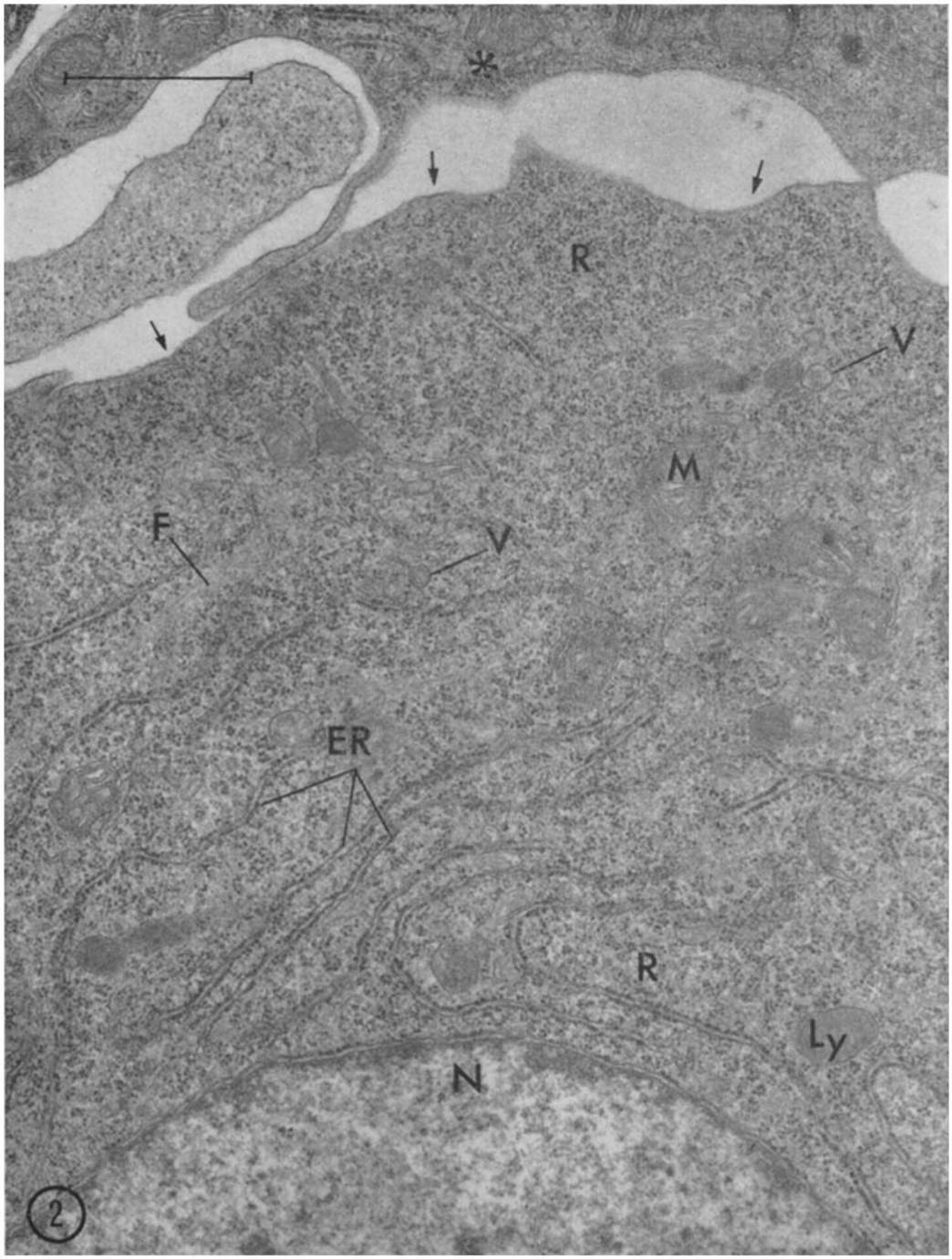


FIGURE 2 Supranuclear region of intestinal epithelial cell prior to differentiation of the brush border (stage 61). Vigorous synthetic activity is evidenced by the abundance of ribosomes. Some are bound to cisternae of the endoplasmic reticulum (*ER*), but most are free in the cytoplasm (*R*). Dense bodies, probably primary lysosomes (*Ly*), and mitochondria (*M*) are also present. The nucleus (*N*) possesses a thin layer of peripheral heterochromatin, an appearance consistent with the interpretation that this cell is metabolically active. The apical surface of the cell (arrows) does not, at this stage, possess microvilli, nor does a well-developed surface coat occur at this or at the lateral surfaces. Although bundles of fine filaments (*F*) are present throughout the supranuclear cytoplasm, there is no evidence that a terminal web is beginning to form. However, small vesicles (*V*), which we believe will eventually participate in the formation of the apical cell surface, can be detected. A degenerating larval cell (*) overlies the developing one. × 27,500. Scale marker = 1 μ.

In view of the designation of the surface coat as an integral part of the plasma membrane limiting the microvilli, we consider that experiments dealing with the formation of the coat are in truth dealing with the formation of the cell surface. Thus morphological events in surface coat formation should afford clues to the origin of the specialized apical plasma membrane and to the mechanism of development of microvilli.

The formation of the coat has been studied by radioautography at the light (Neutra and Leblond, 1966) and the electron microscope levels (Ito and Revel, 1966). These experiments reveal that the mucoproteins are probably synthesized in part or at least assembled in the Golgi region, and they later become concentrated in the surface coat where they remain for a limited time. Employing Thorotrast staining (Revel, 1964) as a means of localizing acid mucopolysaccharide, Berlin (1967) reached a similar conclusion and showed that the transfer of material from the Golgi complex to the coat was effected by cytoplasmic vesicles. A similar role for vesicles was also proposed by Wetzel et al. (1966).

A more general role for small vesicles in the formation of microvilli had previously been proposed by Röhlich (1962), following his study of the regenerating planarian eye, and by one of us (Bonneville, 1961), based on observations of the intestinal epithelium of the bullfrog during metamorphosis. In the latter interpretation, the vesicles were designated as preformed units of the plasma membrane, destined to form part of the cell surface. The purpose of this study was to determine whether the small vesicles are equivalent to secretory granules or whether they play a broader, morphogenetic role in establishing the macromolecular architecture of the cell surface.

MATERIALS AND METHODS

Preparation for Microscopy

Metamorphosing tadpoles of *Xenopus laevis* were raised in the laboratory from embryos that resulted from matings induced in adults by administration of gonadotropin (Antuitrin S, Parke, Davis & Co., Detroit, Mich., administered as suggested by Nieuwkoop and Faber, 1956, and by Gurdon, 1967). Animals were anesthetized with chloretone, and a segment of the small intestine just posterior to the entrance of the hepatopancreatic duct was removed from animals at each stage (56–65) of metamorphosis, as described for this species by Nieuwkoop and Faber (1956). The tissue was bathed in fixative, and fixative was also injected into the gut lumen before removal of tissue from the animal. During fixation the gut was cut transversely with a sharp razor blade.

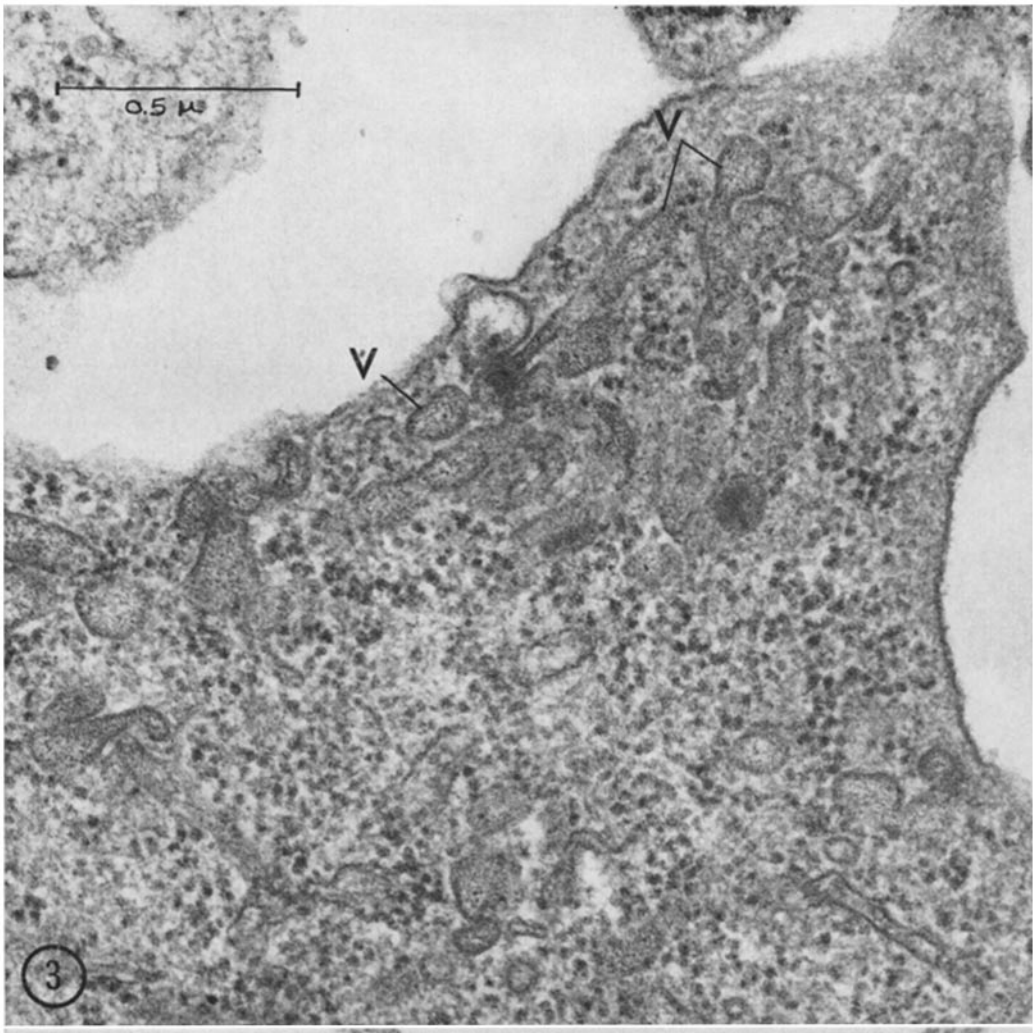
Samples of tissue were fixed in 6.25% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4 (Sabatini et al., 1963), for 1.5–2 hr, rinsed thoroughly in the same buffer, and postfixed in 1% OsO_4 in 0.1 M Sorensen's buffer, pH 7.4, for 1–1.5 hr.

