FINE STRUCTURE OF CELL SURFACE SPECIALIZATIONS IN THE MATURING DUODENAL MUCOSA OF THE CHICK

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ABSTRACT

Cell surfaces in the duodenal mucosa have been studied in maturing tissue of the chick from incubation until hatching. Changes in the distribution of mitoses in this tissue give an indication of its rate of maturation. This rate is paralleled in developmental changes in microvilli and junctional complexes. Concentration of mitotic figures towards the base of villous folds is gradual from day 9 to day 16, then rapid to day 19, after which the mature pattern is acquired. By day 11, microvilli appear in a regular pattern which does not alter through hatching. Their height remains the same to day 16 when it increases gradually to day 19, then very sharply. The angle formed between the microvilli and the cell surface increases gradually to day 16, giving evidence of advancing internal structure. Changes in cell adhesion also occur at day 16. Thereafter, following trypsin treatment cells are held together in patches by the tight junctions of the terminal bar, although the desmosomes are separated. The timing of these morphological changes is compared with that of alkaline phosphatase accumulation in this tissue as reported by Moog (13). Increase in the surface area of the microvilli parallels the increase in the activity of the enzyme.

INTRODUCTION

As a tissue matures, conditions at the surface of a cell or at patches within the same surface can become increasingly different (28). This may be reflected in a variety of surface specializations (6, 18, 27). In cells of the mature intestinal mucosa, microvilli are regularly spaced over the free cell surface, and in any one region are uniform in height. A core of longitudinally oriented material extends from each microvillus as a rootlet into a fibrous surface layer of cytoplasm associated with desmosomes and tight junctional complexes between cells (7, 1, 4, 11, 20, 24, 5). In the present study, the development of this rather elaborate and precise surface structure has been examined in the duodenal mucosa of the chick and related to the state of maturation of the tissue as evidenced by the mitotic pattern. Mitotic figures become concentrated towards the base of villous folds or villi, gradually at first, then more rapidly. These same periods are apparent in the development of microvilli. With respect to their spacing, height, width, angle formed with the cell surface, and changes in shape, microvilli have been compared from the 7th day of incubation through hatching. Morphological detail of this kind is of particular interest in the light of the extensive work of Moog on biochemical changes in the developing duodenum in which alkaline phosphatase, esterase, and mucopolysaccharides were shown to be associated with the developing brush border (13–16).

MATERIALS AND METHODS

White leghorn chicks were used in all observations. Eggs were incubated at 38°C. Chicks maintained after hatching were not fed.

The change in spatial distribution of mitoses was assessed by counting mitotic figures in 10μ sections of duodenum stained with iron hematoxylin. Mitoses were counted in every other section for a minimum of 10 sections. They were scored as near to either the tip or the base of the villus or villous fold. The data were expressed as a percentage of mitoses occurring nearer to the base than to the tip. Fifty per cent of the mitoses scored as nearer to the base would represent a roughly uniform distribution of dividing cells if the number of cells in the two regions were approximately the same. In sample counts of mucosal cells covering 10 villi or villous folds on days 12, 16, and 20, it was found that 52 per cent, 51 per cent and 54 per cent, respectively, of the cells were nearer to the base.

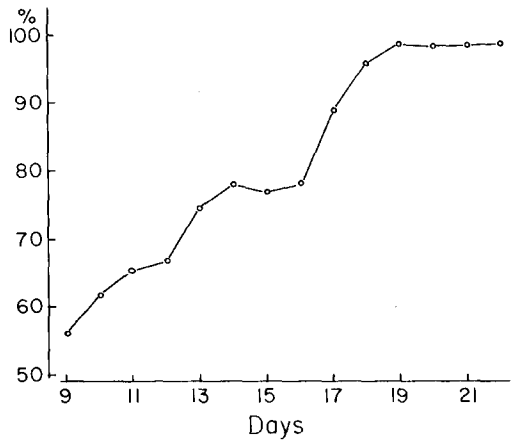


FIGURE 1 Distribution of mitotic figures in the developing intestinal mucosa. Abscissa, days from start of incubation. Ordinate, percentage of mitoses nearer to the base than to the tip of villous folds or villi (see text).

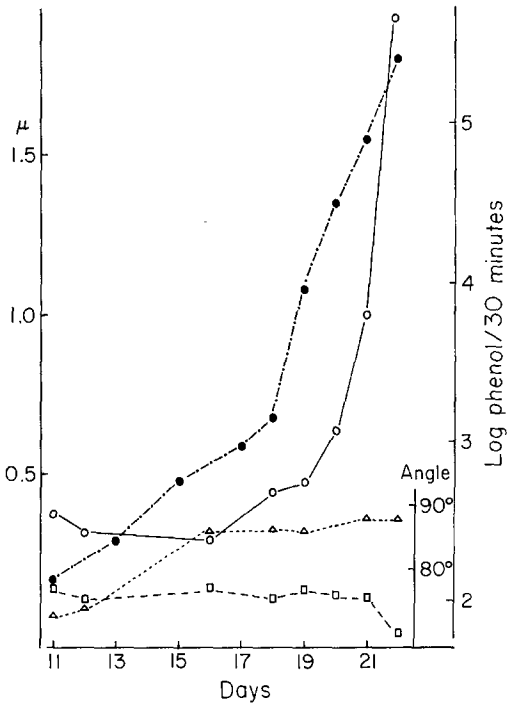


FIGURE 2 Changes in microvillus diameter (squares, dotted line), height (open circles, solid line), and angle (triangles, short dash), compared with data adapted from Moog (13) for alkaline phosphatase content of whole tissue, substrate phenylphosphate (solid circles, dot and dash). See text.

