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Development of the Structural Components of the Brush Border in Absorptive Cells of the Chick Intestine

Carolyn Chambers* and Robert D. Grey

Department of Zoology, University of California, Davis, California, USA

Summary. The spatial and temporal relationships between cytoplasmic filaments and the morphogenesis of the intestinal brush border were examined by transmission electron microscopy of normally developing tissue and of tissue exposed to a variety of experimental conditions in organ culture. Distinct stages in the development of the brush border were identified: (1) Irregular projections of the apical plasma membrane that contain a network of microfilaments are converted to uniform projections filled with a core bundle of straight microfilaments (7–11d of incubation). (2) Rootlets form by an elongation or aggregation of filaments (11–15d). (3) The terminal web forms first as a network of short filaments just below the apical plasma membrane, then secondarily stratifies into two layers (19d of incubation to 3d posthatching). (4) Core filaments elongate as microvilli achieve their maturity (21d of incubation to 5d posthatching). Microvillus formation was not perturbed by culturing 9d tissue in high concentrations of Ca^{++} or Mg^{++}, either with or without the ionophore, A23187. Rootlet formation was stimulated by high Mg^{++}, with or without A23187, and, for reasons unknown, by ethanol. Terminal web formation was not stimulated by Mg^{++} or Ca^{++}, but the integrity of the terminal web was lost when 21d embryonic tissue was cultured with EGTA or cytochalasin B. After stratification, the terminal web could not be disrupted by EGTA, but instead was aggregated to the center of the apical end of the cell.

Key words: Brush border – Microvilli – Core microfilaments – Terminal web – Intestine.

The brush border of absorptive cells of the vertebrate intestine consists of a remarkably uniform array of microvilli and the subjacent terminal web. The microvilli contain bundles of parallel actin filaments (Ishikawa et al., 1969; Tilney

Send offprint requests to: Dr. Robert D. Grey, Department of Zoology, University of California, Davis, CA 95616, USA

* Present address: Biology Department, Xavier University, Cincinnati, Ohio 45207, USA

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and Mooseker, 1971) which extend into the cell as so-called rootlets (see Fig. 6). The terminal web, which surrounds the rootlets, consists of a meshwork of thinner and shorter filaments combined with intermediate (10 nm diameter) filaments. At the lateral boundary of the cell the terminal web appears to join the plasma membrane at the tight and intermediate junctions. The ultrastructure of the intestinal brush border of the adult has been the subject of several previous studies (Palay and Karlin, 1959; Brunser and Luft, 1970; Mukherjee and Staehelin, 1971; Hull and Staehelin, 1979).

Earlier descriptions of the developing intestinal brush border in the chick (Overton and Shoup, 1964) and other animals (Bonneville and Weinstock, 1970; van der Starre-van der Molen and dePriester, 1972) either did not focus on the filamentous structures, which were poorly preserved, or emphasized the role of vesicle fusion with the apical plasma membrane. As a start toward understanding how a developing intestinal cell may regulate the formation of microfilaments, we have examined the ultrastructural events that occur during the formation of various types of filaments in the brush border of the chick embryo using improved fixation procedures.

An important question to be answered about this system concerns the mechanisms by which the cells regulate the time of production, placement, and organization of the microfilaments that form the brush border. The formation and stability of intracellular filaments in a number of cell types is affected by the concentration of divalent cations, especially calcium and magnesium (Fay and Cooke, 1973; Isenberg and Wohlfarth-Bottermann, 1976; Clarke and Spudich, 1977; Hitchcock, 1977). These ions also affect polymerization properties of purified actin (Oosawa and Kasai, 1972). We have therefore explored the possibility that calcium and magnesium play important regulatory roles in brush-border development.

Materials and Methods

Embryos

White leghorn chicken eggs were obtained from a local hatchery and incubated in a forced draft incubator at 37°C at 60% relative humidity. All stages were counted as days postincubation or posthatching. Embryos were sacrificed by decapitation.

Organ Culture

Embryos were dissected in warm, Millipore-filtered, Minimum Essential Medium (MEM) with Hanks' salts. The proximal half of the duodenum was cut into 1 mm^2 fragments and cultured in 5 ml of MEM containing 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Pacific Biologicals or Grand Island Biological Company). In some cultures, Biostat 100 (Pacific Biologicals) was used; the resulting medium then also contained 0.25 µg/ml Fungizone and 60 µg/ml Tylocine. Tissue fragments were cultured in a 35 mm plastic culture dish (Falcon Plastics) at 37° C. If cultures continued for longer than 30 min, proper pH was maintained (detected by phenol red) with an atmosphere of 5% CO_2 in air.

To increase calcium of magnesium concentration to 5 or 10 mM, the medium was augmented with small volumes of 0.25 M $CaCl_2$ or $MgCl_2$. The divalent cation ionophore, A23187 (Eli Lilly), was dissolved in dimethylsulfoxide (DMSO) and added to some cultures to a final concentration of 5 µg/ml

(final DMSO concentration, 0.25 %). Controls consisted of cultures with no additions or to which 0.25 % DMSO was added.

To chelate calcium in the medium, EGTA (ethylene glycolbis (β-aminoethyl ether) N,N,N',N'-tetraacetate) (Sigma Chemical Company) was added from a 100 mM stock solution, pH 7.3, to reach a final concentration of 5 mM. Some of the cultures with 5 mM EGTA also contained 10 mM $CaCl_2$. In some experiments, EGTA was replaced by EDTA (ethylenediaminetetraacetate) (Sigma Chemical Company). Reversibility was tested by removing tissues from the EGTA-supplemented medium after 20 min, washing them in two changes of MEM, and continuing the culture for one hour in MEM with 10 mM $CaCl_2$.

When used, cytochalasin B (CB), (Imperial Chemical Industries, Ltd. or Calbiochem) was added to cultures from a stock solution of either 1 mg/ml or 5 mg/ml in DMSO to a final concentration of 5 μg/ml. Control cultures with DMSO (0.1 to 0.5 %) were also made.