Some specimens from each aninal were embedded in Epon (Luft, 1961), and others were embedded in a prepolymerized methacrylate mixture (Pease, 1964) which was made from butyl and methyl (85:15) monomers and contained 0.8% benzoylperoxide as catalyst.

Preparation of thin sections for the electron microscope was carried out by routine methods. Epon-embedded blocks were sectioned with diamond knives on Porter-Blum MT-1 and MT-2 ultramicrotomes (Ivan Sorval, Inc., Norwalk, Conn.), mounted on copper grids, stained with uranyl acetate (2%

FIGURE 3 Apical tip of differentiating intestinal absorptive cell during metamorphosis (stage 61). An accumulation of small "surface-forming" vesicles (*V*) in the supranuclear cytoplasm is characteristic of the developmental period just preceding the first appearance of microvilli. The unit membrane structure of the membrane limiting the vesicles is more easily resolved than is the structure of the plasma membrane at this stage. Fine filamentous material fills the vesicles, which appear in general to be short tubules, measuring as much as 370 mμ in length and having a median diameter of approximately one quarter that value. There is not a well-developed surface coat at this stage. × 67,000.

FIGURE 4 Area comparable to that shown in Fig. 3. Small vesicles (*V*) have a filamentous coating closely associated with the inner layer of their limiting membranes. Multivesicular bodies (*MVB*) have a limiting membrane and inner matrix that resemble, respectively, the membrane and contents of the small vesicles. Ribosomes and fine filaments (*F*) occur in the cytoplasm, but no evidences of brush border formation at the apical cell surface (arrows) are present. × 58,000.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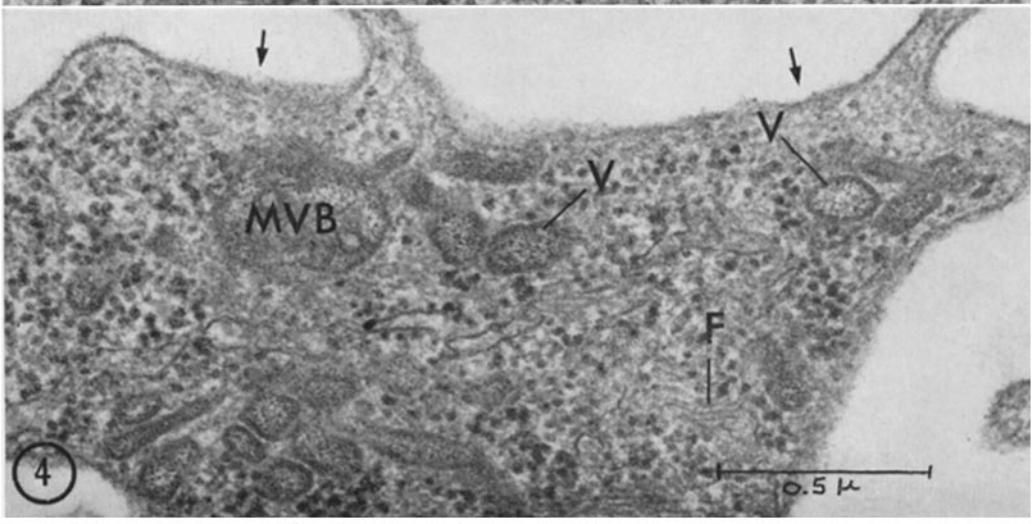
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aqueous for 3–5 min), and then stained with lead citrate (Reynolds, 1963). Sections from methacrylate-embedded blocks were cut with glass knives, supported on grids coated with parlodion, stained with lead citrate, and then covered with a carbon film. Specimens were examined with the Siemens Elmiskop I and the Philips 200 Electron Microscope.

For purposes of orientation and evaluation of specimen quality, light microscope examination of plastic-embedded tissue was carried out routinely on 1–2-μ thick sections, cut with glass knives on a Porter-Blum MT-1, mounted on glass slides, and stained with 0.5% toluidine blue in distilled water or borate buffer.

Thorotrast Staining

Localization of acid mucopolysaccharides was accomplished by staining with colloidal thorium (Revel, 1964) on thin sections of methacrylate-embedded tissues. The sections subsequently were stained with lead citrate before examination with the electron microscope.

OBSERVATIONS

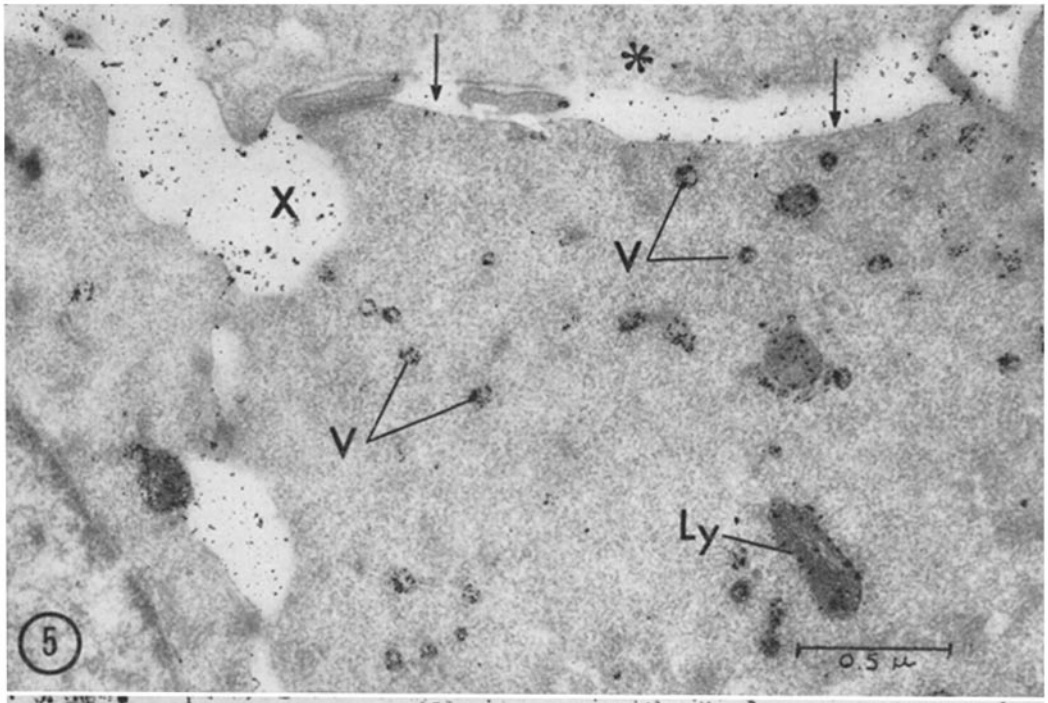
Metamorphic changes in the intestinal epithelium of *Xenopus laevis* parallel closely those already outlined for the bullfrog (Bonneville, 1963). In brief, the adult epithelium arises from undifferentiated basal cells that are present in the larval tissue and that undergo proliferation and differentiation during metamorphosis. Small groups or "nests" of new cells appear within the epithelium near the basal lamina, while the absorptive and mucous cells that functioned during larval life undergo degeneration. Soon the "nests" coalesce to form a complete cell layer, a simple columnar epithelium. The dying larval cells at first cover this new layer, but soon they are lost, leaving the new layer as a lining for the lumen of the gut. The epithelial cells thus exposed possess junctional complexes and desmosomes similar to those seen in the adult epithelium. They then elongate, and the brush border differentiates so that it is fully formed (Fig. 14) at the end of metamorphosis (stage 65, Nieuwkoop and Faber, 1956). The entire differentiation after loss of the larval cells occurs within a period of 2–3 days at 22–25°C. The rates of absorptive cell renewal in adult *Xenopus* are not known.

