Organization of Actin, Myosin, and Intermediate Filaments in the Brush Border of Intestinal Epithelial Cells

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ABSTRACT Terminal webs prepared from mouse intestinal epithelial cells were examined by the quick-freeze, deep-etch, and rotary-replication method. The microvilli of these cells contain actin filaments that extend into the terminal web in compact bundles. Within the terminal web these bundles remain compact; few filaments are separated from the bundles and fewer still bend towards the lateral margins of the cell. Decoration with subfragment 1 (S_1) of myosin confirmed that relatively few actin filaments travel horizontally in the web. Instead, between actin bundles there are complicated networks of fine fibrils. Here we present two lines of evidence which suggest that myosin is one of the major cross-linkers in the terminal web. First, when brush borders are exposed to 1 mM ATP in 0.3 M KCl, they lose their normal ability to bind antimyosin antibodies as judged by immunofluorescence, and they lose the thin fibrils normally found in deep-etch replicas. Correspondingly, myosin is released into the supernatant as judged by SDS gel electrophoresis. Second, electron microscope immunocytochemistry with antimyosin antibodies followed by ferritin-conjugated second antibodies leads to ferritin deposition mainly on the fibrils at the basal part of rootlets. Deep-etching also reveals that the actin filament bundles are connected to intermediate filaments by another population of cross-linkers that are not extracted by ATP in 0.3 M KCl. From these results we conclude that myosin in the intestinal cell may not only be involved in a short range sliding-filament type of motility, but may also play a purely structural role as a long range cross-linker between microvillar rootlets.

The brush border of the intestinal epithelial cell has been a favorite subject for study of the organization and biochemistry of actin filaments and their related proteins in nonmuscle cells. This is in part due to the ease with which this portion of the cell can be isolated, in part due to the quantities which can be made, and in part due to the fact that microvilli occur on the surface of nearly all nonmuscle cells of the body, and thus, by studying the brush border, one can investigate a very basic cellular differentiation. Furthermore, there have been two in vivo studies (39, 40), albeit poorly substantiated, and two in vitro studies (27, 37) that together indicate that microvilli on intestinal cells may move or wave about. Also it is now clear that within the brush border are all the components necessary for movement, including actin, myosin, tropomyosin, α-actinin, calmodulin, a myosin light chain kinase,[1] and a calcium-regulated actin cross-linking protein, villin (3–5, 8, 11, 16, 20, 29). Nevertheless, it is still not clear if in fact microvilli move in vivo and if they do, what type of motion they undergo. Likewise, it is still unclear exactly how the in vitro movements described in previous reports (27, 37) are actually generated. Several models have been proposed, but currently there is not enough structural information available on the organization of actin filaments and their associated myosin in these cells to determine how plausible these models are.

To obtain more structural information on the organization of actin filaments in the terminal web region of the brush border and on the precise localization of myosin relative to these filaments, a prerequisite to testing these models, is by no means a trivial task. It requires a method that preserves the three-dimensional structure of the terminal web and at the same time allows one to identify precisely where each contractile protein is located and to what it is attached. Due to several inherent technical limitations, conventional fixation and thin-sectioning for electron microscopy fails to fulfill these requirements. First and most important, OsO_4 promotes the break-

[1] Keller, T. C. S., III, C. L. Howe, and M. S. Mooseker. 1981. *J. Cell Biol.* 91(2, Pt. 2):305*a* (Abstr.).

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down of actin filaments unless these actin filaments are associated with other proteins such as tropomyosin or the fragments of myosin (subfragment 1 [S_1] or heavy meromyosin) (24) or compounds such as phalloidin (46). In the case of the terminal web, this would mean that the core filament bundles which contain actin and tropomyosin ought to be adequately preserved, but free actin filaments would probably be, to some extent, destroyed. Second, Small (42) has recently demonstrated that dehydration itself results in extreme damage to actin filaments, even actin filaments protected by tropomyosin. Third, a point stressed by Wolosewick and Porter (47) is that the plastic embedding needed for thin-sectioning creates a background electron density that makes a clear identification of individual filaments difficult, even if they have not been destroyed by OsO_4. Fourth, because of superposition of cellular structures in individual thin sections or even in serial sections, it is difficult to determine three-dimensional relationships between objects as thin as actin filaments, e.g., whether two filaments are attached to each other or whether they simply pass by each other. Therefore, since a detailed description of the deployment of individual filaments in the terminal web is needed to test the various motility models, conventionally fixed, dehydrated, and sectioned material cannot be used.

In a recent study (14) we showed that replicas made from quick-frozen, freeze-fractured and deeply-etched samples allowed one to obtain three-dimensional information about the brush border at reasonably high resolution and without whatever artifacts would be induced by osmification, dehydration, or plastic embedding. Furthermore, other work with this technique has demonstrated that it will permit one to identify actin filaments, intermediate filaments, microtubules, and other filamentous structures simply by their surface details (12). This has given us some hope of being able to reconstruct exactly how actin and myosin are arranged in the intact intestinal cell.

In this study we carefully examined the distribution of the actin filaments in the terminal web region of these cells, using stereo pairs of replicas made from quick-frozen and deep-etched brush border fractions, to see if the distribution of these filaments is compatible with models of motility proposed to explain the movements of brush borders in vitro. We have also localized myosin molecules relative to the actin filaments by selective extraction of the myosin and, in other experiments, by examining replicas from brush borders which had been incubated with antibodies against myosin. This has substantiated our earlier speculation (14) that some of the thin fibrils which extend between actin bundles in the terminal web could be composed of myosin. Finally, we have found that the actin filament bundles are also connected to a bed of intermediate filaments by another sort of thin fibril. This morphological analysis of actin filament organization and of myosin localization appears to be incompatible with most of the existing models of the brush border for movement in vitro (8, 30, 37).

MATERIALS AND METHODS

Experimental Subject

Mice were decapitated and their small intestines were dissected out and cut into short lengths. The lumen was flushed with calcium-free mammalian Ringer (155 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 3 mM EGTA, and 5 mM HEPES at pH 7.0).

Preparation of Segments of Intestine

After washing out the lumens of intestinal segments, the intestine was cut into 400 μm thick rings with a Sorvall tissue chopper (Dupont Instruments–Sorvall Biomedical Div., DuPont Co., Newton, CT); these rings were further cut into small pieces by hand. The small pieces were immediately quick-frozen in mammalian Ringers to observe unfixed cells.

Preparation of Brush Borders

After washing out the lumens of the intestinal segments with calcium-free Ringer, they were opened longitudinally and transferred to a homogenizing solution, in which their luminal surfaces were scraped with a glass cover slip to obtain sheets of epithelial cells. These sheets were further dissociated into individual cells by passing them through a 22-gauge needle. The cells were then broken open by passing them repeatedly through a 27-gauge needle. The preparation was then examined with a phase-contrast microscope to make sure the cells were broken. If not, they were passed several more times through the 27-gauge needle. The resulting suspensions were centrifuged at 1,000 *g* for 3 min and washed three times in fresh homogenizing media before further treatment. The solutions used for homogenization were either the one we formerly called "artificial cytosol" (see reference 14), which consisted of 100 mM KCl, 30 mM HEPES at pH 7.2, 5 mM $MgCl_2$, and 2 mM EGTA, or in later experiments, a more standard stabilization buffer which consisted of 60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole at pH 7.2, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis. We could not recognize any differences in samples prepared in one versus the other of these solutions, but we used the latter for all experiments described in this paper. Most of the samples were fixed with 1% glutaraldehyde and 1% paraformaldehyde in 60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, and 10 mM imidazole at pH 7.2, for 1.5 h on ice, washed with distilled water to remove salt and quick-frozen. Other samples (called fresh samples) were quick-frozen without fixation and without washing in distilled water.

Decoration of Actin Filaments by S_1

The unfixed brush border preparations were incubated with myosin S_1 kindly provided by Dr. Annemarie Weber (University of Pennsylvania). This was applied to a suspension of brush borders at a concentration of 1 mg/ml in stabilization buffer. Incubation was carried out for various amounts of time (see Results) at room temperature. For heavier decoration the brush borders were incubated in 2 mg/ml of S_1 in the same solution for 30 min or longer at 22°C. The samples were then washed in stabilization buffer to remove unbound S_1 and then fixed for 30 min at 22°C in 1% glutaraldehyde in stabilization buffer which contained 0.2% tannic acid (2). Then they were washed briefly in 100 mM KCl and 5 mM $MgCl_2$ to remove the aldehyde and buffer, block stained for 1 h in 1% uranyl acetate in the same Mg-KCl solution, washed in this solution again to remove unbound uranyl acetate, and, immediately before freezing, washed briefly in distilled water. (The purpose of this method was to enhance the visibility of the polarity of the decorated actin filaments as will be explained in a subsequent publication by J. Heuser and R. Cooke.)

In some cases the brush borders were fixed before decoration with 1% formaldehyde in stabilization buffer for 30 min at 0°C, then washed several times in stabilization buffer containing 10 mM lysine before S_1 decoration. This also leads to a very successful actin decoration, as will be described in the Results. After decoration and washing the sample was postfixed in glutaraldehyde and processed as outlined above.

Extraction of Myosin from the Brush Border

Freshly prepared brush borders were incubated for 15 min at 0°C in an "extraction solution" which contained elevated KCl (0.3 M) and 1 mM ATP in addition to the 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole buffer at pH 7.2, and 0.1 mM PMSF.

Another part of the samples was incubated with the above extraction solution containing no ATP. All samples were then washed with stabilization buffer and split into two portions, one for light microscope immunocytochemistry of myosin and the other for deep etch electron microscopy. The latter was fixed with 1% glutaraldehyde, 1% paraformaldehyde in stabilization buffer for 1.5 h on the ice, washed with distilled water and quick-frozen.