Electron Microscopy

The following fixation procedures were tested to find the optimal conditions for the tissue and developmental stages to be studied: Karnovsky's fixative (1.3 % formaledehyde, 1.7 % glutaraldehyde); 1–2.5 % glutaraldehyde, 1–2.5 % acrolein; 1–2.5 % glutaraldehyde in 0.1 M cacodylate of phosphate buffer, pH 7.3, with or without 5–10 mM $CaCl_2$ or $MgCl_2$. The best preservation was consistently obtained with the fixation conditions described by Mooseker and Tilney (1975): tissues were fixed in 1 % glutaraldehyde (Electron Microscopy Sciences), 0.1 M phosphate buffer, pH 7.0 for one hour at room temperature and postfixed in 1 % OsO_4 in 0.1 M phosphate buffer at pH 6.0 for 90 min at 0°C. After dehydration in alcohol or acetone and embedding in a mixture of epon and araldite plastic (52.1 % dodecenyl succinic anhydride, 34.7 % Araldite 6005, 1.7 % dibutyl phthalate, 11.5 % Epon 812), thin sections were cut and stained with 2 % alcoholic uranyl acetate and lead citrate and examined with a Hitachi HU-11E electron microscope.

Calcium Localization

In order to visualize locations of calcium binding, duodenal tissues from 11 day embryos were washed in Earle's Balanced Salt Solution (EBSS) and fixed in 2.5 % glutaraldehyde supplemented with 5 mM $CaCl_2$ (Oschman and Wall, 1972). Osmium and wash buffers also contained 5 mM $CaCl_2$. Control tissues were either washed in EBSS and fixed in glutaraldehyde, or washed in calcium- and magnesium-free EBSS and fixed in solutions supplemented with 3 mM EGTA.

The pyroantimonate precipitation technique of Spicer et al. (1968) was also used to localize calcium. After glutaraldehyde fixation, 11 day tissue was postfixed in 1 % OsO_4, 2.5 % potassium pyroantimonate and processed as described above.

Results

Surface Contours of the Developing Epithelium

The apical region of the chick intestinal epithelial cell was examined from 5 days of incubation until 6 days after hatching. During this period, the overall shape of the apical surface of each epithelial cell exhibits three distinct configuations. A brief review of these configurations provides a useful perspective for the description of the ultrastructural events to follow.

From 5 to 10 days of incubation, the apical surface of each individual cell bulges into the lumen (Fig. 1A). At 5 days, when the general shape of the embryonic epithelium is still cylindrical, the bulging is so extensive that the lumen of the gut is occluded. The ballooned configuration persists over the next 4 days (Overton and

Shoup, 1964) as the lumen enlarges (Burgess, 1975) and as the epithelium is thrown into the ridges that precede the definitive villi (Grey, 1972). The second configuration of the apical surface is established by day 11, when the bulges are replaced by lower domes covered with numerous microvilli (Fig. 1B), each containing a bundle of core filaments (Burgess, 1974). The shape of the surface remains dome-like until the terminal web makes its appearance at 19–20 days, i.e., just prior to hatching. As the terminal web forms, the flattened apical configuration (Fig. 1C), typical of the mature epithelial cell, is attained.

Formation of Microvilli

From five to nine days of incubation, the ballooned apex of each intestinal cell is covered with a variety of irregularly shaped projections containing a filamentous network and a variety of small cytoplasmic inclusions. In any given cell during this period, there is considerable heterogeneity in the shape of the projections, and the microvilli do not develop synchronously. At 5 days, only a few of the projections on each cell have the uniform diameters characteristic of microvilli, but by 9 days, projections of more uniform diameter (~0.1 μm) and length (~0.5 μm) are numerous, as also observed by Overton and Shoup (1964).

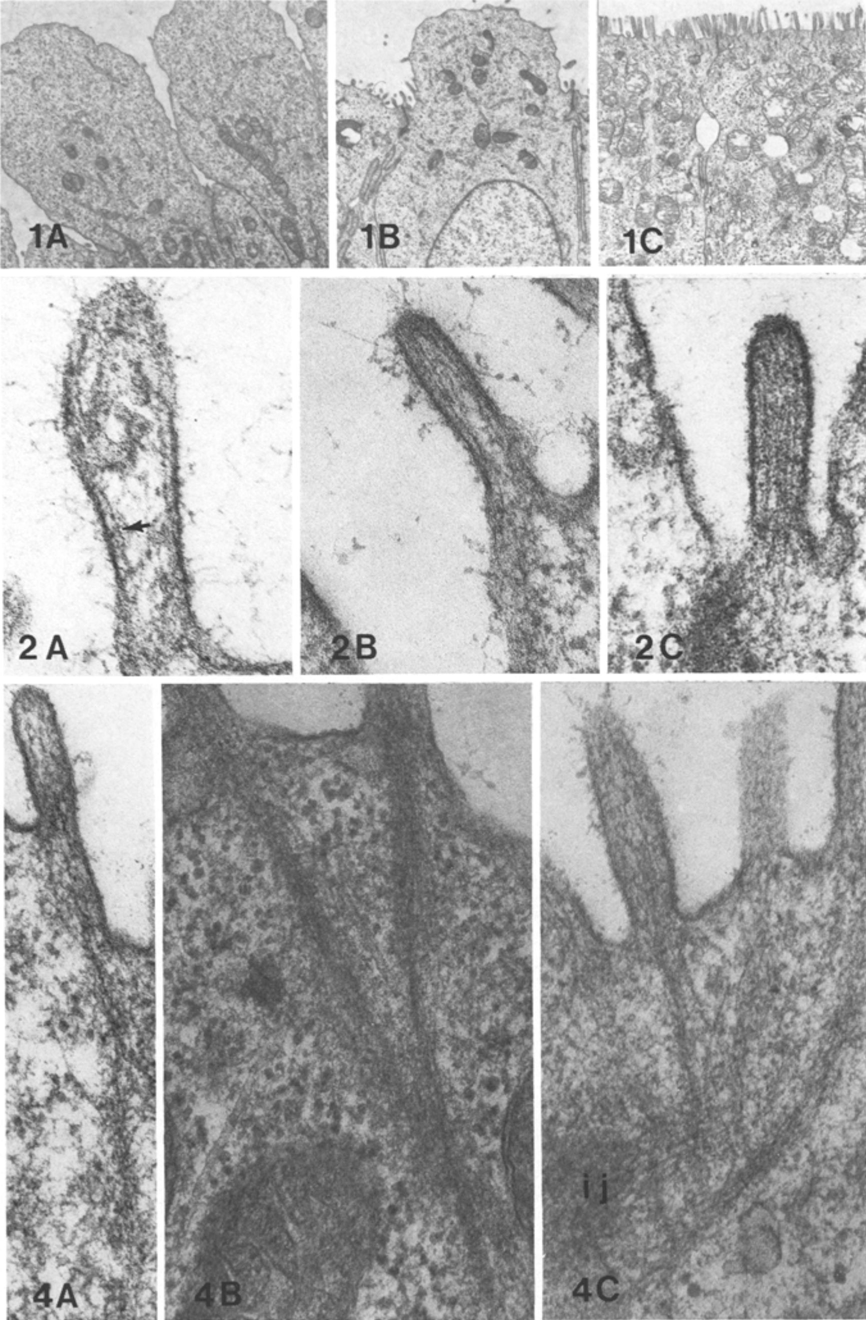
The absence of developmental synchrony makes it impossible to define with certainty the earliest sequence of events involved in formation of microvilli. Assuming, however, that the irregular projections, which predominate at 5 days, are the precursors of the uniformly shaped microvilli, which are dominant at 9 days, a transitional sequence can be reconstructed (Fig. 2).