The "nests" referred to above can be identified readily in light micrographs of intestinal epithelium (Fig. 1) taken from developmental stage 61. Groups of cells in the basal region of the epithelium have large nuclei with prominent nucleoli, and their cytoplasm appears homogeneous and of medium density when stained with toluidine blue. Another characteristic of this developing epithelium in all preparations examined is the extensive extracellular space, evident also in electron micrographs (Figs. 2, 5, and 8).

Simultaneously, as "nests" develop, the functional larval cells suffer degenerative changes, which are detectable first at stage 58. By stage 61, these changes have progressed so that in many cases the formerly columnar cells are round structures, contain large dense bodies (probably lysosomes), and lie below the free surface of the epithelium (Fig. 1). Larval cells with recognizable brush borders are still present and form a coherent

FIGURE 5 Apical cytoplasm of differentiating intestinal absorptive cell (stage 61), embedded in methacrylate and stained with colloidal thorium (Revel, 1964). Prior to the formation of the brush border, when the degenerating larval epithelium (*) covers the developing adult epithelium, small vesicles (*V*) containing acid mucopolysaccharide are detected. These are interpreted as being identical to the vesicles described in Epon-embedded preparations (Figs. 3 and 4). Larger structures, believed to be lysosomal in nature (*Ly*), are also stained. Some stain is usually localized in the extracellular space (*X*), but there is little association of stain with the apical (arrows) or lateral surfaces of these cells. × 20,000. Scale marker = 1 μ.

FIGURE 6 Differentiating intestinal absorptive cell (stage 61). Preparation similar to Fig. 5. Microvilli (*Mv*) have begun to form on the apical surface, and colloidal thorium micelles seem to be more concentrated on this surface of the plasma membrane (arrows) than in the extracellular space (*X*). (At this stage, the degenerating larval epithelium still lines the gut lumen, forming a coherent layer covering the developing epithelium that will function after metamorphosis.) Vesicles (*V*), stained with colloidal thorium, accumulate just beneath the cell surface where the brush border has begun to form. × 45,000.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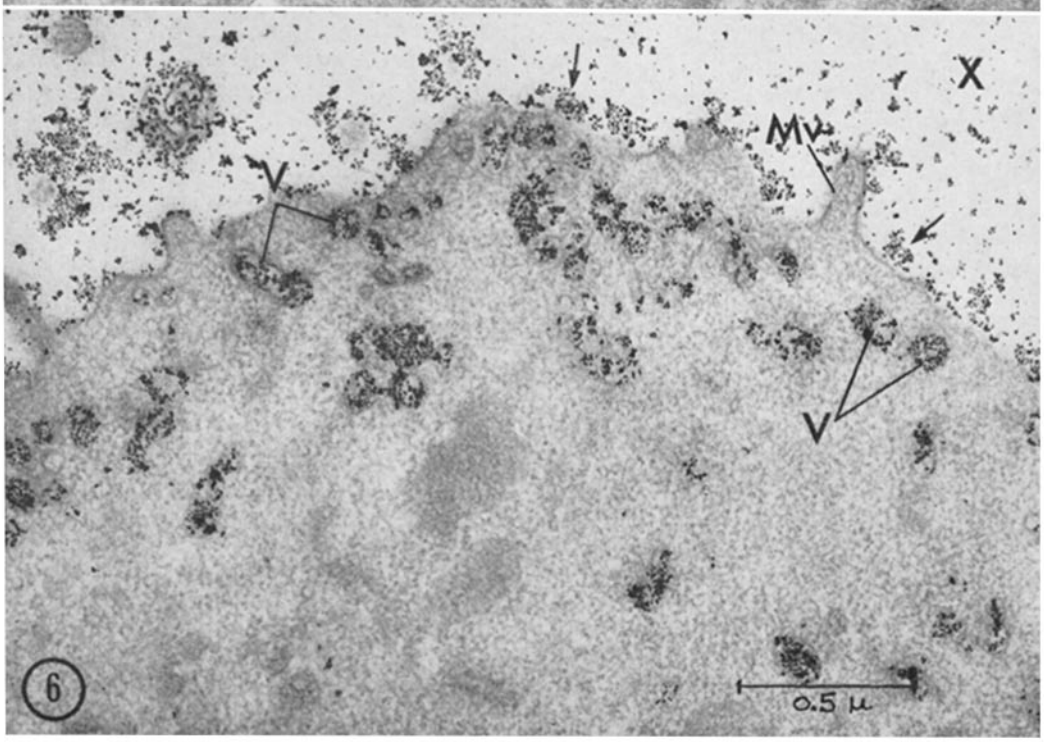
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lining of the gut lumen. When examined with the electron microscope, however, they too display signs of incipient degeneration. In addition to a general loss in cell height, their microvilli are reduced in size and in number, and dense bodies are abundant in the supranuclear cytoplasm.

Elongation of the newly developing epithelial cells begins within the "nests," and in electron micrographs, these units exhibit characteristic cytological features associated with differentiating cells (Fig. 2). For example, within the nuclei there is little peripheral clumping of heterochromatin. The large nucleoli have already been mentioned. In regard to the cytoplasm, it is readily apparent that some ribosomes are attached to flattened cisternae of the endoplasmic reticulum, but free ribosomes occur in great abundance (Fig. 2). Golgi complexes may be supranuclear in position, but usually they are lateral, and sometimes even basal, to the nucleus at this stage.

Thus the cytological picture is one indicating vigorous protein synthesis, but few indications of cell specialization are manifest. Cell elongation and the aggregation of mitochondria near the basal surface of the cell attest to the initiation of cell differentiation. Although thin, curved projections extend from the lateral cell surfaces into the extracellular space, microvilli, which are typical of fully differentiated brush borders (Fig. 14), are not yet present at the apical surfaces of such cells. Bundles of fine filaments (Figs. 2 and 4) are present in the cytoplasm. They do indeed resemble the filaments making up the cores of mature microvilli (Fig. 14), but they are not assembled into bundles of uniform size. Therefore one cannot predict with confidence whether or not these same filaments will become part of the microvillous cores, although they are sometimes observed lying close to and parallel to the presumptive apical cell surface (Fig. 4). Finally, it is to be noted that a well-developed surface coat, characteristic of the apical free surface of fully differentiated absorptive cells (Fig. 14), is not detectable at this stage (Figs. 2–4).

A noticeable feature of the absorptive cells at this early point in their development is a population of small vesicles in the apical region of the cell. We have studied these structures in detail because we believe that they play a significant role in the differentiation of the microvilli. Even at low magnifications these vesicles may be identified (Fig. 2) in all "nest" cells, but in some cells, presumably those farther along in development, considerable numbers can occur (Figs. 3 and 4).

When examined more closely, the vesicles are seen to be limited by a unit membrane that is greater in width and density than that limiting nearby elements of the endoplasmic reticulum (Fig. 4). At this stage too, the vesicular membrane is thicker and denser than the plasma membrane at all surfaces of the cell. Moreover, it resembles more closely the plasma membrane of mature microvilli (Fig. 14) and that membrane which limits multivesicular bodies. Moreover, it should be mentioned that each vesicle contains fine filamentous material that is closely associated with and is perhaps more concentrated near the inner dense layer of its trilaminar limiting membrane. In contrast, the plasma membrane does not have any comparable coating on its apical surface as it will at later stages (see below). In shape, the vesicles appear to be short tubules, so that in any one field some profiles in cross section and a variety of profiles in oblique section are present. Measurements of apparent cross sections yielded a wide range of diameters, from 53 to 183 mμ. Approximately 80% of the measurements fell below 110 mμ, and it was judged that the larger diameters, which overlapped with measurements of tubule length, represented oblique sections through curved tubules. The length of the vesicles was also difficult to determine exactly, but tubules up to 370 mμ in length were observed.

In Fig. 5, the apical region of a "nest" cell is seen in a methacrylate-embedded specimen that has been stained with Thorotrast (Revel, 1964). Acid mucopolysaccharide localization seems to be confined primarily to small vesicles and to larger structures, probably primary lysosomes and/or multivesicular bodies. Although colloidal thorium micelles are scattered in the extracellular space, the outer surface of the plasma membrane, as well as nuclear and other cytoplasmic structures, are almost completely free of stain before there are any evidences of formation of microvilli.

Occasionally, cells within the "nests" have already begun to develop small microvilli on their apical surfaces, although it is more usual for such development to begin only after the apical cell surface is free of its overlying cover of degenerating larval epithelium. When such cells are subjected to the Thorotrast stain (Fig. 6), an aggregation of small vesicles in which the stain is localized can be observed just beneath the cell surface. It is to be

noted that few vesicles lie below the best-formed or longest microvilli. In addition, aggregation of stain occurs on the outer cell surface once differentiation of the microvilli has begun. As in all specimens treated with colloidal thorium, the intercellular space is fairly heavily stained.