SDS Gel Electrophoresis

This work was conducted in the laboratory of Mark Mooseker (Yale University), using his techniques for preparing brush borders from mouse intestines (29). The preparations were subsequently divided into three aliquots. The first was extracted with 0.3 M KCl, the second was extracted with 0.3 M KCl containing 1 mM Mg-ATP, and the third was saved as a control. The preparations were then centrifuged at 15,000 *g* for 3 min and the resulting supernatants and pellets were dissolved in SDS buffer, after which PAGE was performed by the method of Laemmli (21). Gels were stained with 0.1% Coomassie Brilliant Blue.

Incubations of the Brush Border with Calcium ATP or Magnesium ATP

Freshly prepared brush border preparations were incubated for 30 min at 0°C in a solution containing calcium chloride at a concentration of 0.5 mM, 0.2 mM, 1 μM, or 0.1 μM. Each solution also contained 0.1 mM ATP, in addition to 60 mM KCl, 10 mM imidazole buffer at pH 7.2, and 0.1 mM PMSF. $CaCl_2$/EGTA buffers (32) were used to obtain 1 μM and 0.1 μM concentrations: the 1 μM level was consisted of 6.41×10^{-4} M $CaCl_2$, 1.01 mM $MgCl_2$, and 1.0 mM EGTA, while the 0.1-μM level contained 1.51×10^{-4} M $CaCl_2$, 1.03 mM $MgCl_2$, and 1 mM EGTA. After incubation in one of the above solutions, each brush border preparation was fixed for 1 h at 0°C by adding 1% glutaraldehyde and 1% paraformaldehyde to the same calcium containing solution used for extraction, washed with distilled water and quick-frozen.

Brush borders were also incubated in Mg-ATP. For these experiments, we included 10 mM Mg-ATP in all stages of the preparation of the samples. The Mg-ATP was made up in our standard stabilization buffer. After isolating the brush borders in the Mg-ATP solution, they were incubated for an additional 10 min in the Mg-ATP solution at 0°C before fixation with 1% glutaraldehyde and 1% paraformaldehyde in stabilization buffer which also contained Mg-ATP. The samples were then washed in distilled water and quick frozen. Portions of each of these calcium ATP or Mg-ATP samples were also examined immunocytochemically for myosin without any fixation.

Preparation of Myosin Minifilaments

Myosin prepared from rabbit skeletal muscle was generously sent to us by Dr. Annemarie Weber (University of Pennsylvania). We prepared minifilaments of myosin (composed of 16–18 monomers) by the method of Reisler et al. (36). These minifilaments were quick-frozen without fixation in 10 mM citrate, 35 mM Tris at pH 8.0.

Immunocytochemistry of Myosin

LIGHT MICROSCOPY: Rabbit antibodies were prepared against the rod portion of human platelet myosin (10). The unfixed brush borders were incubated in stabilization buffer containing a 50-fold dilution of the rabbit serum or preimmune serum from the same rabbit. Incubation was carried out for 30 min at room temperature. After four washes with stabilization buffer the brush borders were reacted with a 50-fold dilution of fluorescein conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories, Cochranville, PA) for 30 min at room temperature. After washing, the brush borders were mounted on slides and examined with a Zeiss epifluorescent microscope with a × 63 objective lens. Micrographs were taken with Kodak Plus X film and developed in diafine.

ELECTRON MICROSCOPY: Freshly prepared brush borders were prefixed with 1% paraformaldehyde in stabilization buffer for 30 min on ice. This prevented structural deterioration of the sample during the long periods of incubation and washing needed for the indirect method, yet did not seem to diminish the samples antigenicity to any noticeable extent. Before incubation in antibodies, they were then washed extensively with stabilization buffer containing 10 mM lysine. This was followed by a 1.5-h incubation at roomeperature in 20-fold diluted rabbit anti-human platelet myosin or preimmune serum in stabilization buffer which in both cases contained 0.1% bovine serum albumin. After washing four times, the samples were reacted for another 1.5 h at room temperature in 30-fold diluted ferritin-labeled goat anti-rabbit IgG (Miles-Yeda, Elkhart, IN) in stabilization buffer which also contained 0.1% bovine serum albumin. They were washed three times in stabilization buffer, fixed with 1% glutaraldehyde and 1% paraformaldehyde in stabilization buffer for 1 h on ice, washed in distilled water, and quick-frozen.

Quick-freezing, Freeze-fracture, and Deep-etching

Samples were quick-frozen with a liquid helium cooled machine and freeze-fractured in a Balzers apparatus (Balzers, Hudson, NH) as previously described (13, 14). When the fixed specimens were positioned on the specimen holder, a small drop of water containing 15% methanol was placed briefly on the specimen. Excess fluid was absorbed with filter paper. The unfixed specimens were not rinsed in this way, but were frozen in the media indicated in the appropriate section. Deep-etching was carried out at −95°C for 5 min. Replicas were made by rotary shadowing with a mixture of platinum and carbon after the sample had been recooled to either −120°C or −196°C. (The lower temperature did not improve replica quality in any obvious way.) The replicas were cleaned in chrome sulfuric acid for several hours and then picked up on Formvar-carbon-coated 75-mesh grids. They were examined with a JEOL 100CX or 200CX electron microscope operated at 100 kv and photographed in stereo at ±10° tilt.

RESULTS

Basic Anatomy of the Terminal Web Region

From the literature we know that the apical cytoplasm or terminal web of intestinal epithelial cells contain at least three types of filaments. First and most prominent are the actin filament bundles that extend from the microvilli into the apex of the cell. These bundles are in essence "rootlets" of the core filament bundles in the microvilli proper (see references 2, 6, 17, 20, 27, 31). Second is the population of distinct intermediate filaments which forms a more-or-less distinct stratum beneath the rootlets with a minimal degree of intermingling of the two filament types (17, 31). Third is the more intriguing mass of less well-defined filaments that appear to run horizontally among the rootlets and between the rootlets and the underlying intermediate filaments. Earlier observers concluded that these were individual actin filaments, derived either from a splaying of the rootlet filaments (17) and/or from actin filaments that attach to the zonula adherens (17, 37). More recently Begg et al. (2) demonstrated that thinner filaments exist in this region which do not decorate with S_1 and are therefore not actin. Hirokawa and Heuser (14) extended this observation by showing that in deep-etch preparations these thinner nonactin filaments extend horizontally and mostly connect rootlets. Here, we will provide a more detailed description of this region of the cell and present evidence that these thinner filaments could be in part myosin.

When we rapidly isolated small segments of mouse intestine and quick-froze them without any fixation or other treatment, epithelial cells were so filled with granular material that the cytoskeleton was obscured. We presume that the obscuring material was soluble cytoplasmic protein (14) since it disappeared when the cells were broken open. Because we wished to preserve the terminal web region in as natural a state as possible, we attempted to break cells by very mild methods. Indeed, examination of our preparations by light microscopy revealed that in most cases the brush borders remained attached to their nuclei (see Fig. 6), a situation which does not occur if the epithelial sheets are homogenized by more vigorous methods.

The basic structure of the terminal web as seen in replicas of quick-frozen brush borders is illustrated in Fig. 1. The rootlets, known to be composed of actin, appear as compact bundles of finely striped 8-nm filaments which extend from the microvilli into the terminal web. Interconnecting the rootlets is a population of smooth surface fibrils which are obviously thinner than the actin filaments and are making very complicated networks between rootlets. These fibrils often appear to branch, a feature most easily confirmed by observing replicas three-dimensionally. The bulk of these interconnecting fibrils lies near the basal end of the rootlets, but some connect rootlets with the apical plasma membrane. Beneath the rootlets is a large population of smooth-surfaced filaments ~12 nm in diameter. These can be identified as intermediate filaments (see reference 12). They do not branch, a feature readily confirmed by observing stereo pairs. Often they curve up and loop around the fibrils that interconnect the rootlets.

In the present series of experiments, we carefully examined three-dimensional views of fields such as those presented in Figs. 1 and 2, to ascertain how much fraying of the rootlets occurs at their basal ends. From these fields and many others we gained the overall impression that rootlets do not fray out at their basal ends; or if they do, it is to such a limited extent