This sequence also illustrates the probable order in which the core filaments align in the interior of a developing microvillus. As seen in Fig. 2A (*arrow*), the filaments in the irregular protrusions are aligned only at the periphery of the microvillus and are parallel to the plasma membrane. In microvilli that appear to be more advanced developmentally, the central core is also filled with filaments (Fig. 2B, C), suggesting that the alignment process begins at the plasma membrane and proceeds centripetally. The core microfilaments can often be traced for short distances into the cortical cytoplasm which is composed of a meshwork of filaments up to 0.2 μm in depth. The alignment process appears to be completed by 11 days, when all microvilli in a given cell acquire uniform dimensions (Overton and Shoup, 1964).

Fig. 1 A–C. The apical end of intestinal cells showing the change in surface contour during development. **A** Ballooned configuration at 9 days. **B** Dome-shaped surface with microvilli at 11 days. **C** Apical flattening and terminal web formation at 21 days. ×4300

Fig. 2. Apical microvillus projections from 8- to 9-day embryos showing the variation in internal filament structure. In **A**, the microvillus is not of even width and the aligned core filaments are restricted to the periphery. In **B**, core filaments are better aligned. In **C**, alignment is complete. ×70,000

Fig. 4. Rootlets of an 11-day embryo **A** are less dense and contain fewer longitudinally organized filaments than those of a 16-day embryo **B.** ×60,000. **C** Rootlet at the lateral cell margin from a 14-day embryo showing an association with the intermediate junction (*ij*). ×70,000


1A
1B
1C
2 A
2B
2C
4A
4B
4C
ij

Since the dense cap seen at the apex of mature microvilli (Fig. 6) has been suggested to be a nucleating site for core filaments (Tilney and Cardell, 1970; Mooseker and Tilney, 1975), we examined the temporal relationship between the presence of the dense cap and core filaments. Distinctly aligned filaments are present in surface projections that lack a cap at their apex (Fig. 2A, B), indicating that the first appearance of aligned filaments in what are believed to be developing microvilli precedes the formation of a morphologically distinct cap. Caps are invariably found at the apex of the uniformly shaped microvilli that are frequent at at 9 days (Fig. 2C). After about 11 days, nearly all surface projections have dense caps.

Typically, the most advanced microvilli are found at the lateral edges of the cell, where the base of each microvillus is closely associated with the junctional complex and its filamentous structures (Fig. 2C). It should be noted that the junctional complex becomes more prominent at 11 days after the ballooning of the cell surface has subsided. However, there are no rootlets and no terminal web present at this time.

Formation of Rootlets

Rootlets are formed as extensions of the basal ends of core filaments of the primitive microvilli. The pattern of core filament elongation was determined by examining sections made perpendicular to the surface of the epithelium and measuring the lengths of filament bundles extending into the cytoplasm at various ages. Both the average rootlet length and average microvillus length during development are presented in Fig. 3. The results show that the rootlets attain their maximum length during a single phase of growth from 10 days to 15 days of incubation, confirming the observations of Overton and Shoup (1964).

The morphology of developing rootlets is shown in Fig. 4. Microvilli are oriented at various angles to the apical surface, so that the core filaments often pass through a distinct cortical region before diverging from the plasma membrane and passing into the cytoplasm (Fig. 4A). In early rootlets (e.g., at 11 days) not all filaments are aligned in parallel, and at some points filaments splay into a meshwork configuration (Fig. 4A). Later, when growth is completed, rootlets are more clearly organized as tapered bundles of longitudinal filaments distinct from the surrounding cytoplasm (Fig. 4B).

The rootlets of microvilli near the lateral cell boundary are in close proximity to the filaments associated with the intermediade junction, and direct association of the two classes of filaments is suggested by the bending of filaments toward a junction (Fig. 4C).

Formation of the Terminal Web

The terminal web can first be distinguished at 19 days, when the apical cortex first becomes distinct from the cortex of the remainder of the cell. Randomly located patches of fine fibrous material become more extensive in the apical cortex, varying

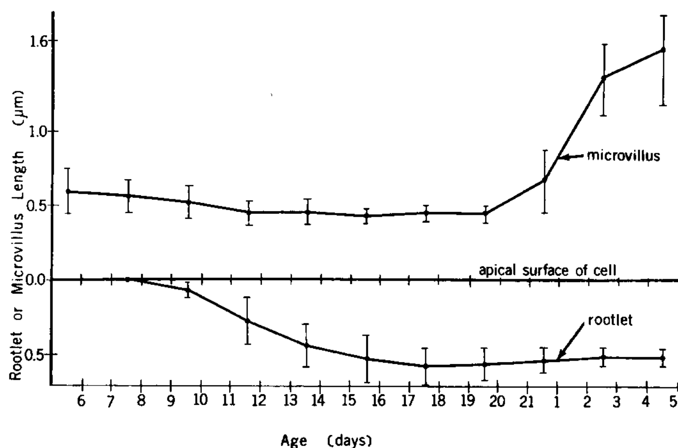


Fig. 3. Change in microvillus and rootlet length, measured from the cell surface, during development. In order to limit measurements only to rootlet bundles that had been sectioned along their entire length, measurements were made of rootlets extending from microvilli sectioned along their entire lengths. Care was also taken to avoid measuring the considerably longer rootlets in developing goblet cells which become distinguishable from absorptive cells at about 16 days. For microvilli, minimum sample size (N) was 20, except for the 5–6d embryonic period ($N=7$) and the 8d-adult period ($N=11$). Minimum sample size for rootlets was 17 for embryonic stages and 10 for posthatching stages. Bars represent one standard deviation

in depth below the apical plasma membrane and interrupted by small regions of glycogen particles, ribosomes, and small vesicles (Fig. 5A). By 21 days the dense network of randomly oriented thin (4–9 nm) filaments surrounds the rootlets, completely excluding all small organelles, reaching to the apical plasma membrane and to the tight and intermediate junctions.