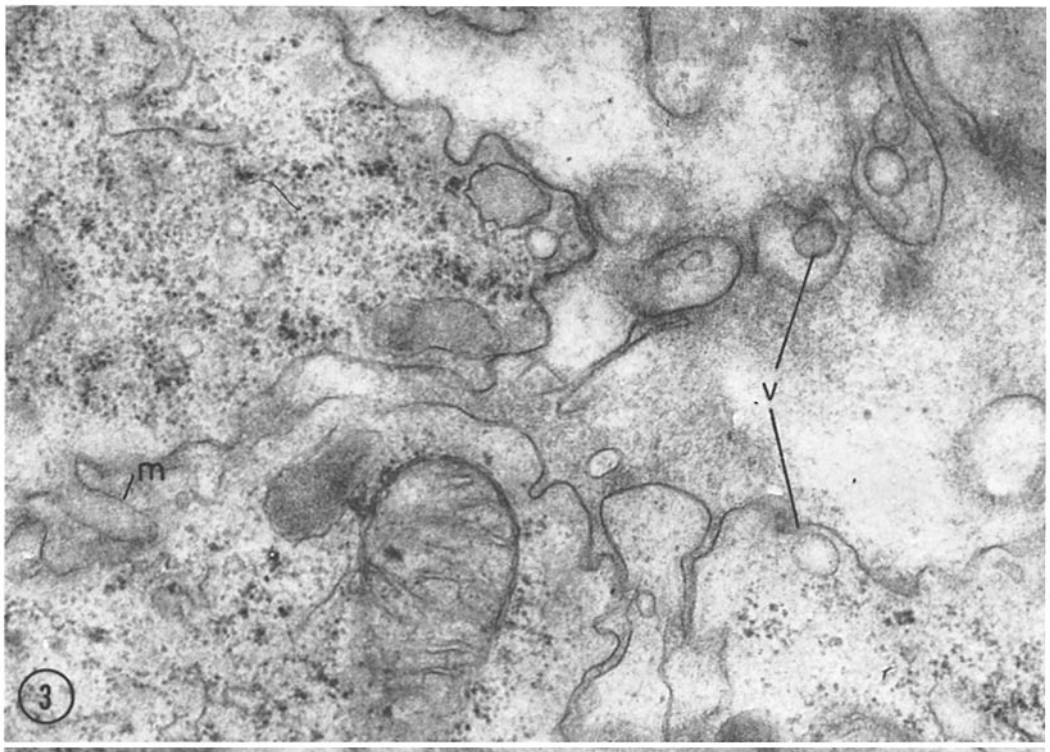
For electron microscopy, tissue was fixed in 1 per cent osmium tetroxide in Veronal-acetate buffer (pH 7.9) for ½ hour at 4°C, carried through a graded series of alcohols, brought to room temperature, and embedded in Epon (10). Sections were cut on a Porter-Blum microtome and stained in 3 per cent uranyl acetate (26) for 24 hours. Preparations were examined with an RCA EMU-3.

Tissue was dissociated by incubating 1 to 2 mm fragments in Ca- and Mg-free Tyrode solution for three 10 minute changes at 37°C, followed by ½ hour incubation in 0.1 per cent trypsin (Nutritional Biochemicals Corporation, Cleveland). The tissue was then transferred to Tyrode solution containing 5 per cent horse serum and dissociated by pipetting 15 times. The suspension was centrifuged and the pellet fixed for electron microscopy. Dissociation was carried out using either the whole duodenal wall or only the mucosa which had been stripped off.

Measurements of the height of microvilli were made

FIGURE 3 Free surface of cell of duodenal mucosa at 7 days. Irregular projections are occasionally microvillus like (*m*), and rounded extensions of the cell surface may contain vacuoles (*v*). × 40,000.