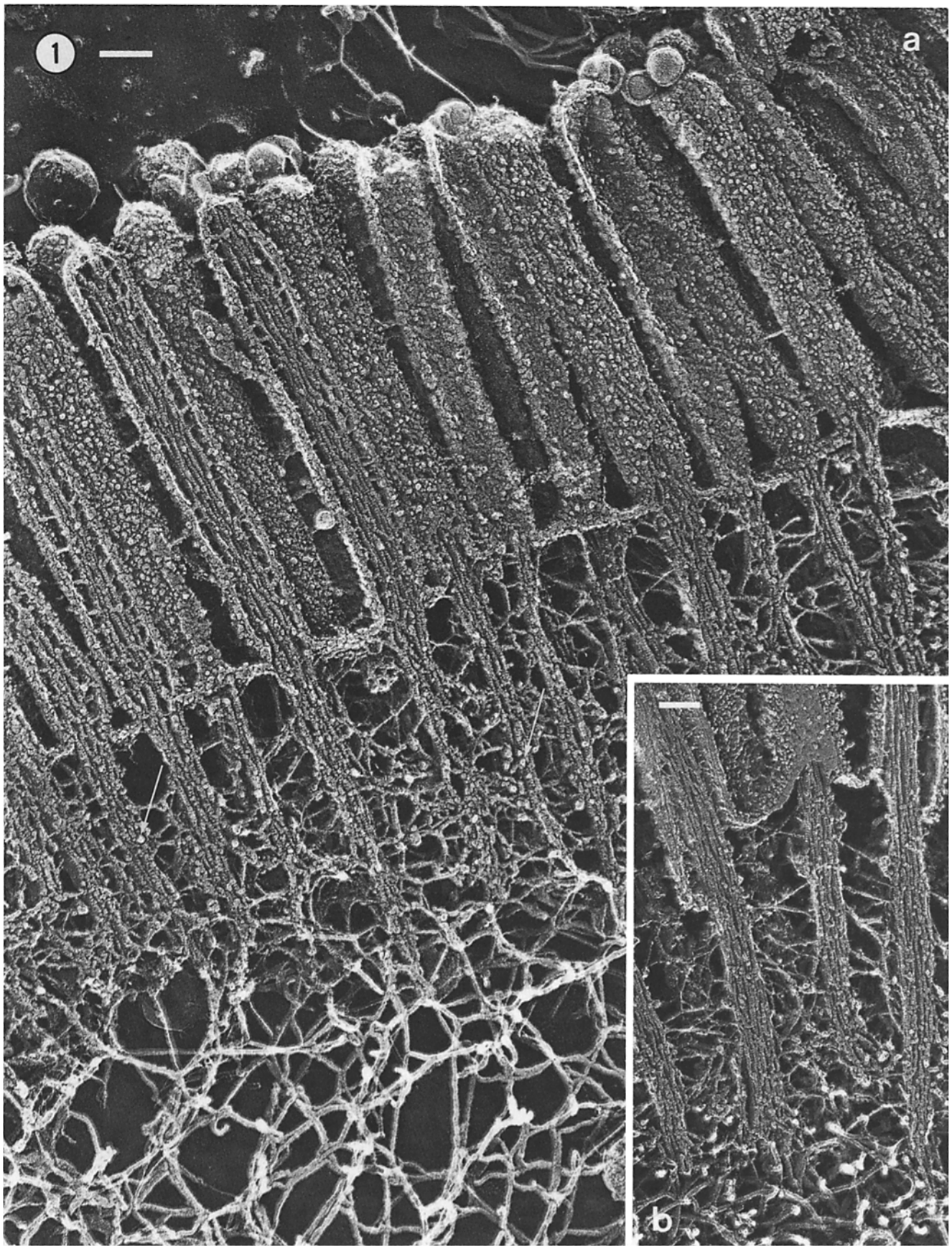


FIGURE 1 (*a* and *b*) The terminal web of a control brush-border preparation fixed, washed with distilled water, and quick frozen. Tight bundles of actin filaments extend out of the microvilli to form straight "rootlets." In between the rootlets are found a number of delicate cross-links which appear as fine fibrils. Note that fibrils tend to form very complicated networks at the basal part of the rootlets. Small dots (arrows) on the rootlets appear to be the remnants of fine fibrils that were cross-fractured. The rootlets rest upon a tangle of thicker intermediate filaments located at the bottom of this field. Notice that the membrane covering the microvilli is a studded with numerous, irregularly shaped bumps. Bar, 0.1 μm. (*a*) × 97,000. (*b*) × 77,000.

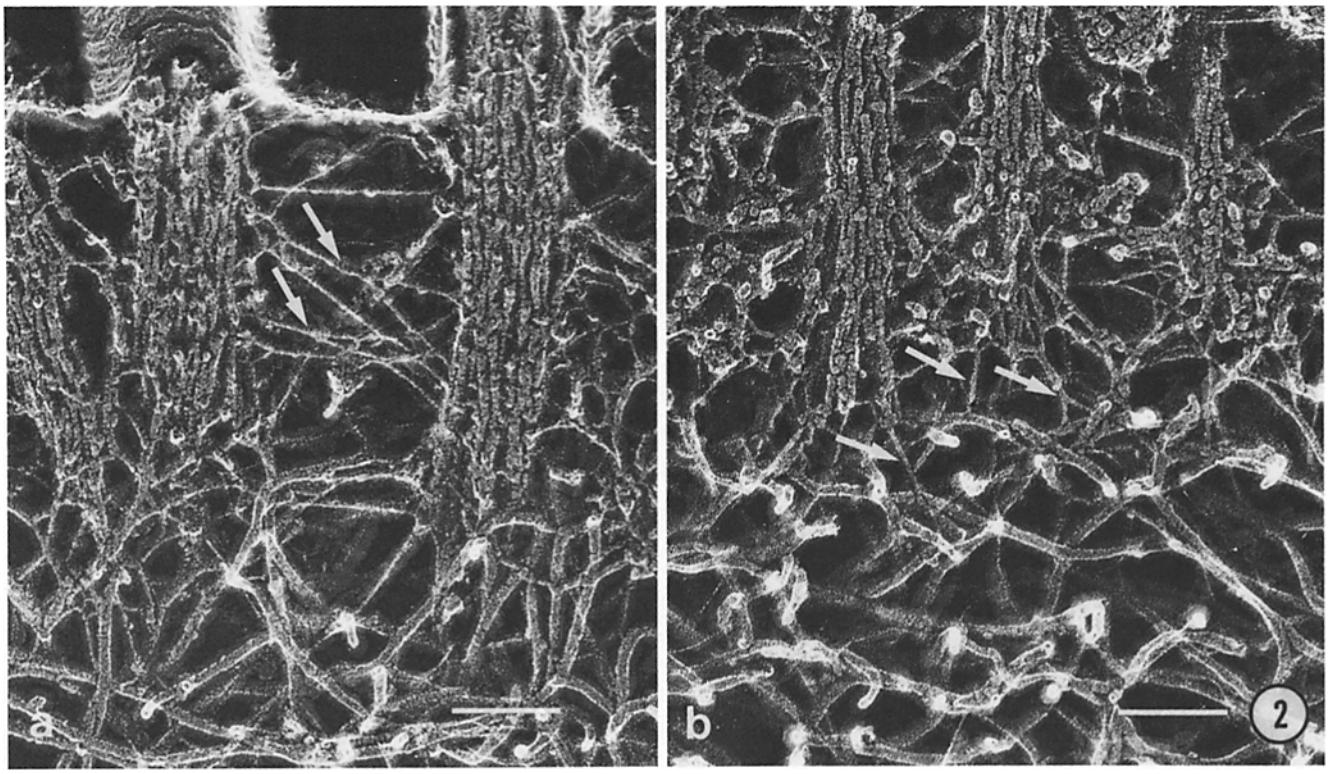


FIGURE 2 High magnification views of control brush borders prepared as in Fig. 1, to illustrate the thin connecting fibrils which run between adjacent microvillar rootlets (arrows in *a*) and between the rootlets and underlying intermediate filaments (arrows in *b*). These fibrils measure ~6 nm in diameter and from 0.1–0.2 μm n length. × 144,000.

that actin filaments from adjacent rootlets never overlap with each other as postulated in certain models of motility (30, 37). To convince ourselves that such compactness of the rootlet bundles was not due to fixation, we also examined unfixed brush border preparations, which looked the same, and we examined intact cells that had been frozen while alive. In these, a granular material tended to obscure the individual filaments; nevertheless, we could see that the rootlet bundles were compact throughout the terminal web. Thus we conclude that rootlet filaments form compact bundles in the living cell and do not bend enough to overlap with each other.

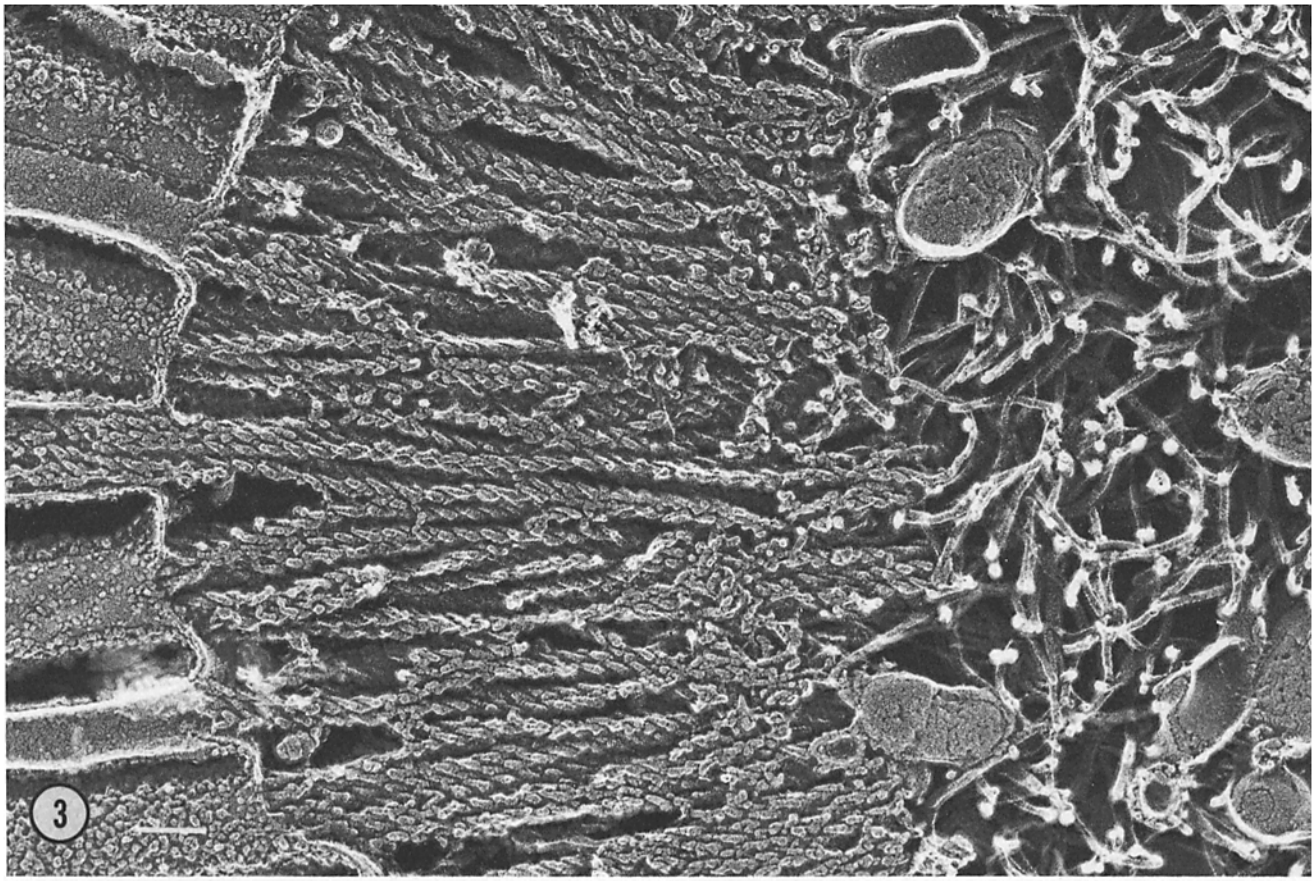
A second conclusion that we could reach from three-dimensional examination of our replicas was that the number of actin filaments that run horizontally across the terminal web is less than was formerly thought (17, 37). Rather than filaments, we find instead numerous finer fibrils that interconnect rootlets (Fig. 2*a*), connect rootlets to intermediate filaments (Fig. 2*b*) or connect rootlets to the plasma membrane. Sometimes we observed horizontally oriented filaments which had the appropriate diameter for actin. However, the number of such "candidates" was low, so even if all of them were actin, there would still be fewer horizontally deployed actin filaments that had been supposed in earlier publications (8, 17) (cf. Fig. 20 of [17]). The only place where we could unequivocally identify a significant concentration of actin filaments was in the discrete bundles which circle each cell just inside the zonula adherens. This ring courses perpendicular to the rootlets and will be analyzed in a subsequent publication (15).