Along with the formation of the terminal web at the apical end of the cell, the junctional region at the lateral boundary of the terminal web also continues developing. A ring of intermediate filaments found just inside the intermediate junction enlarges, increases in density, and becomes continuous with the remainder of the terminal web (Fig. 5B).

The last morphological change in the terminal web is a stratification into a lower dense layer, and an upper, more sparse layer by 2–3 days after hatching (Fig. 5C). The stratification is consistent with the description by Hull and Staehelin (1979) of the mature terminal web in *Xenopus laevis* intestinal epithelium.

The Mature System

Concomitant with the elaboration of the distinct terminal web between 19 and 21 days, the brush border takes on the precise organization that characterizes the mature system (Fig. 6). The core filament bundles become aligned parallel to each other and perpendicular to the cell surface which is now completely flat. The microvilli begin their final phase of elongation, which continues until one week after hatching, when they reach adult length, (2.5 to 3.0 μm, see Fig. 3).

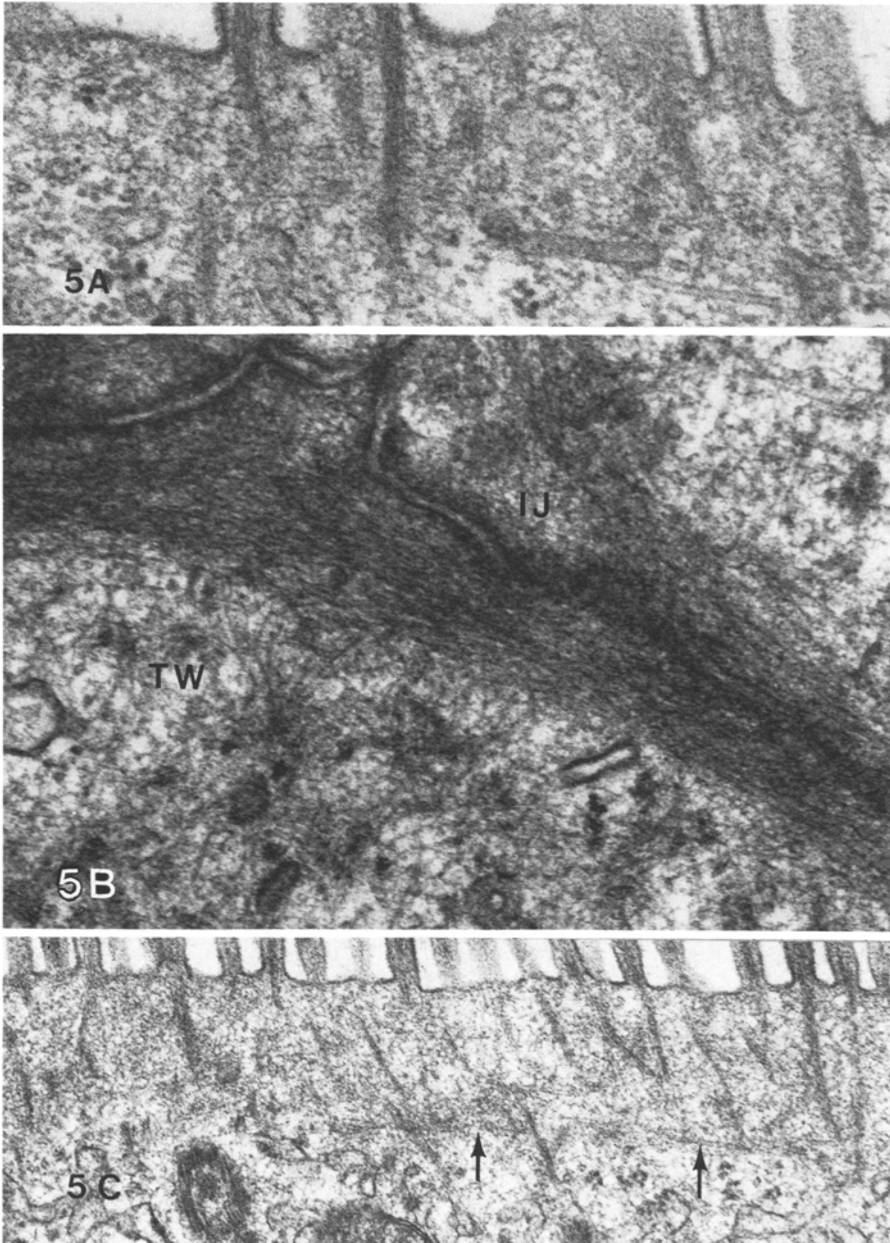


Fig. 5. A The developing terminal web appears at 19 days. × 70,000. **B** A frontal section through the terminal web (from a 2-day chick) at the intermediate junction (*IJ*) clearly shows the distinct ring of intermediate filaments in longitudinal section associated with the junction. Some filaments appear to diverge from the bundle and pass into the terminal web (*TW*), possibly connecting to rootlets. × 70,000. **C** The terminal web of a 3-day chick showing its stratification into a dense lower layer (*arrows*) and sparse upper layer. × 40,000