Localization of the vesicles in question is chiefly in the supranuclear cytoplasm, but they are not confined in all instances to the region of the cell just beneath the apical plasma membrane. In some cases they are found throughout the supranuclear region of the cytoplasm, including the peripheral zone of the cell center (Fig. 7), and near the Golgi cisternae. In all cases observed they can be stained with colloidal thorium, which also can be found in association with Golgi membranes and vesicles. A relationship between Golgi elements and polysaccharide-rich vesicles therefore seems to exist in *Xenopus* intestinal epithelium. Detailed analysis of this relationship, however, has not been carried out.

We should mention at this point the multivesicular bodies (MVB's) and their relationship to the vesicles that have been discussed in detail above. The MVB's are found near the vesicles wherever they occur in the supranuclear cytoplasm of the epithelial cells which are destined to line the intestine of the adult frog (Figs. 4 and 7). However, they are rarely seen in the terminal web region, either when it is developing or after it is com-

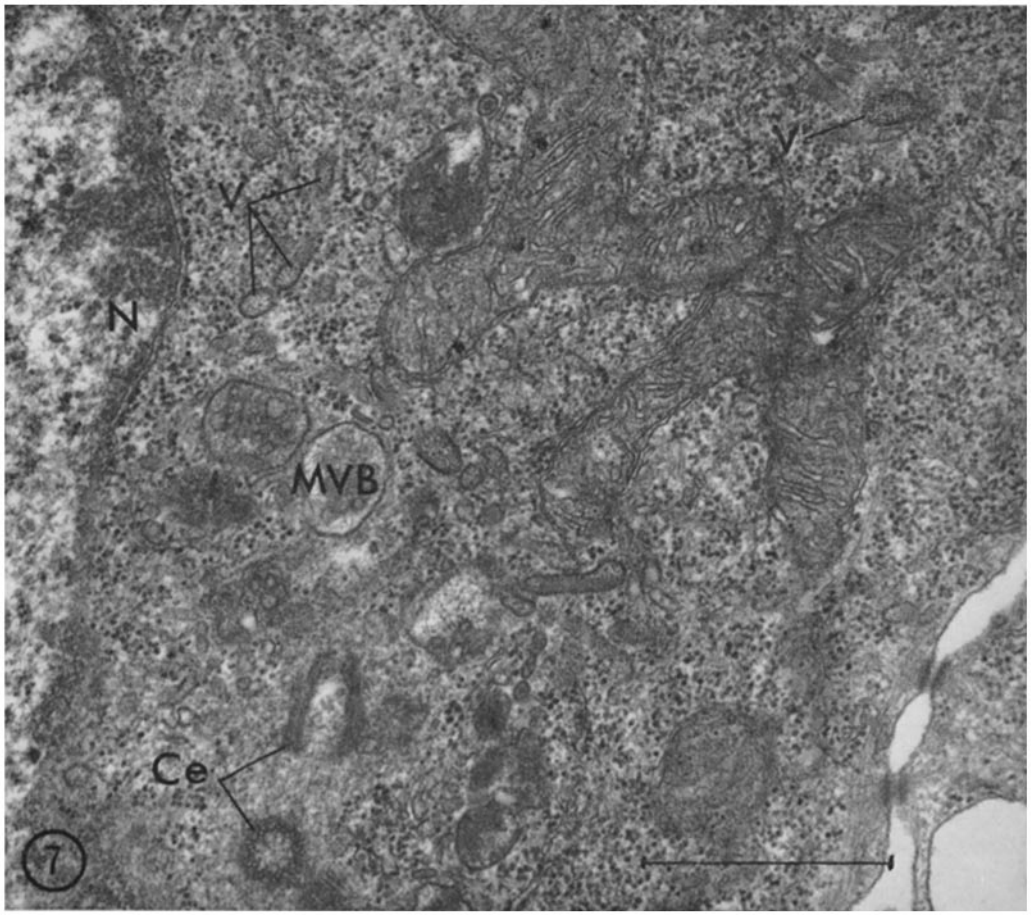


FIGURE 7 Cytoplasm, lateral to nucleus (*N*) of differentiating intestinal absorptive cell (stage 61). Small vesicles (*V*), believed to take part in the formation of the apical cell surface, are distributed throughout the region. Near the centrioles (*Ce*) of the cell center, both the vesicles and multivesicular bodies (*MVB*) are abundant. × 33,600. Scale marker = 1 μ.

pletely formed. Both the vesicles and the bodies also occur near Golgi membranes. Furthermore, the membrane limiting the MVB is comparable in width, as we have noted, to that enclosing the vesicle, and the filamentous material within the matrix of the complex body is morphologically similar to the material within the vesicles (Fig. 4). Finally, the multivesicular bodies are regularly stained after exposure to Thorotrast, and it is our impression that the stain is deposited most heavily

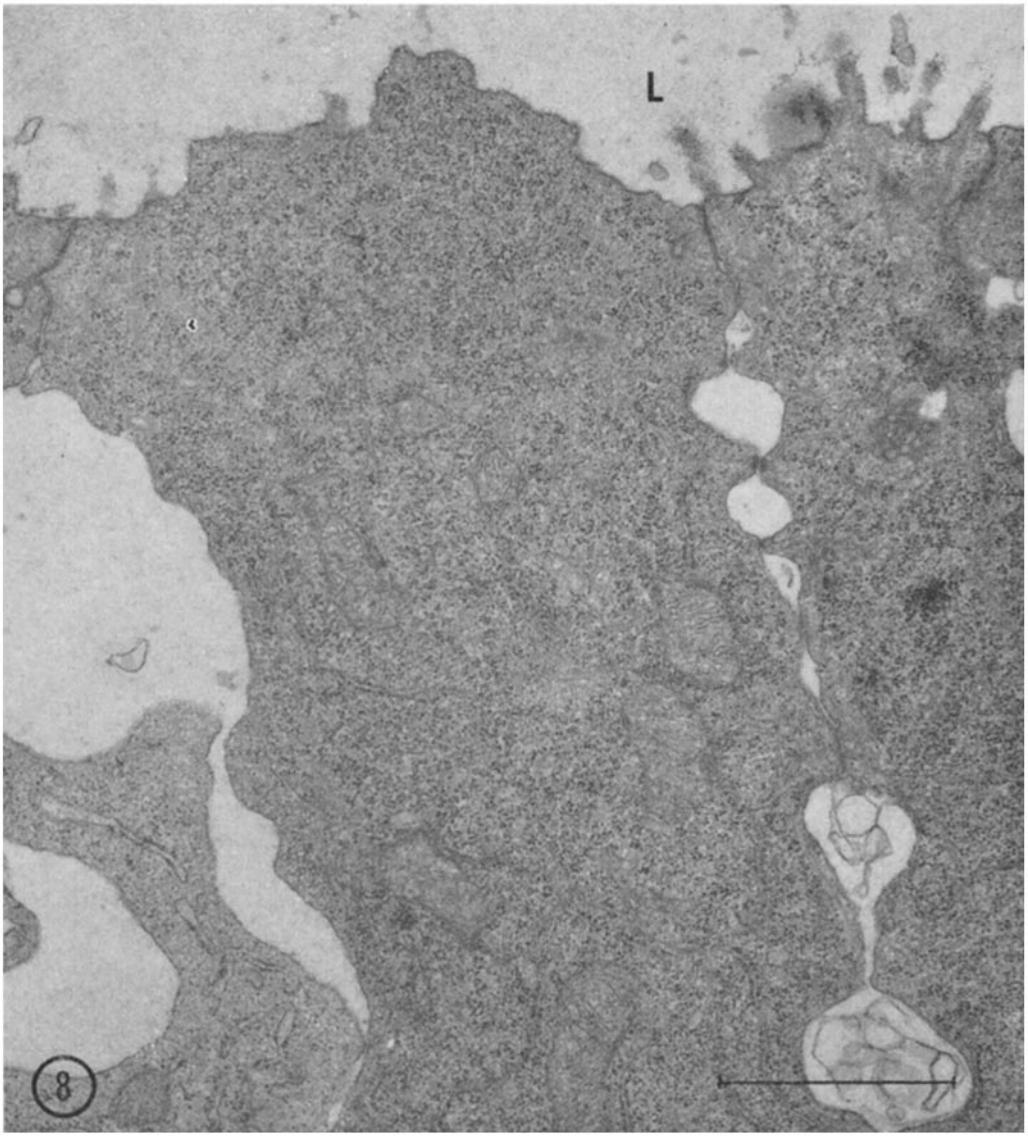


FIGURE 8 Differentiating intestinal absorptive cell is characterized at stage 62 by expansion of the cell surface to form an irregular projection into the intestinal lumen (*L*) but a brush border has not yet formed. Considerable cell elongation has occurred since the previous stage, and the developing epithelium is no longer covered by degenerating larval cells. As at the previous stage, however, the supranuclear cytoplasm, including the apical projection, is rich in ribosomes. Closer examination of cells in this state of differentiation has indicated that small vesicles, such as those seen in Figs. 3–7 (stage 61), are present but fewer in number than before expansion of the surface has taken place. × 31,500. Scale marker = 1 μ.

in areas corresponding to the filamentous material rather than over the small inner vesicles.