The fine cross-linking fibrils in the terminal web are more numerous at the basal end of the rootlets than at their apical ends (Figs. 1 and 2). They vary in length from 100 to 200 nm. We measured the lengths of 168 examples in 50 different stereo micrographs. This yielded an average length of 176 nm with a standard deviation of 22 nm. The widths of these fibrils also varies, particularly because they frequently branch, but they yield an average value of 6 ± 2 nm in diameter, which is less than the 8–9-nm diameter of actin filaments in deep-etch replicas. (These parameters were the same in fixed and unfixed samples.) Fractures that travel through the terminal web parallel to the apical surface illustrated that these fine fibrils tend to run between nearest neighbor rootlets (see Fig. 9). However, we failed to observe any thicker filaments running between adjacent rootlets.

As already mentioned, intermediate filaments frequently loop up among the rootlets. Three-dimensional viewing also revealed that intermediate filaments are often connected to rootlet filaments by similar fine fibrils (Fig. 2*b*; arrows).

Decoration of Actin Filaments with S_1

Because there is always some uncertainty involved in identifying actin filaments in replicas by their thickness and by their fine striping, we also decorated them with S_1. In particular, we were anxious to confirm our earlier claims (14) that the fine fibrils in the brush border are not actin, and that relatively few actin filaments run perpendicular to the rootlet filaments. 20 min of incubation in 1 mg/ml S_1 turned out to be sufficient to decorate the brush border's actin completely (Fig. 3). With S_1 decoration the actin filaments in replicas look like twin-stranded twisted ropes (see references 12, 14). We found that a few of the elements that run horizontally assume the twisted rope configuration and thus must be actin, but these are fewer than most investigators have suspected (8, 17, 37). In fact, less than one filament in ten lies either horizontally or <45° to the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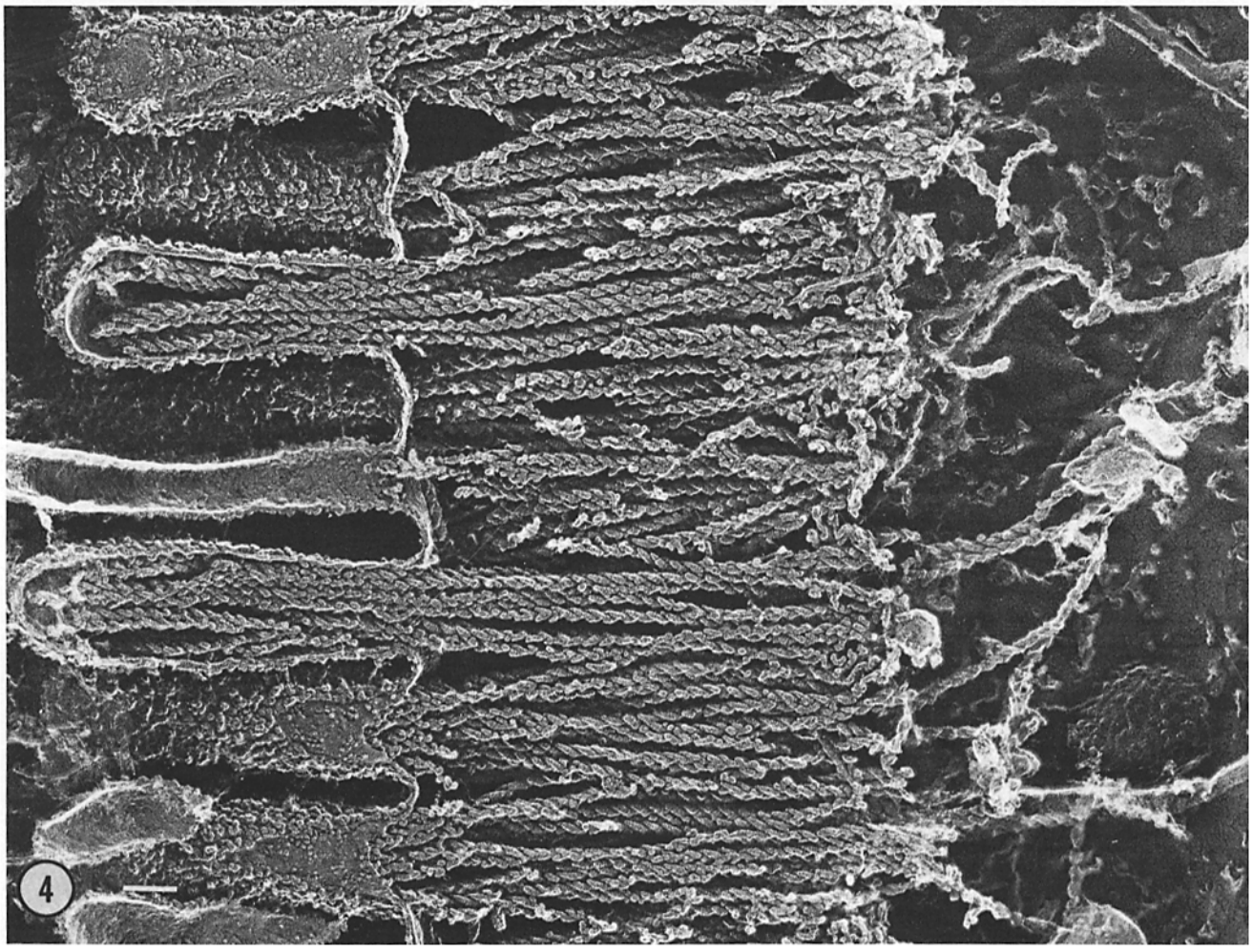
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rootlet axis. However, it was difficult to confirm that the thin cross-linkers were not actin because most of them were completely removed by the S_1 treatment (Figs. 3 and 4). The few that remained were clearly not decorated, as was expected, but their complete removal was certainly not expected. We attempted to prevent this phenomenon by prefixing brush-border preparations with formaldehyde before exposure to S_1, on the presumption that fixation would stabilize the cross-links; but to our further surprise, after 30 min of incubation in S_1, the decoration looked as complete as an unfixed preparations and the fine cross-linking fibrils were again completely gone. The only way we could prevent their loss was to reduce the duration of S_1 exposure to 10 min, in which case the actin filaments in fixed samples were not completely decorated and appeared somewhat knobby. In this case, many of the fine fibrils remained, but they did not assume the knobby texture of the adjacent actin filaments (Fig. 5), further confirming that they are not actin.

We also observed that when the S_1 incubation was prolonged to 1 h or the concentration of S_1 was increased to 2 mg/ml for 30 min, the thin cross-links between actin bundles were completely removed, the actin bundles were splayed wide apart, and the actin filaments within each bundle were no longer associated with the plasma membrane above or the intermediate filaments below (Fig. 4). Presumably, these prolonged incubations in high concentrations of S_1 eventually displaced all of the molecules that normally associate with actin and thereby hold the brush border together. With shorter times of incubation, e.g., 10–20 min at 1 mg/ml concentration, enough actin-intermediate filament associations persist so that the brush borders do not fall apart.

Three points emerged from these decoration experiments. First, fewer actin filaments run horizontally through the terminal web than was formerly thought. Second, the fine fibrils that cross-link the rootlets are not actin. Third, prolonged incubation in S_1 displaces all such fine cross-linking fibrils and separates the actin bundles from each other and from the underlying intermediate filaments.