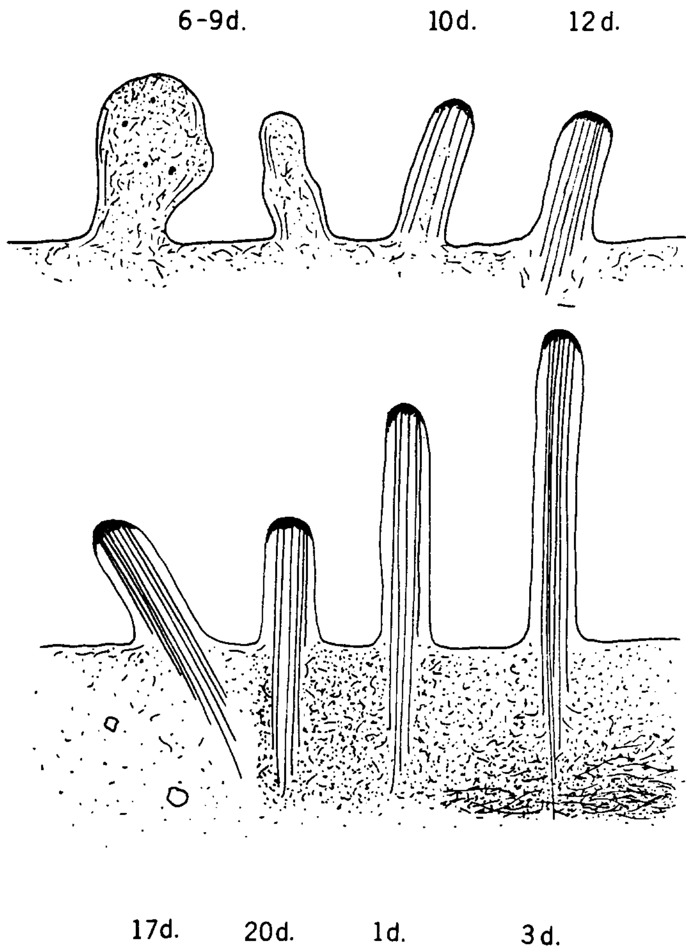


Fig. 7. Diagram summarizing the major morphological events in brush border development: 6–9 days of incubation – Microvillus projections appear with increasing numbers of longitudinal filaments below the plasma membrane. 10 days – Complete microvillus core bundles are forming. 12 days – Some microvilli with complete core filament bundles possess short rootlets. 17 days – Rootlets of maximum length have formed. 20 days – The terminal web forms around the rootlets which are positioned perpendicular to the cell surface. 1-day chick – Microvilli are elongating. 3-day chick – The terminal web is stratified

The overall sequence of events in brush border development is summarized in Fig. 7.

Increasing Calcium or Magnesium Ions Stimulate Rootlet Formation

The genesis of the intestinal brush border involves the formation of several sets of filaments in a precise sequence and in specific locations in the apical portion of the cell, but the stimulus that causes a particular type of filament to form at a precise

Table 1. Effect of increased levels of Ca^{++} or Mg^{++} on filament formation at different ages

Ion	Addition to culture medium (mM)	A23187 (μg/ml)	Age		
			9 days	11 days	19–20 days
Ca	0	5	0	0	0
	5	0	0	0	0
	5	5	0	+	0
	10	0	0	0	–
Mg	0	5	0	0	0
	5	0	0	+	0
	5	5	0	+	–
	10	0	0	+	0

0 = no effect; + = stimulation of filament formation or aggregate of dense material in at least 5% of cells; – = disruption of terminal web filaments

position and time in this system is unknown. To explore the possibility that calcium or magnesium ions act to regulate filament formation, duodenal tissue was cultured in media with high ion concentrations, with or without the ionophore, A23187, which allows divalent cations to reach equilibrium across membranes (Reed and Lardy, 1972). Tissues were then examined by transmission electron microscopy for alterations in filament development. The results are shown in Table 1.

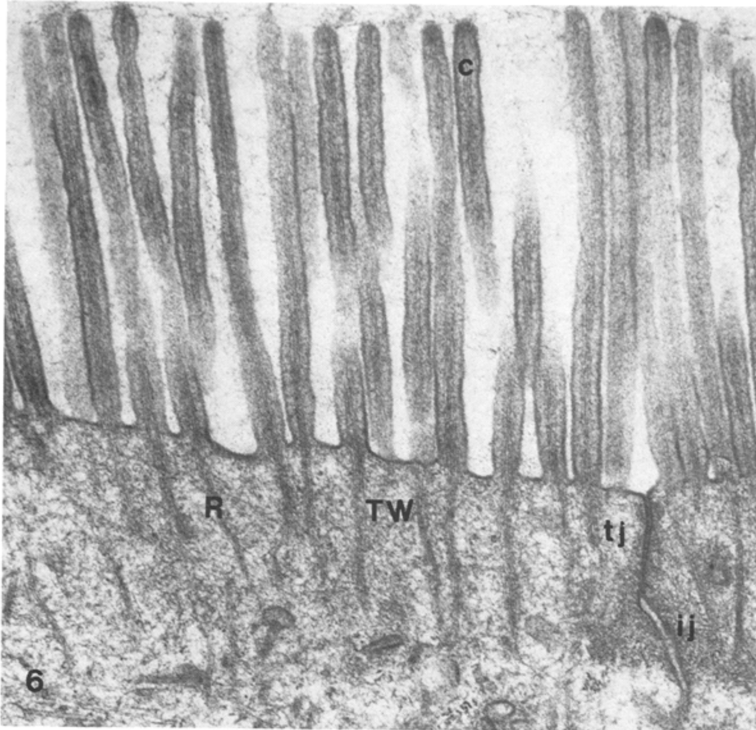
Tissue from 9-day embryos gave no response to altered ionic concentrations, but at 11 days, when rootlet formation is beginning, the tissues are clearly responsive to an altered ionic environment. Media with elevated levels of magnesium ions produced either large aggregates of filaments in the position of rootlets (Fig. 8B) or unusually long rootlets (Fig. 8A). Calcium, by contrast, was effective at this stage only when ionophore was also present in the medium, and produced similar results.

Localization of Calcium

To determine if stored calcium was localized near incipient microvilli or rootlets, an attempt was made to visualize any calcium deposits either by fixation in high calcium (Oschman and Wall, 1972) or by precipitation with potassium pyroantimonate (Simson and Spicer, 1975). Both techniques produced small densities randomly located in the plasma membrane of the microvilli (Fig. 8C). Similar densities were also seen in controls, though less frequently. We conclude that storage of calcium is probably not a significant factor in the development of microvilli.

EGTA Alters the Terminal Web

To determine the consequence of decreasing the calcium ion concentration in the external medium, tissues were incubated in medium containing 5 mM EGTA.