The vesicles observed at stage 61 also may be abundant at stage 63 in cells that have elongated, reached the luminal surface, and are no longer covered by a layer of degenerating larval cells. It has been noticed, however, that vesicles occur in such numbers only in cells whose apical profiles appear as tapered tips rather than with the fan-shape characteristic of fully differentiated cells. Comparable areas can also be found in methacrylate-embedded sections, where the vesicles stain with Thorotrast.

The next important event in the differentiation of the brush border appears to be the expansion of the free (luminal) cell surface. This step and succeeding steps in the formation of microvilli all occur slightly asynchronously so that several different stages in the process are found within one developmental stage (63), as defined by Nieuwkoop and Faber (1956). The observer must therefore assign to each event its order within the sequence. Keeping this pitfall in mind, we have designated the earliest event as the formation of an apical cytoplasmic bulge (Fig. 8). The plasma membrane limiting the ribosome-rich cytoplasm is folded in an irregular manner, resulting in a series of small bulges and depressions. This state is apparently short-lived, however, because more frequently, when such irregular protrusions are seen, microvilli are beginning to form on the same cell surface adjacent to them (Fig. 9). Small vesicles of the type already described lie beneath the more irregularly folded areas of the surface. Nearby, bundles of fine filaments, 40–70 mμ in diameter, are attached to the plasma membrane and descend into the underlying cytoplasm (Figs. 9 and 10). The entire free surface possesses a surface coat once it has undergone expansion. Thus the irregular apical bulges and the microvilli, when

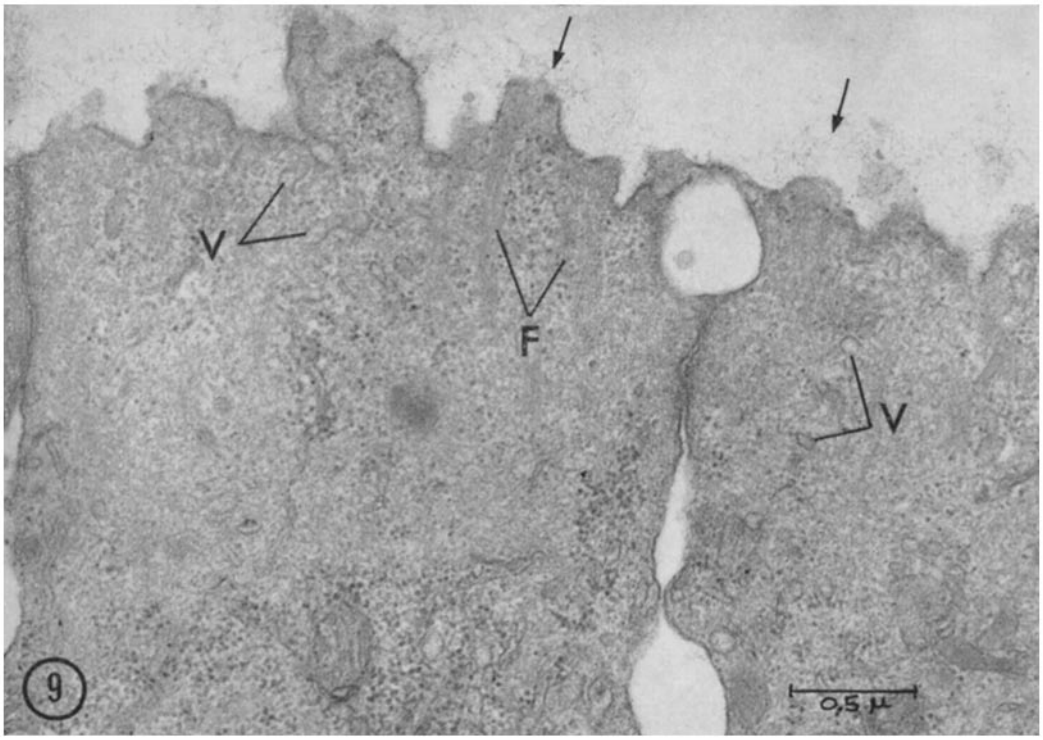


FIGURE 9 Early evidences of brush border formation can be identified at stage 63. As at earlier times, small vesicles (*V*) are present beneath irregular bulges of the cell surface as well as in deeper areas of the cytoplasm. In some regions, however, bundles of fine filaments (*F*) are attached to the surface and descend into the underlying cytoplasm. These are interpreted as cores of the differentiating microvilli. Unlike the apical surface of absorptive cells at stages prior to its expansion, the free surface is covered by a surface coat (arrows). × 33,000.

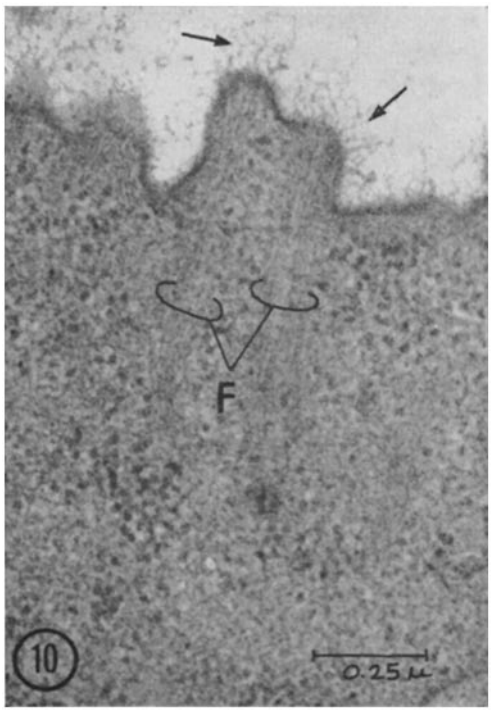


FIGURE 10 Early development of microvilli (stage 63) on free surface of intestinal absorptive cell. Two bundles of fine filaments (*F*), each approximately equal in diameter to the filamentous cores of mature microvilli, are attached to the plasma membrane and extend into ribosome-rich cytoplasm. An outer surface coat (arrows) is present. The image suggests that two incompletely separated, developing microvilli have been cut in profile. Note that differentiating microvilli may be longer on one side than on another. × 60,000.

they are first detectable, are limited by a plasma membrane that is thicker and denser than the membrane at the lateral and basal cell surfaces and that is complete with a well-developed surface coat.

The bundles of filaments deserve special mention for several reasons. First of all, we have not detected a marked difference between the diameters of these bundles and those constituting the cores of fully differentiated microvilli (40–60 mμ). Furthermore, the bundles are of uniform thickness and apparently show no tendency to fuse into larger aggregates. At this point they are embedded in the ribosome-rich ground cytoplasm, which, in the absence of a terminal web, extends as far as or at least very near to the plasma membrane (Fig. 10).

In other cells of the epithelium at stage 63, differentiation is further advanced. Short microvilli, measuring 70–100 mμ in width and between 100 and 500 mμ in length, are present (Figs. 11 and 12). The plasma membrane limiting them, as well as that between the young microvilli, is of course covered by a surface coat. As is the case with the fully differentiated cell (Fig. 14), this coat is more extensive on the tips of microvilli than on their lateral borders. Filament bundles make up the cores of the microvilli, as they do in the fully formed brush border; but in certain respects the core is different from that of the mature cell, in addition to being of different length. For example, a microvillus is frequently longer on one side than on the other (Fig. 12). As a result, the filamentous core passes through a widened, pyramidal base, but it maintains a constant width and does not take the shape of such a base. Furthermore, at this stage, the core does not usually extend into the underlying cytoplasm at a 90° angle as it does in the mature brush border. It may enter at an oblique angle (Figs. 11 and 12) and curve as it descends into the cytoplasm.

As already noted above, the terminal web is incomplete before the microvilli attain their full length. Thus there is no separate cortical region at the free surface of the cell. The filament bundles extend into the presumptive terminal web area, but they are not as rigidly aligned with respect to the cell surface as they are in the adult tissue. In addition, ribosomes and the small vesicles extensively described above are found just beneath the intervillous regions of the plasma membrane (Fig. 11). Indeed one gains the impression that the vesicles may be channeled into the cytoplasm between the microvillous cores so that a file of vesicles may form a kind of strand which extends down from the cell surface. Thus it appears that the vesicles move or are moved into cytoplasmic channels between filamentous cores of the growing microvilli, beneath intervillous regions of the cell surface. The immature terminal web, therefore, continues to be characterized by the presence of ribosomes and acid mucopolysaccharide-rich vesicles which lie between groups of filament bundles.