FIGURE 4 Free surface of cell of duodenal mucosa at 9 days. Microvillus like projections (*m*) are much more frequent though still rather irregular in shape. × 40,000.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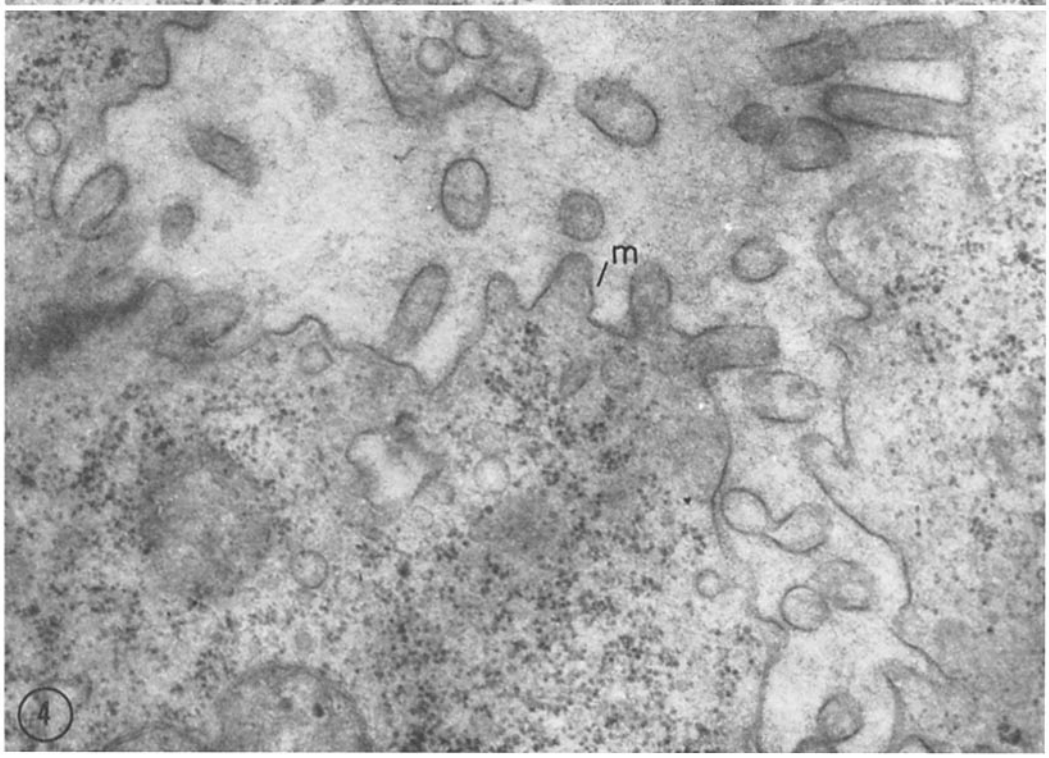
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on microvilli in which the cell membrane could be seen, in cross-section, around the tip and in which the core was clearly connected with the subsurface region of the cell. Measurements of the width of microvilli were made on the same cases, or on sections cut tangential to the cell surface. The angle made between the microvilli and the cell surface could be measured when microvilli were straight. When they were curved, this angle was taken as the angle made between the distal third of the microvillus and the cell surface. The number of microvilli per unit length of cell surface was estimated by counting the number, cut in longitudinal section, in which the core was fully connected with the cell subsurface over a 3.1μ length of surface in a print magnified 32,000 or the number lying squarely across a line of equal length drawn across a field of microvilli cut in cross-section. All averages of measurements were based on a minimum of 25 cases unless otherwise stated.