Extraction of Myosin

EXTRACTION EXPERIMENTS: Brush border preparations were incubated in a variety of solutions in an effort to extract their myosin. They were evaluated for their content of myosin by light microscope immunocytochemistry (Fig. 6), SDS gel electrophoresis (Fig. 7), and by deep-etch electron microscopy (Figs. 8–11). Unexpectedly, several solutions which we thought would remove myosin actually failed to do so. These included Mg-ATP at 1 to 10 mM and sodium pyrophosphate at 20 to 40 mM. Even after brush border preparations had been exposed to these solutions for 60 min, our antimyosin antibody continued to react with them (Fig. 6) and they looked unchanged in the electron microscope (data not shown). Only when we followed a suggestion offered by Tom Pollard (John Hopkins Medical School), which was to incubate brush borders in ATP in a medium that contained a relatively high concentration of KCl (0.3 M), did we observe myosin extraction. After 15 min in such a solution, we could no longer demonstrate any myosin in the brush borders by immunocytochemistry (Fig. 6*c*). This was not simply due to the high ionic strength, because 0.3 M KCl in the absence of ATP did not extract myosin (Fig. 6*d*).

To substantiate the above results obtained by immunocytochemistry, we divided preparations of brush borders from the same animals into three aliquots. One was extracted with 0.3 M KCl, another with 0.3 M KCl and 1 mM ATP, while the third remained as an untreated control. The supernatants and pellets from these extractions were then run in SDS polyacrylamide gels (Fig. 7). These demonstrated that several proteins mostly associated with microvilli (see Fig. 1 of [28]), including some actin, were extracted by 0.3 M KCl. However, there was a clear difference between the high KCl supernatants with and without ATP. Myosin was released in significant amounts only when ATP was present. This substantiated the immunocytochemical data and also showed that the myosin extracted from the brush borders had not been proteolysed, since it did not have a different molecular weight than native myosin (compare lanes *1P* and *3S*, Fig. 7). (Note that the gels in Fig. 7 also show that ATP extracts a 110-kdalton protein from the brush border. This was first observed by Matsudaira and Burgess [22], who presented structural evidence that the 110-kdalton protein forms delicate actin bundle-to-membrane "bridge filaments" within the microvilli proper.)

To determine the location of myosin, we examined deep-etched replicas of myosin-extracted brush borders. These replicas present striking images (Figs. 8 and 10). Most of the thin fibrils normally seen between rootlets are gone. (This can be easily appreciated by comparing Figs. 1 and 9, control, with Figs. 8 and 10, an extracted sample.)

Although cross-fractures through the brush border (Fig. 8 and 9) do not display the intermediate filament layer because it is not in the plane of view, longitudinal fractures such as Fig. 10 illustrate that the intermediate filaments remain even after extraction of the thin fibrils with KCl and ATP. With these fibrils gone, it is possible to examine the physical association between intermediate filaments and rootlet actin filaments without obscuration by another population of filaments. A typical example of this association is shown in stereo in Fig. 11, which illustrates that intermediate filaments are connected to rootlet filaments by another type of thin fibril, one which is obviously not extracted as easily as is the type which runs between rootlets. (Unfortunately, the rootlets in such extracted preparations are somewhat distorted, as would be expected from the partial loss of actin that was apparent in the gels of Fig. 7.)

Examination of Skeletal Muscle Myosin in Replicas

We sought to compare the width and length of the fine fibrils in the brush border with purified myosin molecules or recon-

FIGURES 3 and 4 Fig. 3: A terminal web decorated with myosin S_1 (1 mg/ml for 20 min). The filaments which normally comprise the rootlets have splayed apart and have become completely covered with S_1, each assuming the appearance of a two-stranded twisted rope typical of decorated actin. In contrast, the intermediate filaments to the right have not been decorated. Note that all the thin interconnecting fibrils appear to be gone, yet the actin filaments remain attached to their foundation of intermediate filaments. Bar, 0.1 μm. × 95,000. Fig. 4: A terminal web decorated more heavily with S_1 (2 mg/ml for 30 min) to show the complete separation between actin and intermediate filaments which this treatment inevitably provokes. Often this treatment also causes the apical plasma membrane to separate from the actin bundles, though that has not happened in this particular field. Bar, 0.1 μm. × 70,000.

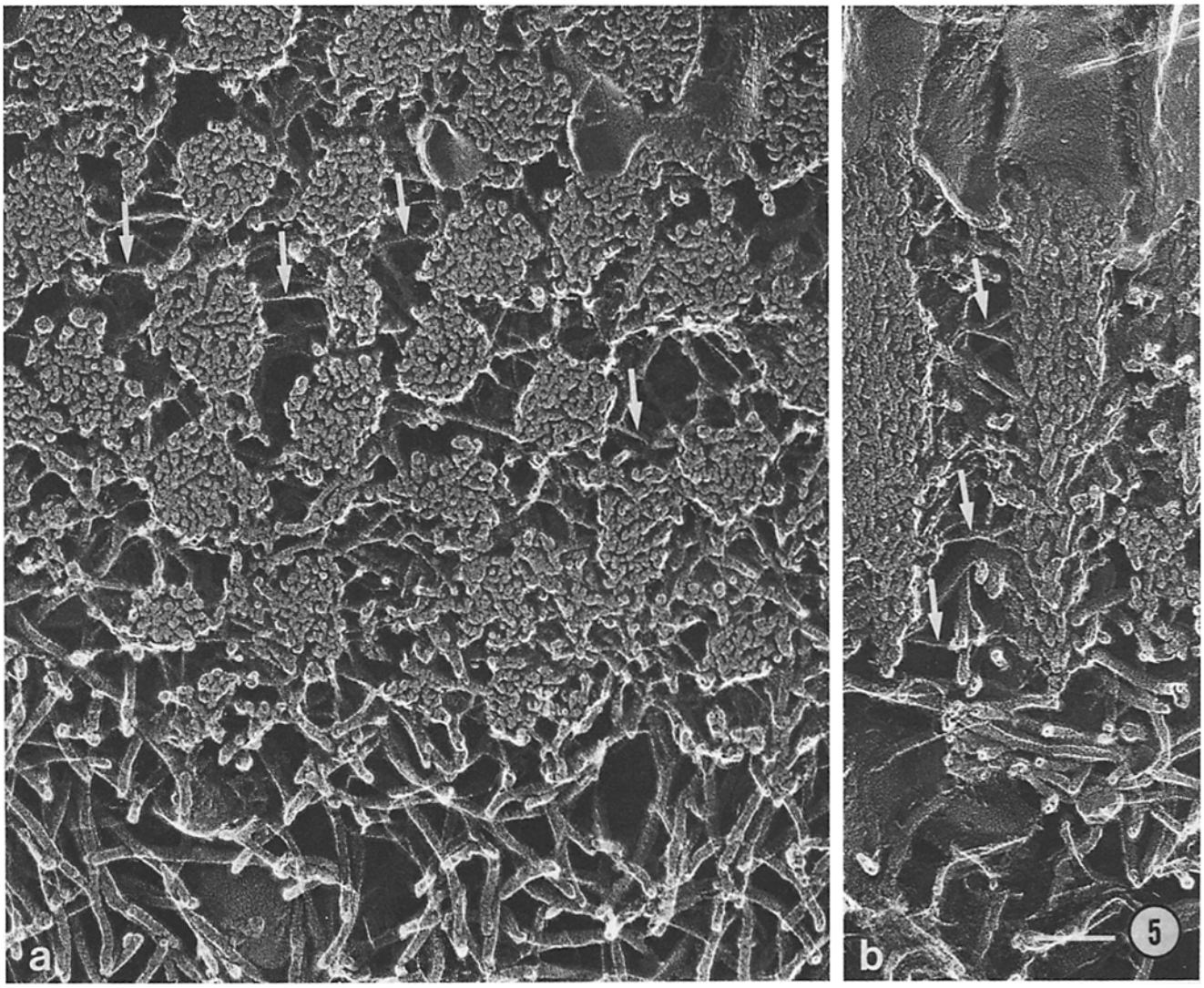


FIGURE 5 Two views of terminal webs prefixed with 1% formaldehyde before brief decoration with S_1 (1 mg/ml for only 10 min), and fractured such that the rootlets are displayed in cross section (*a*) and in longitudinal section (*b*). Although the rootlets have become somewhat thickened, decoration is incomplete, and as a result the thin cross-linking fibrils have not been displaced. A few examples of these cross-links are indicated by the arrows. Note how thin and delicate they are, compared to the thick intermediate filaments at the bottom of each field. Bar, 0.1 μm. × 119,000.

stituted myosin filaments by generating minifilaments of myosin (36) (composed of 16–18 molecules) and preparing them for electron microscopy by the same technique. We found the bare zone of 12 different minifilaments (see Fig. 11, *inset*) was ~150 nm in length and ~9 nm in width. The length of the myosin head appeared to be ~12 nm. In the Discussion, we will compare these dimensions with the fine fibrils seen in brush borders.

Electron Microscope Immunocytochemistry

Although a number of different techniques of antibody labeling were attempted, the best results were obtained by prefixing brush-border preparations with 1% formaldehyde before incubating them in the antimyosin antibody. This prevented structural deterioration of the samples during the long periods of incubation and washing needed for the indirect method, yet did not seem to diminish the samples's antigenicity to any noticeable extent. As depicted in Figs. 12 and 13 *a* and *c*, after incubation with antimyosin followed by ferritin-labeled goat anti-rabbit IgG, ferritin was predominantly found attached to the fine fibrils that existed between microvillar rootlets especially at the basal level (Figs. 12 and 13 *a*). (In the *insert* to Fig. 12 is displayed a replica of pure ferritin at the same magnification so that one can compare the size and shape of the pure molecule with the particle-clumps seen in the decorated brush border.)