As the microvilli increase in length and number, they tend to form a 90° angle with the cell surface, and their filamentous cores are embedded in a partially formed terminal web (Fig. 13). Channels of cytoplasm, containing ribosomes and small vesicles, still occur in the developing web region.

Larger organelles, such as mitochondria, are excluded from the presumptive web region as they are from the beginning of differentiation.

At the completion of metamorphosis, microvilli of the *Xenopus* intestinal absorptive cells (Fig. 14) have widths (70–90 mμ) similar to those of short ones seen at stage 63 and illustrated in Figs. 11–13. Their length, however, is increased to 1.2–1.4 μ. Although the surface coat is present over their entire surface, it is markedly thicker at their tips (Fig. 14). Thus the variation in its thickness observed at immature stages is maintained.

Another feature of microvilli is the apparent presence of a dense region of material just beneath the plasma membrane which limits their tips (Fig. 14). This increase in density of the matrix surrounding the filaments of the core is more clearly seen when tangential sections through the tips are compared with those through more proximal areas (Fig. 15). This is the region where the filamentous cores of the microvilli seem to be attached or are in close association with the inner surface of the plasma membrane. Thus far, in our examination of younger microvilli, we have not been able to demonstrate clearly a similar region of density at their tips.

The cores extend into the terminal web region, which, at the end of differentiation, forms a cortex consisting of fine filaments embedded in an amorphous matrix. A more densely staining layer within the web lies just beneath the plasma membrane at the base of the microvilli (Figs. 13 and 14). Penetration of the web region by ribosomes and small vesicles, as seen during differentiation, occurs very rarely indeed.

This study did not include animals that had begun to feed carnivorously, and we therefore do not know whether further structural modification of the brush border occurs in response to feeding.

DISCUSSION

The hypothesis expressed at the outset of this paper, namely that a specific type of small vesicle plays a central role in the formation of the brush border, has received support from the observations presented. In defining these vesicles and their proposed function, we have described in some detail the morphological events detectable as the complex macromolecular architecture of the cell surface is being assembled. From a conceptual point of view, we suggest that these observations are of interest because they substantiate the idea that specializations of the cell surface arise not through a process of self-assembly of molecules at the surface itself but as a result of an orderly series of events in the cytoplasm, mediated by organelles present therein. More particularly, we hypothesize that the vesicles represent preformed units of the plasma membrane and have all the characteristics of the fully differentiated surface, that they are assembled in the Golgi region, and that they then find their way to and become incorporated into the free cell surface. These "surface-forming" vesicles would owe their specific properties to the enzyme-producing machinery of the cell, the ribosomes. This interpretation further implies ultimate control of cell surface properties by information present in the genome.

At present, we cannot assert that the process as described for *Xenopus* absorptive cells is a general one for other species or for other tissues. However, from our observations of brush border formation in the colon (Weinstock and Bonneville, 1969, unpublished observations), we are encouraged to continue to use this interpretation as a working hypothesis in order to explain the formation of specialized cell surfaces and as a basis for further experimentation.

The Development of the Microvilli

We have so far distinguished four steps in the formation of the brush border and have summarized them in a schematic drawing (Fig. 16). Initially (*A*, Fig. 16) there is an accumulation of small vesicles that are, in effect, precursor units of the cell surface (Figs. 3–6). The concentration of these "surface-forming" vesicles in the supranuclear cytoplasm, and especially just beneath the apical plasma membrane, may be considerable just prior to the second step. Then (*B*, Fig. 16) there is an expansion of the cell surface that is destined to possess microvilli (Figs. 8–10). In such cases fewer of the vesicles are present in the apical cytoplasm. Soon, or perhaps simultaneously, bundles of fine filaments, interpreted as the presumptive cores of the microvilli, are seen attached to the expanded surface. The long axis of the bundles is not strictly perpendicular to the free surface and usually follows a curved path as it descends into the underlying cytoplasm (Figs. 11 and 12). At a later phase (*C*, Fig. 16), the microvilli are molded by the addition of units of surface adjacent to areas where the cores are attached to the free surface (Figs. 11 and 12). At the same

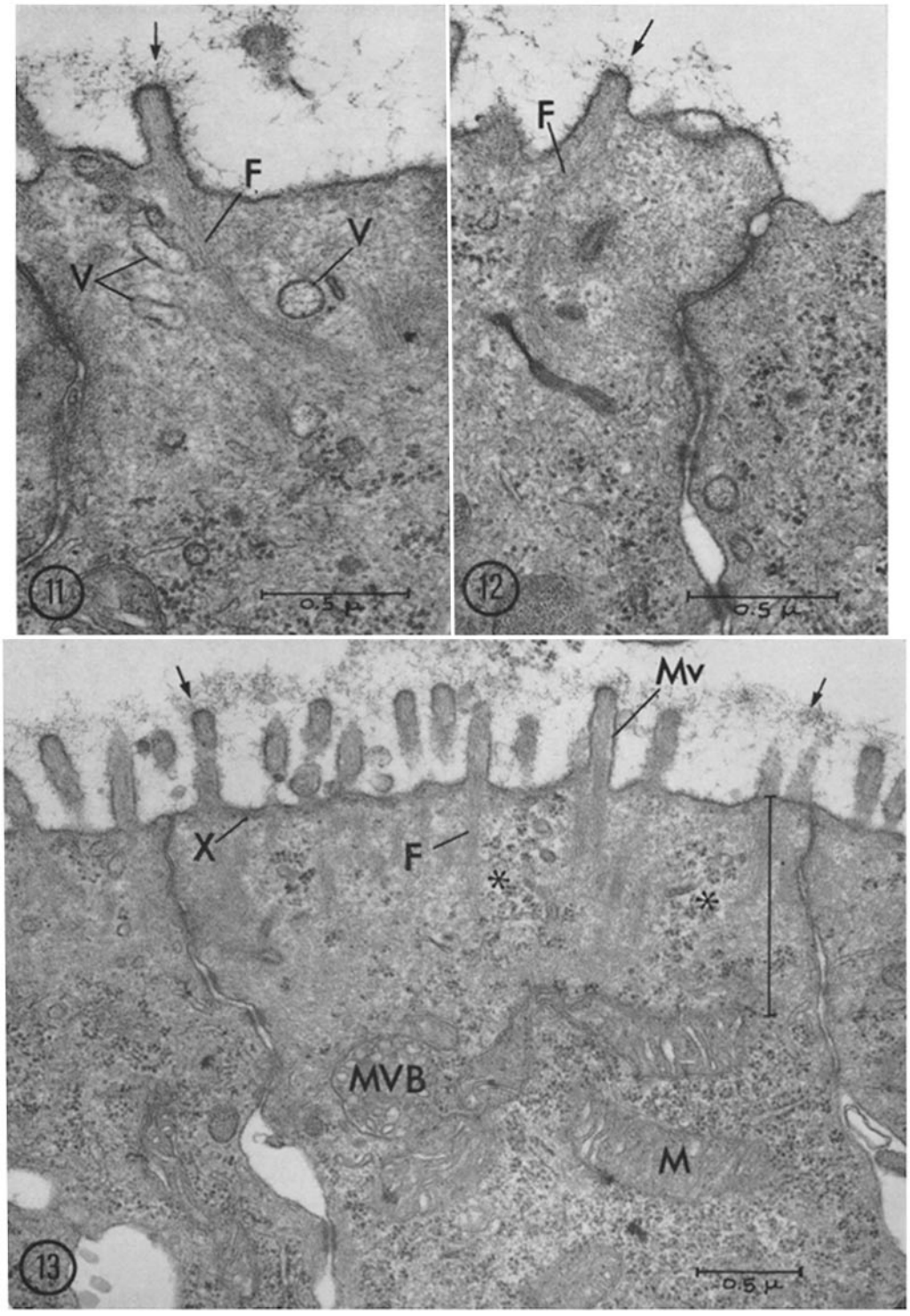
time the cores tend to become highly regular in their orientation, and their long axes assume a disposition perpendicular to the cell surface (Fig. 13). In the terminal web area, channels of ribosome-rich cytoplasm remain for a time (*D*, Fig. 16). Through these channels, vesicles are presumably capable of moving toward the surface until almost the end of the development period. When the microvilli have attained their full height, however, the channels are no longer present, and extremely few vesicles are seen within the terminal web (Fig. 14).

This interpretive discription differs in several respects from that of Overton and Shoup (1964) and of Overton (1965). These authors suggest a morphogenetic role for the core in both chick and mouse tissues. They found that the cores were observed first in short, developing microvilli, and that the extensions of the cores into the terminal web region became organized later. The first phase of organization therefore seemed to proceed from the surface inwards. They further reported that, in a second morphogenetic phase (after hatching in the chick and after a diet change in the mouse), microvilli increase in length while their volumes remain constant and their diameters markedly decrease. This evidence, together with the appearance of the isolated core as a double stranded, twisted rod of filaments (Overton et al., 1965), led to the speculation that this second phase of elongation might be due to a change in the conformation of molecular structure of the filaments.