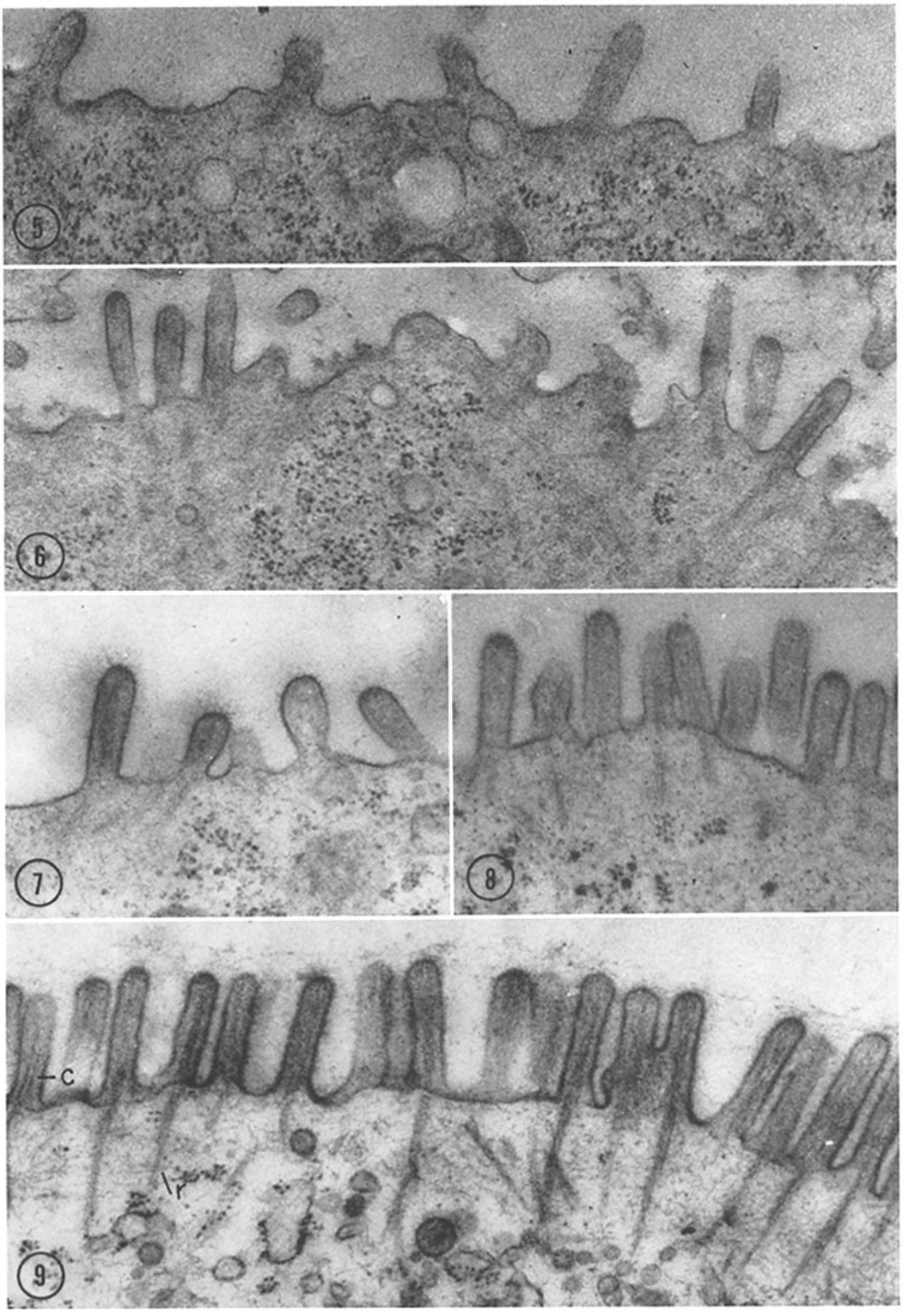
RESULTS

MITOTIC PATTERN: Results of mitotic counts are presented in Fig. 1. Each symbol represents an average of three cases, except on days 10 and 18 through 21 when only two cases were averaged. It is clear that the acquisition of the mature pattern is at first very gradual and is initiated at about the 9th day, shortly after the villous folds begin to originate (3). Before this time mitoses are apparently rather evenly distributed throughout the mucosa. Although there is an increasing tendency for mitoses to be concentrated basally, occasional mitotic figures can still be seen at the extreme villous tip as late as day 15, as noted by Moog (14). After day 16 there is a sharp rise in the curve, and by day 19 virtually no mitoses are present in the distal half of the villus. The change towards the mature generative pattern is thus first gradual, then rapid, and precedes the final chemical and morphological differentiation of the tissue as described by Moog.

MICROVILLI: At 5 days there are no indications of microvilli. The free surface of the cell is irregularly lobate and tends to bulge out into the lumen of the gut so that it may extend as much as 4 μ beyond the junctional complexes between adjacent cells. The cytoplasm toward the distal surface of the cell characteristically contains numerous small vacuoles. Major changes after this time are summarized in Fig. 2 and illustrated in Figs. 3 through 13. By 7 days, lobate projections may bear extensions which in some cases are elongate and of approximately the diameter of microvilli but are as frequently much more irregular in shape (Fig. 3). The cytoplasm within these extensions may contain small vacuoles. By day 9, projections resembling microvilli are more numerous, though still somewhat irregular. Cell surfaces tend to be flatter and junctional complexes usually lie in their characteristic position just below the surface (Fig. 4). By day 11, microvillous projections are much more uniform, and are about 0.45 μ in length and 0.12 μ in diameter. They may lie at various angles relative to the cell surface and are generally straight rather than curved (Fig. 5). At later stages, the angle they form with the cell surface approaches 90° (Fig. 2). Evidence of longitudinal orientation in the core is apparent by 11 days. In these earliest stages the structural part of the core is not localized towards the center as it will be in later stages (Figs. 10 and 12). More evidence of core structure occurs by day 12 when the core is occasionally associated with short rootlets. Rootlets are characteristic by day 16 though not invariably present. The rootlet region becomes progressively regular until day 21 when it forms a layer about half a micron deep in the subsurface of the cell, for the most part uninterrupted by other cytoplasmic entities. By day 22, it is somewhat deeper. The cytoplasm in this region has a fibrous character. Occasional sections suggest a close association between the rootlets and the fibrous components (11) arising from the junctional complexes (Fig. 9), although no actual connections are evident.

Microvilli on any one cell or any group of adjacent cells are rather uniform in length from day 11 on. They show little change through day 16, after which time they increase in length, more and more sharply, to greater than 6 times their

FIGURES 5 to 9 Microvilli on days 11, 12, 16, 18, and 20, respectively. Microvilli become progressively regular with respect to their shape and angle. The rootlets increase in length and the vacuoles and ribosomes are farther from the surface. Rootlets (*r*) are sometimes closely associated with transverse fibers. The microvillous core (*c*) is occasionally double in longitudinal section. All × 40,000.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16-day height by day 22 (Fig. 2). There is no change in diameter of microvilli through day 21, but between days 21 and 22, when increase in height is greatest, the diameter drops by 25 per cent. This reduction in diameter seems to involve the outer, rather homogeneous part of the core of the microvillus. The structural inner part of the core appears to remain about the same in diameter (compare Figs. 12 and 13). A change in shape of the tip of the microvillus also occurs at this time. It is somewhat blunt until day 22 when it becomes more tapered (Fig. 11). The surfaces of microvilli are bounded by double electron-opaque layers separated by a less dense layer (11). In these preparations the double nature of this membrane in cases beyond 16 days is frequently clear, but is rarely observed in earlier stages. Granular fibrous material is associated with the surface of the microvillus, particularly at the tips. This material is present to a slight extent after 12 days, but becomes increasingly conspicuous after 16 days.