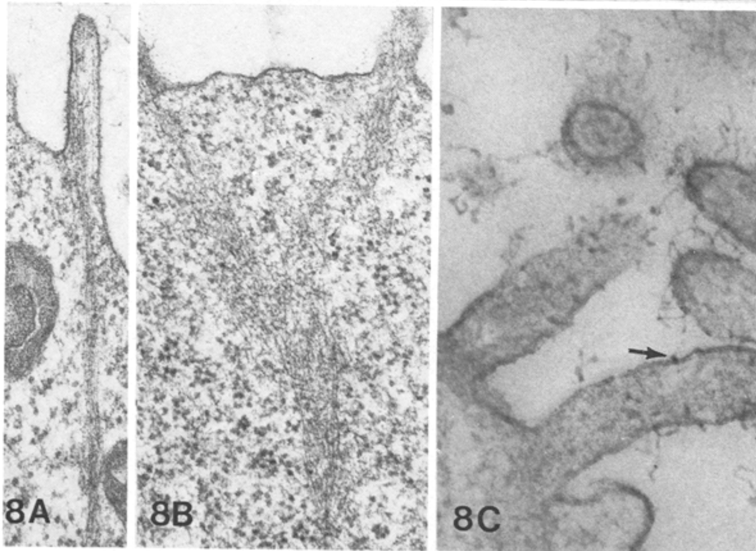


Fig. 6. A portion of the brush border and junctional complex from a 2-day chick intestine showing the core microfilament bundle (*c*) within a microvillus, and the terminal web (*TW*) surrounding rootlets (*R*). Tight junction (*tj*), intermediate junction (*ij*). ×70,000

Fig. 8. A Duodenal tissue from an 11-day embryo cultured with 5 µg/ml A23187 and 5 mM $MgCl_2$ for one hour showing an unusually long rootlet. ×40,000 **B** Duodenal tissue from an 11-day embryo cultured with 5 mM $MgCl_2$ for one hour showing loose filaments in the position of rootlets. ×40,000 **C** Calcium localization (*arrow*) by potassium pyroantimonate precipitation in 11-day duodenum. ×78,000

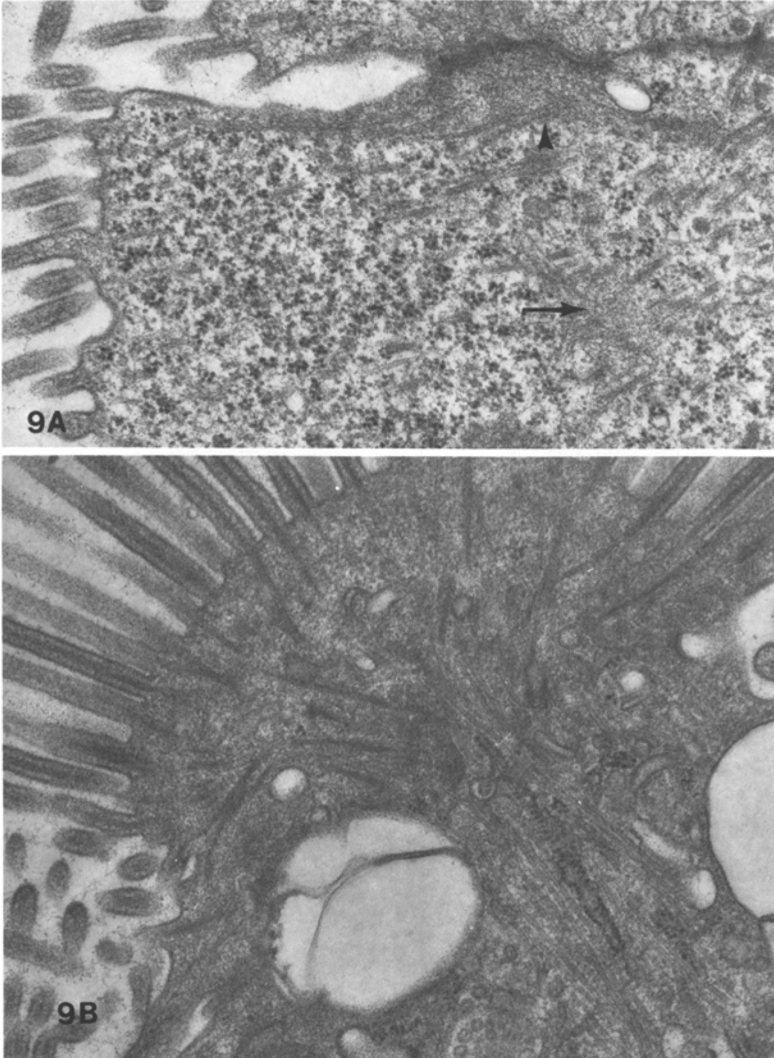


Fig. 9. A The effect of culturing 20-day duodenal tissue with 5 mM EGTA for one hour. The arrow indicates dense material that is at the level of the junctional complex and may be part of the terminal web. The *arrowhead* shows two rootlets embedded in the fibrous material adjacent to the intermediate junction. × 32,500 **B** The brush border from a 4-day chick intestine cultured with 5 mM EGTA for one hour contains a collapsed terminal web, large vesicles (or intercellular spaces), and many microtubules. × 32,500. In no case did EGTA disrupt the epithelial nature of the tissue

Between 11 and 20 days of development, EGTA had no effect on the apical morphology of the cells. At 20 days, however, EGTA caused the apical surface of the cell to balloon out in a manner reminiscent of cells in the 5–9 day period; the terminal web in these cells was clearly disrupted (Fig. 9A). That this effect was due to calcium deprivation is indicated by the fact that tissues from control cultures

(5 mM EGTA plus 10 mM $CaCl_2$) appeared entirely normal, with a developing terminal web and a flat cell surface. Rootlets in calcium-deprived cells were no longer regularly arranged; they were elongated, and could often be traced along a curved pathway into the meshwork of material still associated with the intermediate junction (Fig. 9A, *arrowhead*). There also appeared to be few 10 nm filaments discernible in EGTA-treated tissue. The EGTA effect was not reversible by reculturing without EGTA for one hour; longer reversal times were not examined.

Very different responses were obtained from posthatching stages. The terminal web region of 4-day hatched chicks was not disrupted as in younger stages, but instead detached from the intermediate and tight junction regions at the edges of the cells and condensed at the center of the apical surface (Fig. 9B). The rootlets were still enmeshed in the terminal web, although they were somewhat longer and radiated from the small region of terminal web at the center of the apical surface. Junctions appeared to be intact, although intercellular gaps just below the junctions were prominent. The dense material normally associated with the intermediate junction remained attached to the rest of the terminal web and pulled away from the lateral plasma membrane.

Posthatching-stage tissues cultured in 5 min EGTA plus 10 mM $CaCl_2$ appeared normal. The effects of EGTA were not reversed by reculturing the tissue in medium without EGTA, with or without an additional 10 mM $CaCl_2$. Substitution of EDTA for EGTA produced similar effects, but disrupted intercellular adhesions more completely.