It should be noted that there are also clumps of particles on the membrane limiting the microvilli. These particles are not ferritin because they are found in replicas of all our control and experimental samples (see Figs. 1, 3, 4, and 14) as well as in the replicas of Hirokawa and Heuser (14). They can be distinguished from ferritin because they are smaller and more irregular in shape. More specifically the membrane particles measure 9–12 nm in diameter in contrast to the ferritin particles which are 15 nm in diameter (Fig. 12). We should also mention that it is not uncommon to find small bumps attached to the surface of untreated rootlets (see Figs. 1 and 2). We presume that these bumps are the remnants of fine fibrils that extended to rootlets that were fractured away. These knobs were not confused with ferritin because they are smaller, measuring

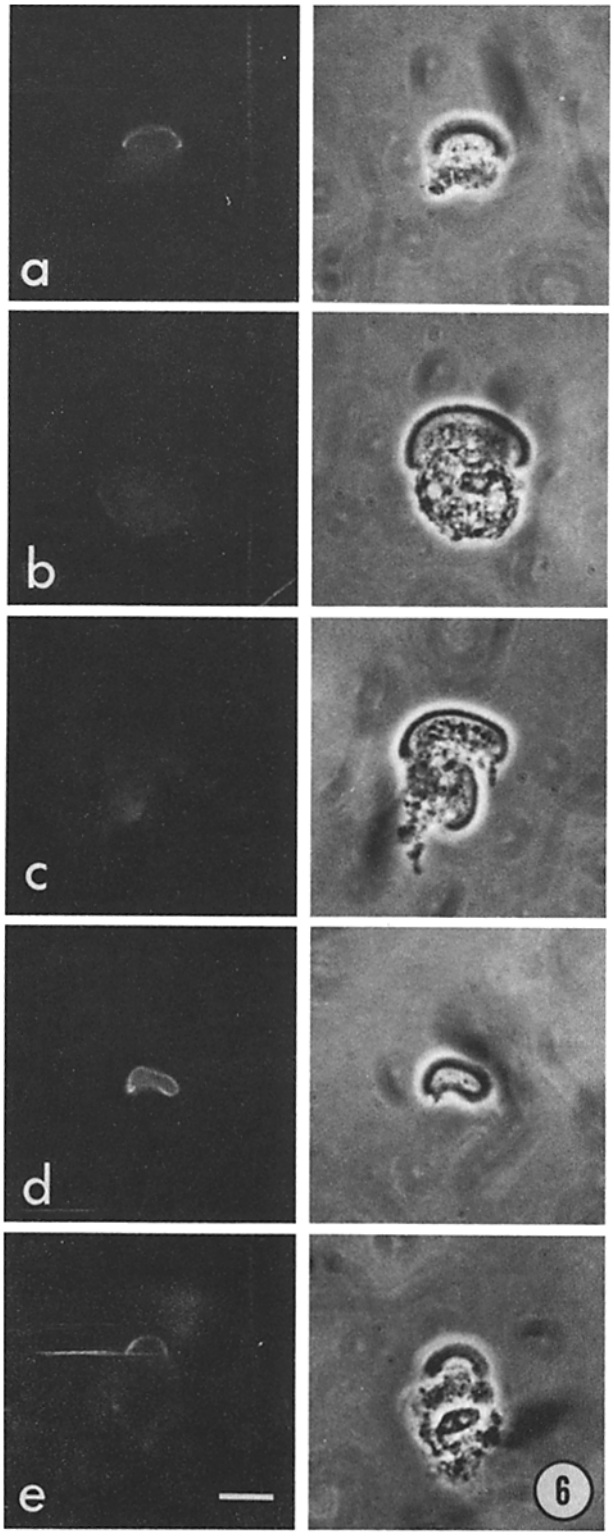


FIGURE 6 Light microscope immunocytochemical localization of myosin in intestinal brush borders, using antibodies to human platelet myosin, followed by fluorescein labeled goat anti-rabbit antibodies. Fluorescent images on the left compared to the same fields seen in phase contrast on the right. Experimental treatments were as follows: (*a*) control brush border with its normal complement of myosin; (*b*) control brush border incubated with preimmune serum to confirm the specificity of our immunocytochemical procedure; (*c*) brush border after exposure to Mg-ATP in high KCl (0.3 M); (*d*) after exposure to 0.3 M KCl alone, in the absence of ATP; and (*e*) after exposure to Mg-ATP in KCl (0.1 M). These results illustrate that the combination of high salt and ATP is required to remove myosin from this cell, and that neither treatment is sufficient by itself. Bar, 10 μm. × 750.

between 5 to 8 nm. We have indicated some of these knobs by the arrows on Fig. 1. (To aid in the identification of ferritin particles, Fig. 13*a* is reproduced at very low density, with ferritins accentuated by black dots, in Fig. 13*b*. Comparison of these figures should help the reader to appreciate what ferritin particles look like in deep-etch replicas.)

Examining the terminal web as a whole (Fig. 12) we concluded that ferritin particles are not found in the zones occupied exclusively by the intermediate filaments, nor are they found near the membranes limiting the microvilli. Instead they are most commonly seen near the bases of the rootlet bundles, where they appear to attach to the fine fibrils. This is especially clear in Fig. 13*c* in which the fracture plane passes in front of a rootlet and exposes its surrounding fibrils distinctly. These are abundantly coated with ferritin particles. Since this antibody is directed against the rod portion of the myosin molecule

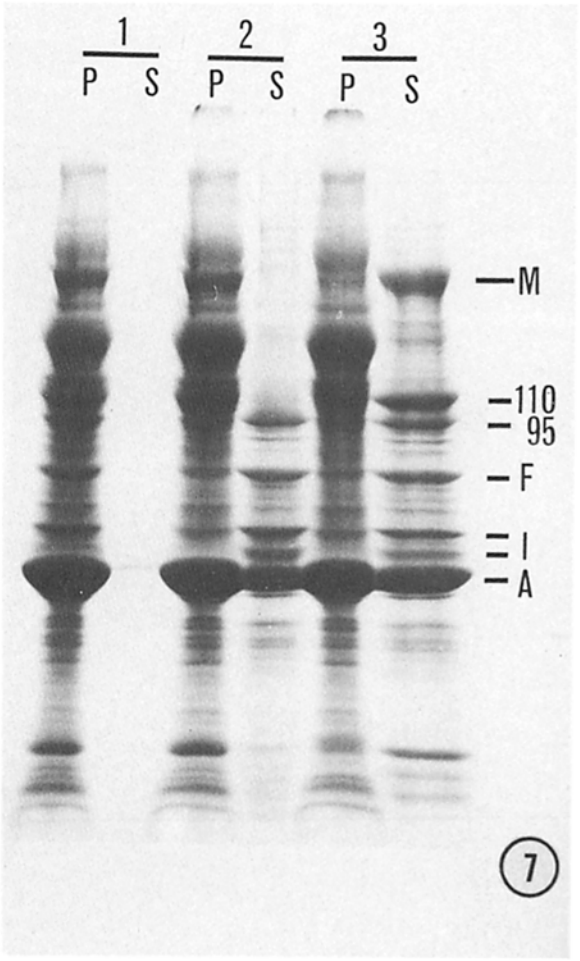
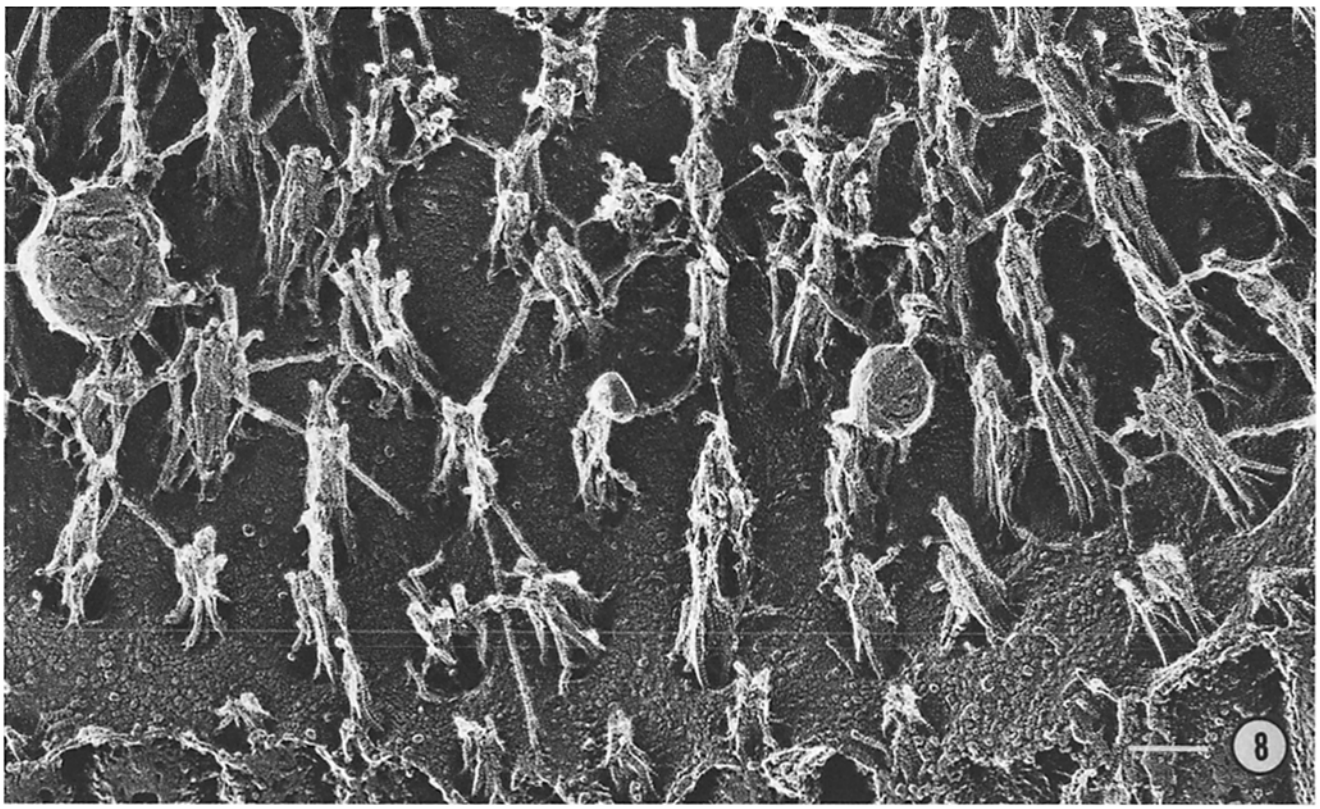
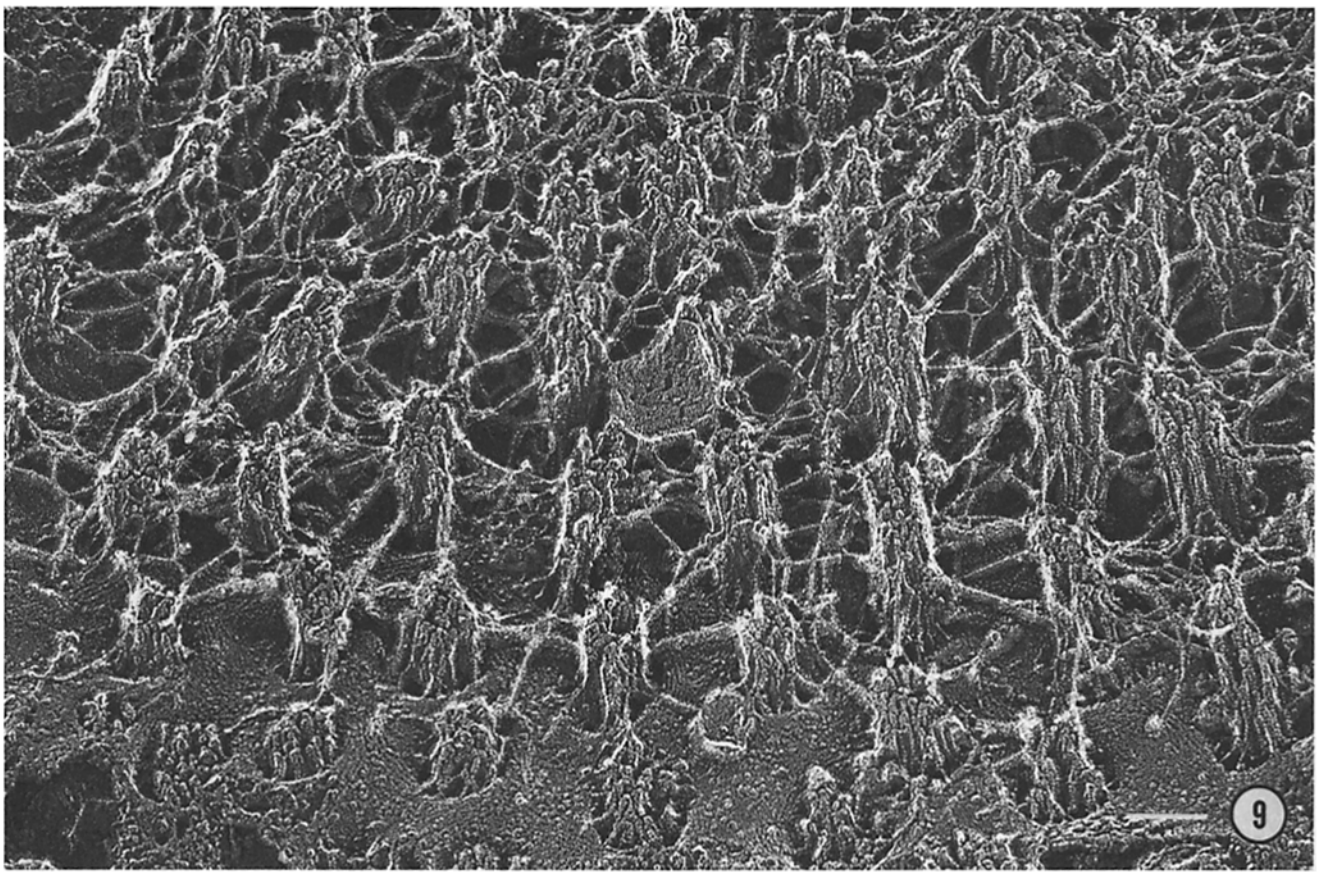