The number of microvilli over the cell surface does not seem to change after day 11. On superficial inspection their number seems to increase. However, if only the measurable cases are considered, no significant increase in number occurs. Fifteen measurements on days 11 and 12 gave an average of 8.5 microvilli per unit length (range 6 to 10), while 21 measurements on day 22 gave an average of 9.1 (range 7 to 13). Twenty-one measurements on intervening days averaged 8.8 (range 7 to 12). The characteristically uniform height of microvilli suggests that they develop simultaneously, confirming these results. A comparison of Figs. 12 and 13 indicates that the distance from the center of one microvillus to that of adjacent ones remains the same between the time the microvilli are beginning to increase in height and the time they reach their final differentiated state. Branching of microvilli at the base (9) occurs infrequently.

JUNCTIONAL COMPLEXES: The junctional complexes between cells in the mucosa consist of an area of close contact or tight junction (5), ca. 0.8 micra deep, extending continuously around the distal border of the cells, and just basal to this region a series of desmosomes. The cytoplasm in the region of close contact is distinct in that it appears as a band relatively low in density and devoid of cytoplasmic organelles such as endoplasmic reticulum or mitochondria. The essential features of these junctional complexes are present in the earliest stages studied. On day 5, tight junctions and desmosomal plaques occur. No definite, morphologically distinguishable changes in these structures appear until the final stages examined near and after hatching (Figs. 14, 15, and 16). In the mature mucosa the low density band of cytoplasm is clearly fibrillar. A prominent band of fibrils is also associated with the desmosomal plaque at later stages (18).

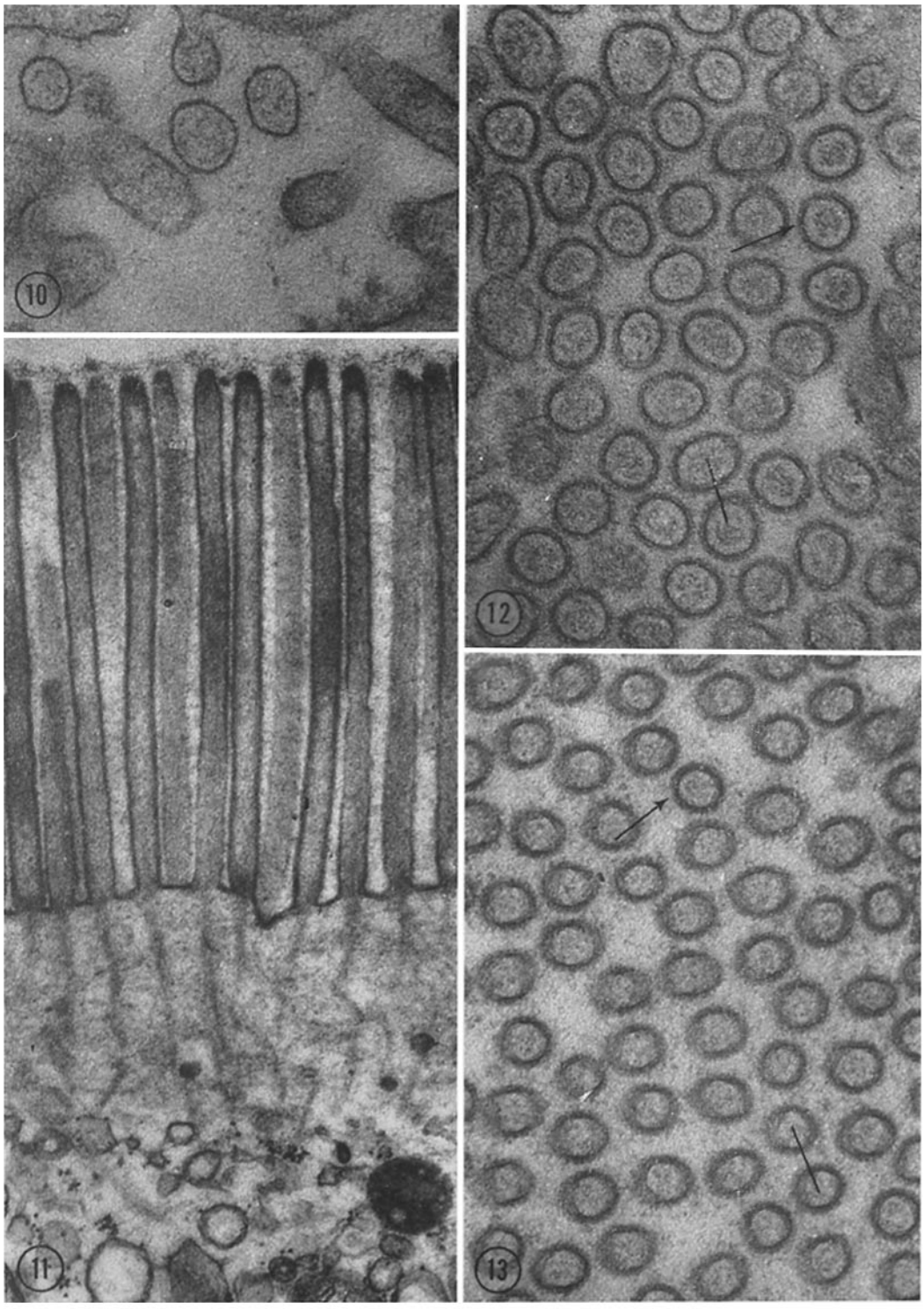
Some indirect evidence for structural changes of junctional complexes was obtained by trypsin dissociation of the tissue. Tissue was dissociated on days 9, 11, 12, 16, and 17. In the three earliest stages, dissociation to a single cell suspension was relatively complete. Separated desmosomal plaques persist in isolated cells (18), but no convincing evidence of persistence of microvilli was