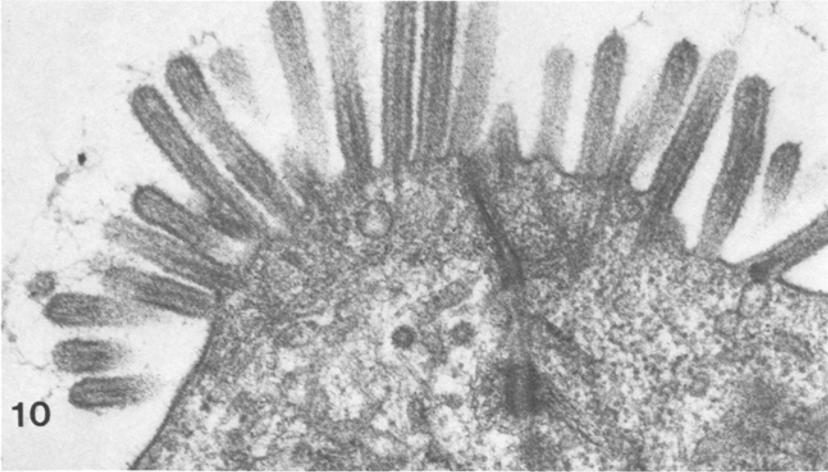
To summarize, the effect of EGTA on the brush border varied with age. At 20 days the apical surface bulged out when the terminal web was disrupted, but in the 4-day chick, when only the lateral border of the terminal web and intermediate filaments were disrupted, the terminal web contracted or collapsed to the center of the cell. At both ages, the core filament bundles were elongated.

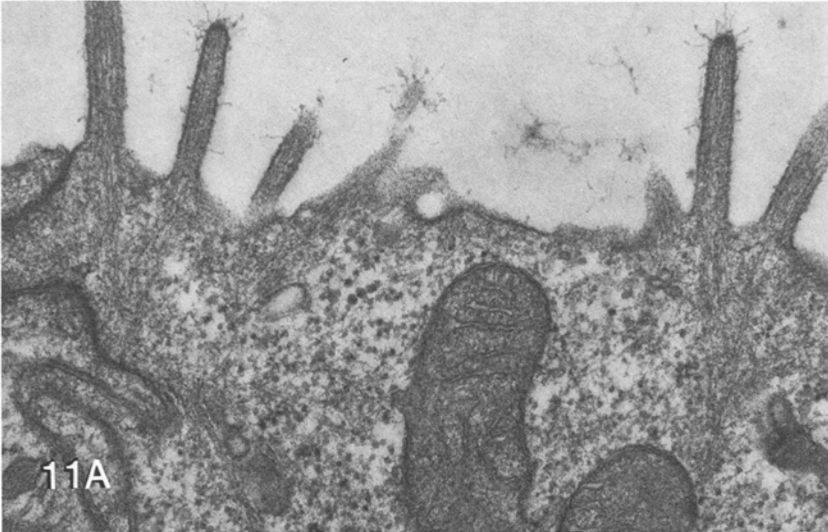
Cytochalasin B Disrupts the Early Terminal Web

Cytochalasin B (CB) is reported to cause the disruption of microfilaments or their detachment from membranes (Wessells et al., 1971), although a variety of other effects have also been documented (Burnside and Manasek, 1972; Pollard and Weihing, 1974).

In the 11-day embryonic chick intestine it has been shown previously that CB not only fails to disrupt microfilaments, but causes elongation of core filament bundles, branching of microvilli, aggregation of cortical filaments, and bulging, of epithelial cells (Burgess and Grey, 1974). These results, which were obtained with tissues cultivated on the chorioallantoic membrane, have been corfirmed in the present study using an in vitro organ culture system. Later stages of the developing intestine were also studied in organ culture to determine the changing susceptibility of various filament systems to the effects of CB.

CB did disrupt components of the terminal web, at 20 days, during the early stage of its development. The early terminal web was disrupted sporadically in the central regions of the cell but was relatively undisturbed at the periphery (including the 10 nm filaments) near the lateral plasma membrane (Fig. 10). In regions where





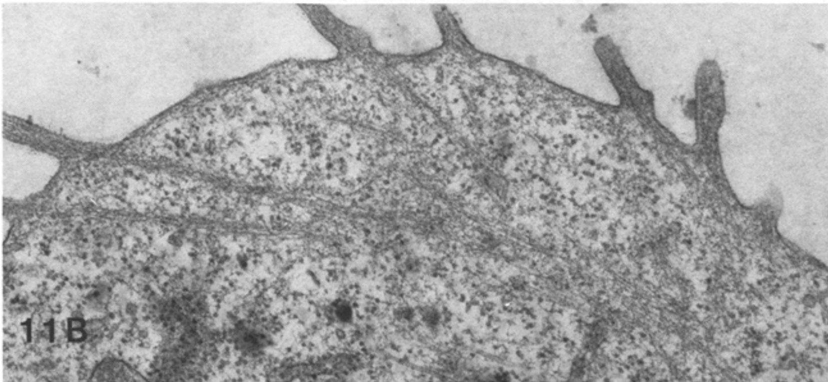


Fig. 10. Culturing 19- to 20-day intestinal tissue in medium with 5 μg/ml cytochalasin B for 30 min results in partial disruption and aggregation of the terminal web. ×40,000

Fig. 11. **A** Nine-day tissue cultured with 2% ethanol results in the formation of rootlets below some microvilli. ×40,000 **B** Extremely long rootlets in 11-day tissue cultured in 0.2% ethanol. ×40,000

the terminal web is disrupted there were few microvilli, but adjacent regions consistently showed clumps of microvilli and an aggregation of the terminal web.

By the time the chick is three days old, the terminal web is completely resistant to CB. It has been previously reported that the adult mammalian brush border is morphologically unaffected by CB at the same concentration used in the present study (Mak et al., 1974).

The Effect of Ethanol

In preliminary experiments which employed ethanol as a solvent, it was discovered that ethanol itself affected microfilament elongation in cells in culture. The capacity of ethanol to promote elongation of filaments in the apical end of epithelial cells of various ages was investigated by culturing in medium containing 2% or 0.2% (v/v) ethanol.