In addition to the most obvious difference between our work and previous studies, i.e., our assignment of an important role in cell surface formation to small vesicles, we have not been able so far to confirm that microvilli undergo a period of rapid growth in length during which their diameters decrease considerably (Granger and Baker, 1950; Brown, 1962; Overton and Shoup, 1964; Overton, 1965). In *Xenopus*, then, there seems to be no marked difference between diameters of the microvilli soon after they first appear (70–100 mμ) at approximately stage 62 and after they are completely differentiated (70–90 mμ) at stage 65. Although stage 65 seems to be an adult form, we must point out that we have not examined the intestine to determine whether morphological responses occur once carnivorous feeding begins, and for this reason we may have failed to see the final differentiative changes. In any event, we imagine that in this tissue the cores with their complement of filaments must play a role in the morphogenesis of the microvilli. As development progresses, the filament bundles first grow longer and then become straighter until finally their distal ends are incorporated into the terminal web. These observations suggest that they, together with the amorphous matrix in which they are embedded, become more rigid, and in this way they play an essential part in establishing the regularity of the microvillous array and the properties of the terminal web that prevent

FIGURES 11 and 12 Progressive differentiation of microvilli at stage 63. Surface coat (arrows) is particularly prominent at tips of microvilli. The microvilli frequently deviate from 90° in the angle at which they extend from the cell surface. The filamentous cores (*F*) may curve as they descend into the developing terminal web. These last two features distinguish the differentiating from the fully developed brush border. Small vesicles (*V*) may be aligned in a manner that suggests their imminent fusion to form channels and moats around the microvillous cores. Such a process is envisioned as providing surface membrane for the lengthening microvilli. Note that the opposite sides of the microvilli may be of unequal length, and that at their bases they may be wider than the filament bundles. Fig. 11, × 42,000; Fig. 12, × 44,000.

FIGURE 13 Further differentiation of the brush border (beyond that shown in Figs. 11 and 12) may be seen at stage 63. Microvilli (*Mv*) have become longer and more numerous. Their filamentous cores (*F*) extend into the developing terminal web. A layer (*X*) of increased density, present beneath the intervillous portions of the plasma membrane, is characteristic of the terminal web in cells of this and subsequent stages of development. The latter includes channels of cytoplasm (*) which contain ribosomes and small vesicles. Mitochondria (*M*) and multivesicular bodies (*MVB*) are excluded from the terminal web, the extent of which is indicated here by the vertical line. Arrows indicate the surface coat. × 30,000.


F
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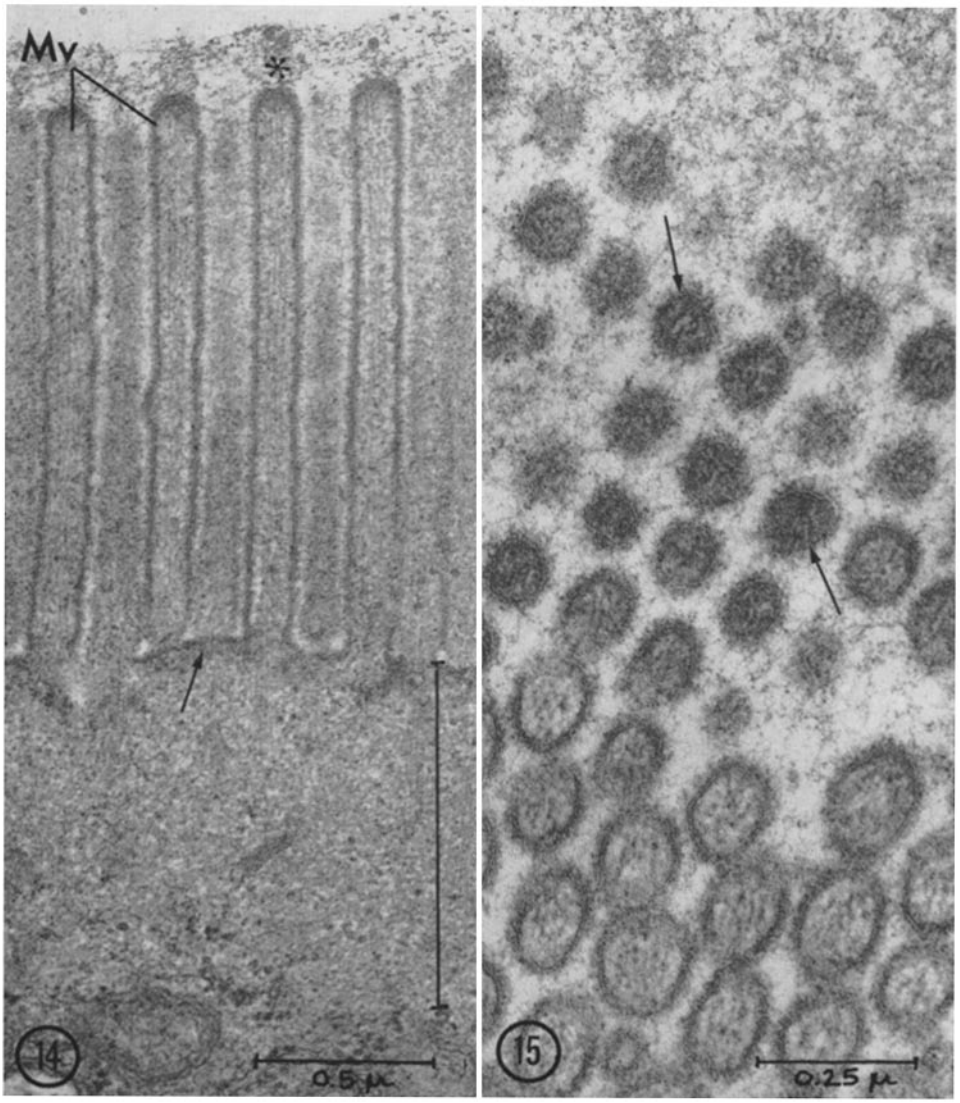

FIGURE 14 Brush border of an intestinal epithelial cell at the end of metamorphosis (stage 65). The microvilli (*Mv*) have attained their full height, and they display their remarkable regularity of shape and frequency. Their plasma membrane possesses a surface coat, most easily seen at the tips (*). Bundles of filaments extend from a region of increased density at the tips of the microvilli into the terminal web. This region, the extent of which is indicated by the vertical line, rarely contains any of the "surface-forming" vesicles. A zone of moderately dense material is present within the terminal web beneath the plasma membrane at the base of the microvilli (arrow). × 55,000.

FIGURE 15 Oblique section through microvilli at the end of metamorphosis (stage 65). Profiles near the top of the field (arrows) show the tapered tips of the microvilli where the filaments of the cores are embedded in a moderately dense matrix. These images contrast with profiles through the more proximal regions of microvilli, seen in the lower part of the field, in which the matrix surrounding the filaments is of lower density. × 79,500.