FIGURE 10 Cross-section through microvilli at day 12. Longitudinally oriented fibers are not concentrated towards the center of the microvilli. The cell membrane in this preparation does not show the unit membrane structure. Compare with Fig. 12. × 64,000.

FIGURE 11 Microvilli at 22 days. The rootlets, as well as the microvilli proper, are considerably longer than at 20 days (Fig. 9). The microvillous tip is now tapered and the densely staining granular material associated with the surface is more prominent. × 40,000.

FIGURE 12 Cross-section of microvilli at 18 days. In the case indicated by the arrow, the the unit membrane structure at the surface and the concentration of oriented fibrils towards the center are particularly clear. The distance between the centers of adjacent microvilli (indicted by a bar) is roughly the same as at day 22 (Fig. 13). × 64,000.

FIGURE 13 Cross-section of microvilli at 22 days. The diameter of the core at this stage is the same as the diameter of the structured core at 18 days. Relationships are clearest in the case indicated by the arrow. The bar indicates the distance between the centers of adjacent microvilli which is roughly the same as at 18 days. × 64,000.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seen in these preparations. The mucosa was still largely dissociable by day 16, but by day 17 it showed a tendency to remain in patches despite the same or prolonged incubation in trypsin. Examination of these patches of tissue by electron microscopy showed that desmosomal connections were consistently disrupted while tight junctions were not (Figs. 17 and 18). These observations indicated that the desmosomes or connecting bodies do not resist dissociation at an early period, but that after day 16 some developmental change in the tight junctions occurs. The present work does not provide evidence as to the nature of this change. However, recent studies indicate that a fusion of the outer electron-opaque layers of the cell membranes of adjacent cells occurs in the area of the tight junction in mature mucosa (5).

DISCUSSION

Time Sequence of Developmental Changes

If the change in spatial distribution of mitoses is taken as a measure of the progress of differentiation, then the process may be seen to consist of three parts: a period of moderate change through day 16, a more rapid change on days 16 through 19, and finally a stage in which mitoses are virtually confined to a generative region. If the microvilli are used as a measure, three corresponding periods are evident. First, a period from day 11 to day 16 when, although there is no increase in height of microvilli, the microvillous core becomes structured, rootlets appear, and the angle formed with the cell surface approaches 90°; second, a period from day 16 to day 19 when moderate increase in height occurs; and finally a period of extremely rapid increase in height and decrease in diameter. Junctional complexes show a corresponding change at 16 days. If the generative pattern and structural changes are now compared to changes in enzymatic activity described by Moog, a further correlation is apparent. Moog's data for alkaline phosphatase of whole tissue are plotted in Fig. 2. Although not confined entirely to the brush border, alkaline phosphatase activity is localized here from day 18 on (13) and occurs at the surface of microvilli (2, 24). The increase in enzyme activity can thus be seen to correspond fairly well with the increase in the surface area of microvilli. It may be noted that a 22 day microvillus (1.9 x 0.09 μ) has approximately the same volume as a 21 day microvillus (1.0 $\times$ 0.12 μ) but there is an obvious increase in surface area. Although a correlation between enzyme accumulation and structure exists the part of the observable structure constituted by the enzyme, if any, is not clear. Since the height of microvilli of the intestinal mucosa can be markedly increased by osmotic shock (11) without change in diameter, an increase in the volume of microvilli can not be equated with protein synthesis. In any event, it is the increase in surface area that seems relevant here.