FIGURE 7 Polypeptides found in the pellets (*P*) and supernatants (*S*) of three brush border preparations: (*1*) not extracted at all (control); (*2*) exposed to 0.3 M KCl and (*3*) exposed to 0.3 M KCl containing 1 mM Mg-ATP. Shown are 4–16% gradient SDS PAGE. Abbreviations are: *M*, myosin heavy chain; *F*, fimbrin; *110*, 110-kdalton protein; *95*, 95-kdalton protein; *I*, 10 nm filament proteins; *A*, actin. (Note addition of ATP to the high KCl extracting solution resulted in the removal of two additional proteins, namely myosin and the 110-kdalton protein.) Gel courtesy of M. Mooseker.





FIGURES 8 and 9 Comparison between a brush border treated with Mg-ATP and high salt (Fig. 8) and a control (Fig. 9), both fractured obliquely through the terminal web and oriented so that microvilli would extend out the bottom of the field. This orientation dramatizes the almost complete disappearance of the cross-linking fibrils (Fig. 8), when compared to the dense network that is seen normally (Fig. 9). Note that without these cross-linkers, the actin filaments in the rootlets tend to become disordered and collapse together. Bar, 0.1 μm. × 107,000.

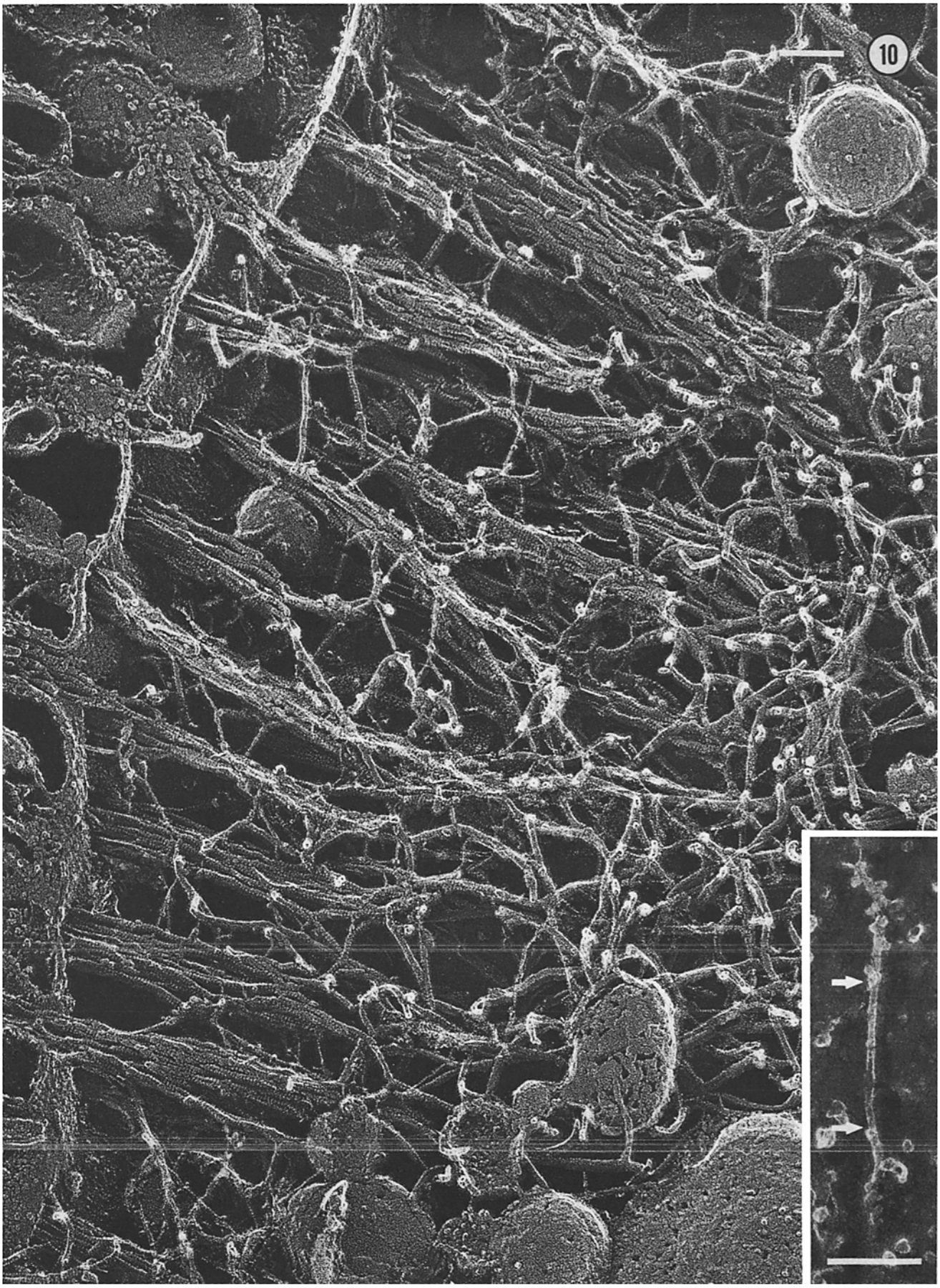


FIGURE 10 Higher magnification of a brush border treated with Mg-ATP and high KCl. The disappearance of cross-linking fibrils makes it easier to discern the relationship between the striped actin filaments and the intermediate filaments. Actin bundles in the rootlets are also somewhat distorted. Bar, 0.1 μm. × 121,000. *Inset:* replica of a minifilament of myosin. The arrows indicate the extent of the bare zone. Bar, 0.1 μm. × 175,000.