At nine days, when rootlets are normally absent, ethanol (2%) caused the aggregation of dense material below microvilli and the production of distinct rootlets in some cases (Fig. 11A). Microvilli are not affected. At 11 days (the period when rootlet formation is beginning) there was a much more dramatic effect of ethanol, producing some long microvilli (not shown) and extremely long rootlets (Fig. 11B). This was also the only stage at which 0.2% ethanol had a noticeable effect. The response of older tissue to ethanol was similar, but observed less frequently.

Discussion

The principal aim of this study was to examine the temporal and spatial relationships between cytoplasmic filaments and the morphogenesis of the brush border. The relationships that have emerged provide some insight into the developmental significance of the axial core and the terminal web.

The development of the axial core of a microvillus appears to comprise two, fairly distinct, sets of events. The first set involves the assembly and alignment of core microfilaments; the second involves elongation of core microfilaments.

The assembly and alignment phase occurs between about 9–11 days of incubation. During this period, the internal structure of a developing microvillus changes from a network of short, randomly oriented, thin filaments, to long, parallel filaments aligned parallel to the plasma membrane. A similar kind of reorganization of filament has been observed in the formation of filopodia in sea-urchin coelomocytes (Edds, 1977).

It has previously been inferred from observations of filaments forming in mature intestinal epithelial cells recovering from treatment with hydrostatic pressure that the dense cap, located at the apex of mature microvilli, functions as the major organizational site for developing filaments (Tilney and Cardell, 1970). Since the cap does not appear prior to formation of aligned filaments, it seems unlikely that the cap plays this role in the initial development of the microvilli. Instead, the first aligned filaments are seen subjacent to the plasma membrane and the axial core is

apparently completed by addition of aligned filaments in a centripetal pattern. These two observations suggest that the plasma membrane that forms the lateral wall of a microvillus may play a dominant role in the initial alignment of the filaments that form the axial core. Elements that link filaments with plasma membrane have been identified in adult systems (Mukherjee and Staehelin, 1971; Tilney and Mooseker, 1976). It will be important to learn whether in normal development these elements appear at a time and place consistent with the hypothesis that they function in the alignment phase of axial core filament formation.

Once formed, the aligned bundle of filaments elongates during two distinct periods (Fig. 3). In the first period (ca. 10–15 days of incubation), microvillar height remains constant while the growing filament bundle penetrates the apical cytoplasm to form rootlets. During the second period of elongation (after hatching), rootlet length remains unchanged as microvillar length nearly triples.

Unlike the alignment phase, the initial period of elongation is sensitive to elevated levels of Mg^{++} and, to a lesser degree, Ca^{++}. It is especially interesting that high levels of Mg^{++} accelerate or exaggerate the process of rootlet formation (Table 1). While these results suggest that the normal elongation process could be mediated by a change in ion concentrations, there is as yet no evidence that such a change occurs during normal development. Magnesium is, however, required for the formation of actin bundles in extracts from several cell types (Spudich and Cooke, 1975; Taylor et al., 1976; Pollard, 1976; Hinssen and D'Haese, 1976; Kane, 1976). Also, the polymerization of purified actin in vitro occurs much faster in the presence of Mg^{++} than Ca^{++} (Mihashi and Ooi, 1965).

The initial period of filament elongation is also sensitive to cytochalasin B (Burgess and Grey, 1974) and ethanol, but the means by which these agents promote precocious elongation are not known.

We have noted that microvilli located near the perimeter of the apical surface, in association with the junctional complex, are the most advanced in terms of core filament alignment and elongation. The significance of this relationship is not clear, but it may be important that junctional regions contain actin (Ishikawa et al., 1969; Rodewald et al., 1974), myosin (Bretscher and Weber, 1978a, b) and α-actinin (Craig and Pardo, 1978; Bretscher and Weber, 1978a, b), proteins which, in high concentrations, may accelerate or stabilize the development of core filaments.

Two phases in the construction of the terminal web were distinguished on the basis of morphological stratification and susceptibility to disruption by EGTA and CB. There is an early phase when a dense and fairly homogeneous meshwork of filaments condenses below the apical plasma membrane. The intermediate filament ring just inside the intermediate junction becomes larger. Three days after hatching, the terminal web is stratified into a lower dense layer and a sparse upper layer, and the terminal web is altered in its susceptibility to EGTA and CB.

A possible role of the intermediate filaments is suggested by comparing the results of EGTA and CB treatments on surface morphology. EGTA treatment produced a bulging surface, a loss of the terminal web, and disruption of intermediate filaments at the junctions. CB, which also disrupts the terminal web, does not affect the intermediate filaments, and the apical surfaces remain flat. Since the terminal web is disrupted in both cases, it does not seem to be responsible for the

surface contour or shape, contrary to the assumptions of other workers (Palay and Karlin, 1959; Tilney and Cardell, 1970). However, since intermediate filaments are found at the cell junctions only when the cell surfaces are flat, the intermediate filaments may have a role in maintaining the flattened cell surface, consistent with their proposed structural role in skeletal, cardiac, and smooth muscle (Lazarides and Hubbard, 1976; Small and Sobieszek, 1977) and in fibroblasts (Goldman and Knipe, 1972; Brecher, 1975).

The earliest terminal web that forms appears to have the function of aligning the microvilli so that they are parallel and a constant distance from each other (0.1 to 0.15 μm), as proposed by earlier investigators. The mechanism of acquiring the precise spacing is unknown, but may rely on linking adjacent rootlets with a filament of constant length. A model of terminal web structure based on its contractile properties includes actin filaments splayed out from the rootlet bundles and connected by myosin filaments (Mooseker and Tilney, 1975; Mooseker et al., 1978). The proposed myosin filaments are in the appropriate position to link rootlets, and could provide regular spacing if they were of a constant length. Filaments resembling myosin were not observed in the present study.

At three days after hatching, the terminal web is stratified into two layers, is resistant to disruption by CB, and apparently contracts in EGTA-treated preparations. The molecular basis for the stratified morphology is unknown, but could possibly be the addition or arrangement of myosin, tropomyosin, α-actinin, or filamin which exist in the mature terminal web (Bretscher and Weber, 1978a, b). The addition of these proteins would be consistent with the newly acquired resistance to disruption and with the ability to contract when attachment to the junctions is broken with EGTA. Contractility has been directly demonstrated in isolated brush borders of the adult (Mooseker, 1976; Rodewald et al., 1976).

Further investigation should clarify the significance of the stratification of the terminal web, as well as the control of the associations among the various filaments described in this study.

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