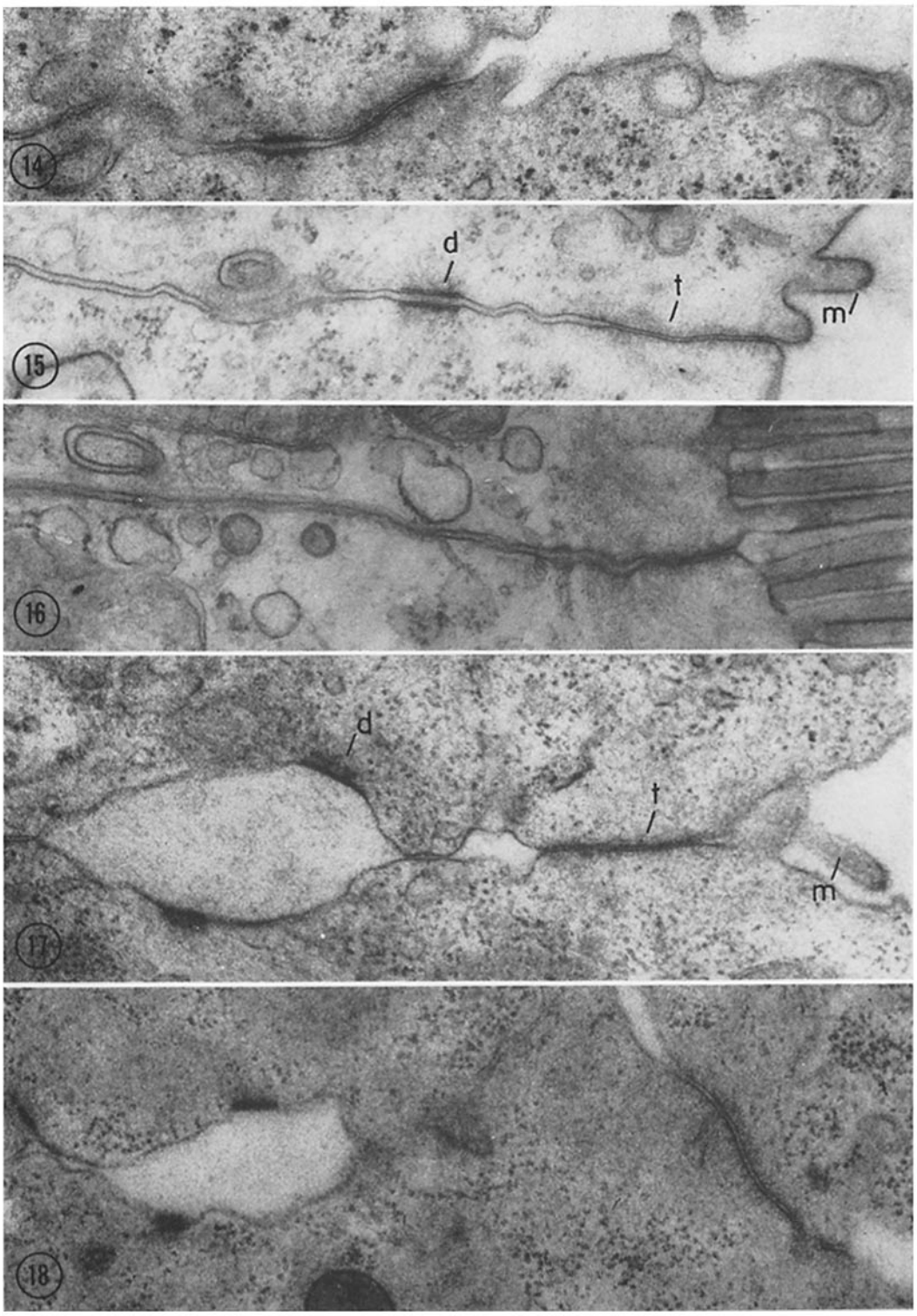
Developmental Changes and Physiological Replacement

In the earliest studies of fine structure of the intestinal brush border, it was noted that the diameter of microvilli varied inversely with the length (7). Height has been found to vary systematically along the villus, being least in the crypts and greatest at the villous crest (1, 4). An increased tapering of the tip in longer microvilli has been noted, in that short microvilli in various tissues have been described as blunt (8, 12, 23). Branching of microvilli, which is common in rodents (9), occurs occasionally in the chick.

Changes in the character of microvilli that occur from the base to the tip of the villus represent a sequence from the generative region of the tissue to the fully functional region; hence this sequence should be similar to the developmental sequence studied here. Brown's observations on human jejunal epithelium (1) are roughly comparable to those made on later stages in the chick, in that they show a doubling of the height of microvilli and a decrease in width by one half. Although a distinction has been made between microvilli and the striated border in mature tissue (22) on the

FIGURES 14, 15, AND 16 Junctional complexes at 12, 16 and 22 days, respectively. This region becomes associated with an extensive fibrillar arrangement. *d*, desmosome; *t*, tight junction. All $\times$ 40,000.