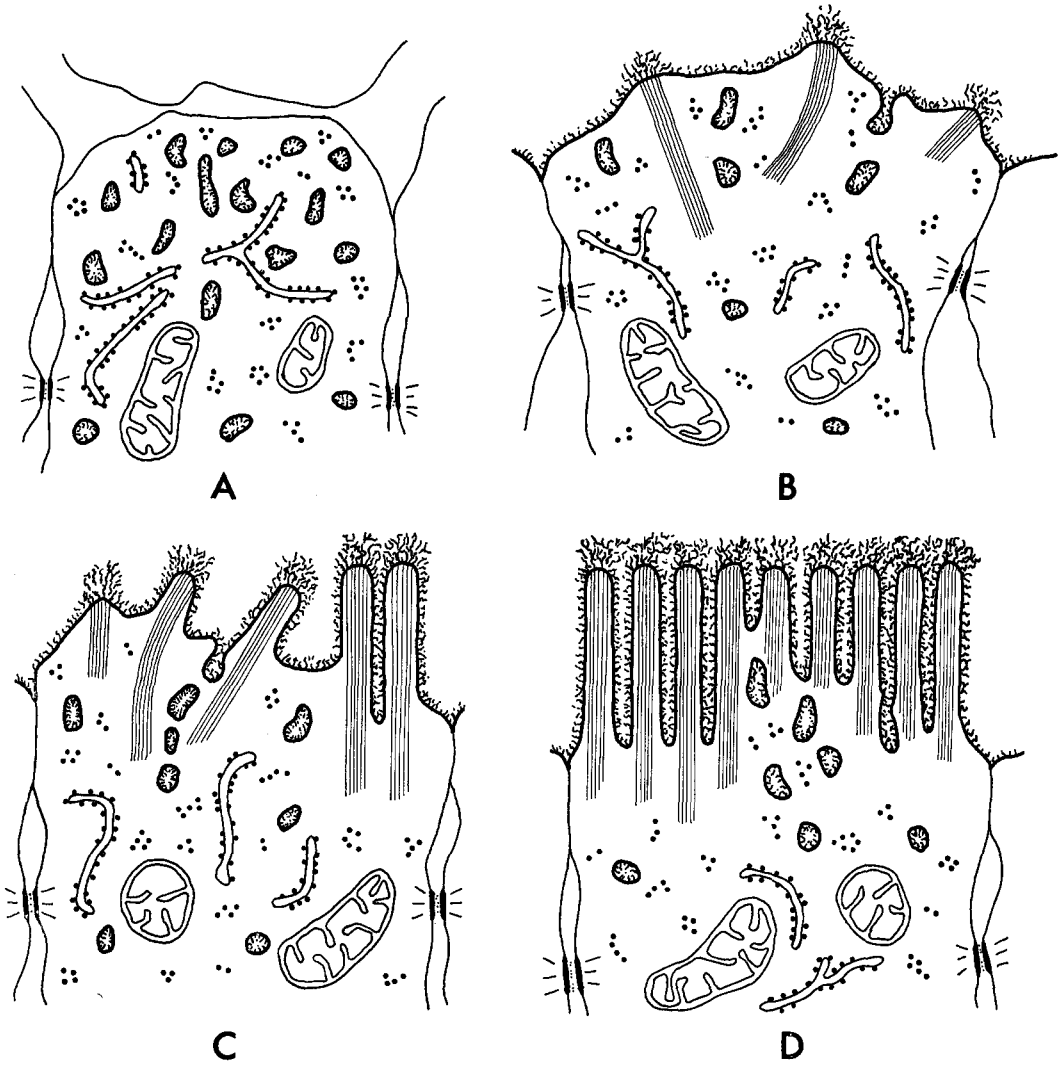

FIGURE 16 Schematic interpretation of brush border formation as observed in intestinal absorptive cells of *Xenopus* during metamorphosis. *A*. Numerous vesicles appear in supranuclear cytoplasm of undifferentiated epithelial cell (stage 61, Nieuwkoop and Faber, 1956). Vesicles are often seen to aggregate just beneath the surface where microvilli are destined to form. At this stage, the developing epithelium is overlain with a layer of degenerating larval epithelial cells. *B*. Expansion of the apical cell surface (stage 62) results in a reduction of the number of "surface-forming" vesicles and the presence of a filamentous surface coat on the apical free border of the cell. This stage of development is believed to occur through the fusion of the vesicles with the preexisting apical plasma membrane. Bundles of fine filaments extend from the membrane into the cell cytoplasm; these bundles, which may be curved or straight, are destined to become the cores of the microvilli. *C*. Microvilli elongate (stage 63) by a process in which strands of vesicles fuse with each other and with the plasma membrane to form "moats" around the filamentous cores. Note that from the time of their first appearance, microvilli possess a plasma membrane that is thicker than the unit membrane at the lateral and basal borders of the cell. *D*. Near the end of metamorphosis most of the microvilli have attained their mature height and the terminal web is partially formed. In certain regions, however, channels of cytoplasm, including vesicles and ribosomes, still extend to the plasma membrane between microvilli. These channels are thought to allow for continued addition of membrane to the surface until the end of metamorphosis.

penetration of the cell cortex by most cytoplasmic organelles.

In regard to the origin of the filaments, the proposal that they become organized from the surface inwards seems to be a plausible one, although we could not discover any additional pertinent information on this point. The tips of microvilli possess a surface coat that is more extensive than that on their lateral surfaces. Furthermore, as other authors have noted (McNabb and Sandborn, 1964), we also observed an aggregation of dense material at the area where the filaments attach to the inner surface of the tip. Thus, some special properties seem to exist at the tips of microvilli. Perhaps these areas serve as initiating or anchoring sites for the filaments.

"Surface-Forming" Vesicles

This interpretation of brush border formation suggests a new role for small vesicles, which we wish to discuss in some detail. In our view these vesicles are not instruments of cell secretion but, as stated above, are preformed units of the cell surface.

Other workers (Wetzel et al., 1966; Berlin, 1967; Rambourg et al., 1969) have all suggested that vesicles, probably homologous to the ones under discussion here, participate in the formation of the surface coat, and this study agrees with their findings. We would add, however, that the function of the vesicles is not simply to carry coat material to the surface but, as Röhlich (1962) also has pointed out, to add area to that surface for expansion during development. We considered that the surface coat is an integral part of the vesicle wall, just as it is thought to be of the cell surface (Ito, 1965; Revel and Ito, 1967), and that its integration into membranes occurs when the vesicles arise within the cytoplasm.

The evidence on which this hypothesis is now based is admittedly indirect. Chief arguments in its favor are (*a*) The vesicles appear in considerable numbers before and during the formation of the brush border; they are exceedingly rare once the border is completely formed. (*b*) They aggregate just beneath the plasma membrane at the surface where microvilli are destined to form. (*c*) The membrane which limits the vesicles resembles morphologically the plasma membrane which limits the fully differentiated microvilli. (*d*) This similarity extends to the possession of a polysaccharide-rich coat that adheres to the outer surface of the plasma membrane and to the inner surface of the vesicles. (*e*) When the vesicles first appear, the presumptive apical free surface of the cell is limited by a membrane that is thinner than that limiting the vesicle, and furthermore, it lacks a well-developed surface coat. This plasma membrane assumes both its mature form and its similarity to the membrane which limits the vesicles subsequent to the period when the vesicles are present in greatest number. (*f*) Once the microvilli have begun to develop, the vesicles are seen beneath the plasma membrane in regions between the microvilli. (*g*) In certain images, the vesicles seem to be aligned in such a way that fusion with each other and with the surface would result in a lengthening of the lateral surfaces of the microvilli. (*h*) In the metamorphosing frog, the vesicles appear when the animal does not eat. At the same time, we see no evidence of pit formation, such as that taking place during fat absorption (Cardell et al., 1967). Therefore the likelihood of the vesicles being derived from the pits and associated with absorptive processes is reduced.

An important objection to this interpretation is the lack of direct evidence that the small vesicles are moving toward, and are not in fact derived from, the apical cell surface. Although both the surface of the apical plasma membrane which arises during development and the contents of the vesicles can be stained by colloidal thorium, this does not mean, of course, that the stained areas contain identical acid mucopolysaccharides, nor does it reveal the direction of movement. In addition, we have not been able to demonstrate that the vesicles are unrelated to multivesicular bodies (MVB's), which share several properties with the vesicles. Evidence has accumulated that MVB's function as digestive vacuoles in which exogenous tracers may become localized as a result of their transfer from the surface by coated vesicles (Rosenbluth and Wissig, 1964; Friend and Farquhar, 1967). It therefore seems unlikely that MVB's contribute to cell surface formation. To distinguish the "surface-forming" vesicles from MVB's and also from any vesicles derived from the cell surface, it is essential to present marker substances to living cells on which microvilli are developing. We are now undertaking such experiments.

A second difficulty arises when one considers radioautographic studies of surface coat renewal (Ito and Revel, 1966; Revel and Ito, 1967). Observations on cat intestine indicate a constant

renewal of surface coat in cells with a fully differentiated brush border. As yet, vesicles have not been identified as participants in this process. For the moment, then, we must regard our scheme as applying only to differentiating absorptive cells and point out that renewal of components of the surface in differentiated cells may occur without morphological manifestations.

Another puzzling problem is the deficiency of the scheme presented in accounting for the increased thickness of the coat at the tips of the microvilli as opposed to the thickness at their lateral surfaces. Seemingly, the membrane at the tips of the microvilli, having special properties, would have to originate from a special population of "surface-forming" vesicles. Thus far such a population has not been identified.

Origin of "Surface-Forming" Vesicles

Where vesicles have been shown to be involved in the formation of the surface coat (Wetzel et al., 1966; Berlin, 1967; Rambourg et al., 1969), the investigators agree that the vesicles arise at the Golgi region, where they are detected at the mature face of the stacks of Golgi cisternae or seemingly as dilated saccules budding off from the cisternae at this face. Radioautographic methods (Neutra and Leblond, 1966; Ito and Revel, 1966; Revel and Ito, 1967) also indicate that surface coat material is assembled in the Golgi region.

Observations of *Xenopus* absorptive cells demonstrate that the small, "surface-forming" vesicles, when present, are distributed throughout the supranuclear cytoplasm and may be seen near the Golgi region. Wherever they occur, the vesicles may be stained by colloidal thorium, as may the inner, presumably more mature, cisternae of the Golgi complex. At present, we are studying in more detail the relationship between the Golgi complex and the "surface-forming" vesicles and are extending these observations to include differentiating colonic absorptive cells in the mouse. Thus far, our observations are consistent with the hypothesis that such vesicles probably arise from dilations of Golgi cisternae at the mature face of this organelle (Weinstock and Bonneville, 1969, unpublished observations). Thus, we are focusing attention on the Golgi complex as a source not only of membrane surrounding secretion droplets but as the site of origin of specialized areas of the plasma membrane.

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Dr. Weinstock is recipient of a Dental Research Fellowship, National Research Council of Canada. His present address is the Department of Anatomy, McGill University, Montreal.

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