FIGURES 17 AND 18 Mucosa at 17 days partially dissociated by trypsin. *m*, microvillus; *t*, tight junction; *d*, desmosomal plaque. Both $\times$ 40,000.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basis of length and regularity of arrangement of surface projections, it is hard to make a sharp distinction on this basis developmentally. Studies of the changes in other tissues which might be considered comparable to the earlier changes studied here are those in which the structure of microvilli has been examined, after hormone treatment, in uterine surface epithelium (17) and in the gastric parietal cell (23, 25). Cells have been found to possess some projections which have the characteristic shape of microvilli, while other projections are broad or deformed. Small bleb-like protrusions also occur in the early stages of hormone treatment, and finally numerous microvilli of rather uniform height are seen (17). In the chick, such changes also appear to be under hormonal control (14, 15). A reverse effect can sometimes occur. In association with secretion, microvilli may be replaced by blunt, irregular pseudopodia (9) or by cytoplasmic "blebs" (21). Thus the organization of microvilli may be rather labile. This is also suggested by the fact that in isolated cells of chick duodenal mucosa no convincing evidence was found of retention of regular structure of microvilli. However, at the stage when these cells were isolated, the internal organization of microvilli appears to be only partially formed. If cells could be readily separated at later stages, the structure of the free surface might be more stable. It should be noted that as the microvilli take on definite internal structure the process progresses from the core to short rootlets to fully formed rootlets in a highly fibrous subsurface region. That is, structural organization of this part of the cell appears to progress from the surface inwards.

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REFERENCES

1. Brown, A. L., Microvilli of the human jejunal epithelial cell, *J. Cell Biol.*, 1962, **12**, 623.
2. Clark, S., The localization of alkaline phosphatase in tissues of mice, using the electron microscope, *Am. J. Anat.*, 1961, **109**, 57.
3. Coulombre, A. J., and Coulombre, J. L., Intestinal development, I., *J. Embryol. and Exp. Morph.*, 1958, **6**, 403.
4. Dalton, A. J., Electron micrography of epithelial cells of gastrointestinal tract and pancreas, *Am. J. Anat.*, 1951, **89**, 109.
5. Farquhar, M. G., and Palade, G. E., Junctional complexes in various epithelia, *J. Cell Biol.*, 1963, **17**, 375.
6. Fawcett, D. W., Structure specializations of the cell surface, *in* Frontiers in Cytology, (S. L. Palay, editor), New Haven, Yale University Press, 1958, 19.
7. Granger, B., and Baker, R. F., Electron microscope investigation of the striated border of intestinal epithelium, *Anat. Rec.*, 1950, **107**, 423.
8. Helander, H., and Ekholm R., Ultrastructure of epithelial cells in the fundus glands of the mouse gastric mucosa, *J. Ultrastruct. Research*, 1959, **3**, 74.
9. Ito, S., The endoplasmic reticulum of gastric parietal cells, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 333.
10. Luft, J., Improvements in expoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
11. Millington, P. F., and Finean, J. B., Electron microscope studies of the structure of the microvilli on principal epithelial cells of rat jejunum after treatment in hypo- and hypertonic saline, *J. Cell Biol.*, 1962, **14**, 125.
12. Moe, H., The ultrastructure of Brunner's gland of the cat, *J. Ultrastruct. Research*, 1960, **4**, 58.
13. Moog, F., The functional differentiation of the small intestine. I. The accumulation of alkaline phosphomonoesterase in the duodenum of the chick, *J. Exp. Zool.*, 1950, **115**, 109.
14. Moog, F. R., The functional differentiation of the small intestine. IX. The influence of thyroid function on cellular differentiation and accumulation of alkaline phosphatase in the duodenum of the chick embryo, *Gen. and Comp. Endocrinol.*, 1961, **1**, 416.
15. Moog, F., and Richardson, D., The functional differentiation of the small intestine. IV. The influence of adrenocortical hormones on the differentiation of the chick embryo, *J. Exp. Zool.*, 1955, **130**, 29.
16. Moog, F., and Wenger, E. L., The occurrence of a neutral mucopolysaccharide at sites of high alkaline phosphatase activity, *Am. J. Anat.*, 1952, **90**, 339.
17. Nilsson, O., Ultrastructure of mouse uterine surface epithelium under different estrogenic conditions, *J. Ultrastruct. Research*, 1958, **2**, 73.
18. Overton, J., Desmosome development in normal and reassociating cells in the early chick blastoderm, *Develop. Biol.*, 1962, **4**, 532.

19. OVERTON, J., Stolon outgrowth and fine structure of intercellular connections in *Cordylophora*, *J. Cell Biol.*, 1963, **17**, 661.
20. PALAY, S. L., and KARLIN, L. J., An electron microscopic study of the intestinal villus, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 363.
21. PARKS, H. F., Morphological study of the extrusion of secretory materials by the parotid glands of mouse and rat, *J. Ultrastruct. Research*, 1962, **6**, 449.
22. RHODIN, J. A. G., An Atlas of Ultrastructure, Philadelphia, W. B. Saunders Co., 1963, 4.
23. SEDAR, A. W., and FRIEDMAN, M. H. F., Correlation of the fine structure of the gastric parietal cell (dog) with functional activity of the stomach, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 349.
24. SHELDON, H., ZETTERQVIST, H., and BRANDES, D., Histochemical reactions for electron microscopy: acid phosphatase, *Exp. Cell Research*, 1955, **9**, 592.
25. VIAL, J. D., and ORREGO, H., Electron microscope observations on the fine structure of parietal cells, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 367.
26. WATSON, M., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 474.
27. WEISS, P., Cell contact, *Internat. Rev. Cytol.*, 1958, **7**, 391.
28. WILMER, E. N., Tissues in culture and in the body, *Symp. Soc. Exp. Biol.*, 1960, **4**, 28.