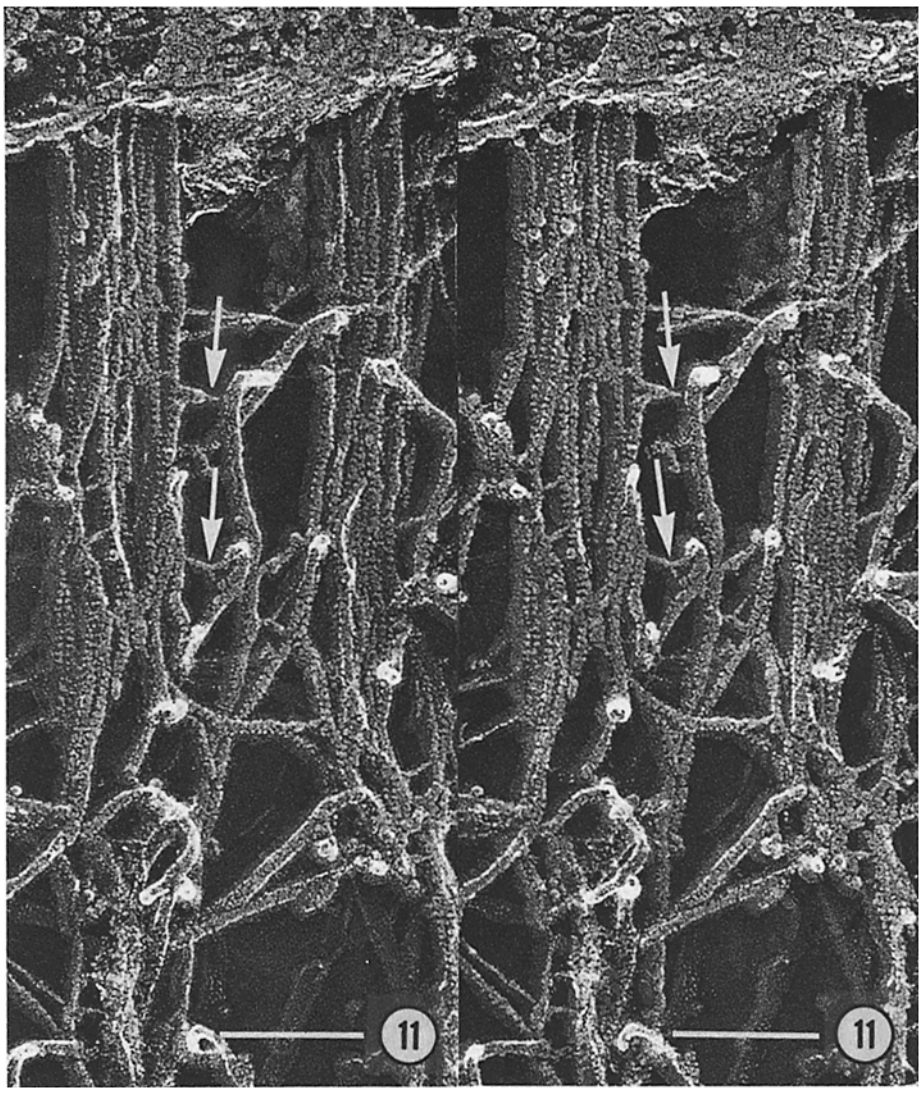


FIGURE 11 High mignification stereo view of a ATP-high salt treated brush border which displays particularly clearly the residual cross-links (arrows) that can be found between actin and intermediate filaments after most of the myosin has been extracted. Bar, 0.1 μm. × 194,000.

rather than its S_1 portion, we should have expected that the decoration with ferritin would be on the fine fibrils rather than on the rootlets themselves where the myosin heads are presumably located.

As a control for this electron microscope immunocytochemistry, brush borders were prefixed and then incubated in preimmune serum before the ferritin-conjugated second antibody (Fig. 13 *d*). Little ferritin was found under these conditions. As in untreated brush borders or brush borders incubated in antimyosin (Fig. 12), small knobs still appear on the surface of the rootlet filaments and particles are present on the membrane limiting the microvilli (Fig. 13 *d*); but neither of these are ferritin. Thus electron microscope immunocytochemistry localizes myosin to the fine fibrils that exist between rootlet bundles.

Effect of Calcium ATP on Brush Border Structure

Recently, several groups have observed that incubating brush borders in submillimolar levels of calcium chloride causes striking morphological changes in the actin filament bundles inside the microvilli proper (23, 28). This we could confirm by showing in our replicas that when brush borders were exposed to calcium chloride at concentrations of 0.2 to 0.5 mM, the microvillar actin bundles completely disappear and the microvillar membranes vesiculate (Fig. 14). We could also confirm that this disruption does not extend all the way down to the bases of the actin bundles, but leaves the rootlets intact. In addition, we found that the fibrils which interconnect these rootlets remain intact, as do the intermediate filaments beneath the rootlets. It was not surprising then, to find that brush border myosin remains demonstrable immunocytochemically after this exposure to calcium. On the other hand, after exposure to 0.1 mM ATP and lower levels of calcium (10^{-6} to 10^{-7} molar), we could not observe any change in the microvilli, their core of actin, nor the overall organization of the terminal web. Thus we could not confirm one recent report (28) that such low levels of calcium will also fragment the actin filaments in microvilli. We did not, however, demembranate our preparations with Triton X-100 before this calcium exposure that was done in the previous report (28). This might have made a substantial difference.

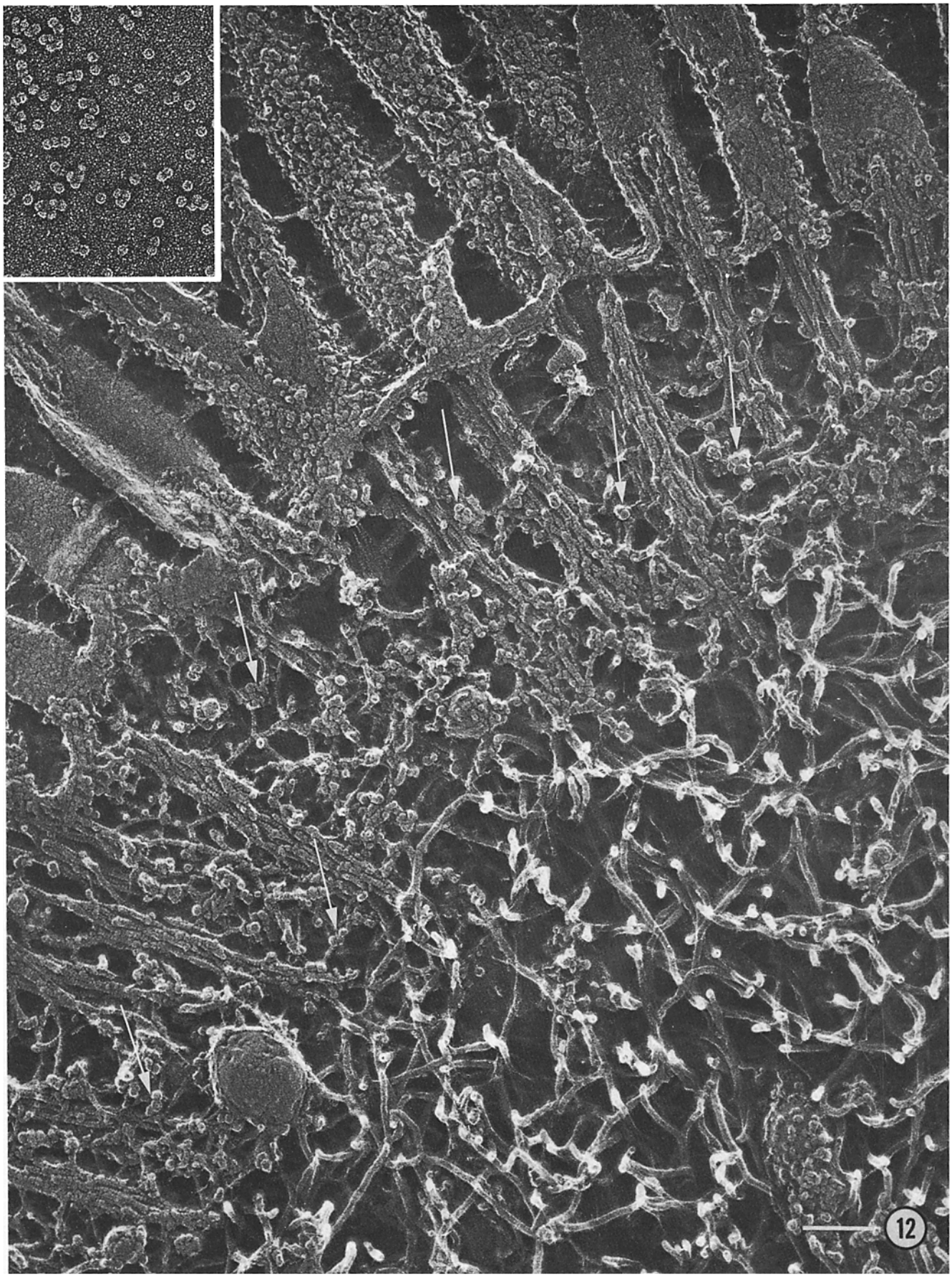


FIGURE 12 Replica of a brush border treated with rabbit antimyosin antibody followed by ferritin conjugated anti-rabbit IgG antibody. Decorating the fibrils between rootlets mainly at the basal level are small particles (arrows) which are the same size as pure ferritin (shown for comparison at the same magnification in the inset). Little or no ferritin is found among the underlying tangle of intermediate filaments, in the lower right of the field. The particles present on the microvillar membrane are not ferritin (see text). A membrane fragment seen at lower right is rough endoplasmic reticulum. Bar, 0.1 μm. × 131,000.

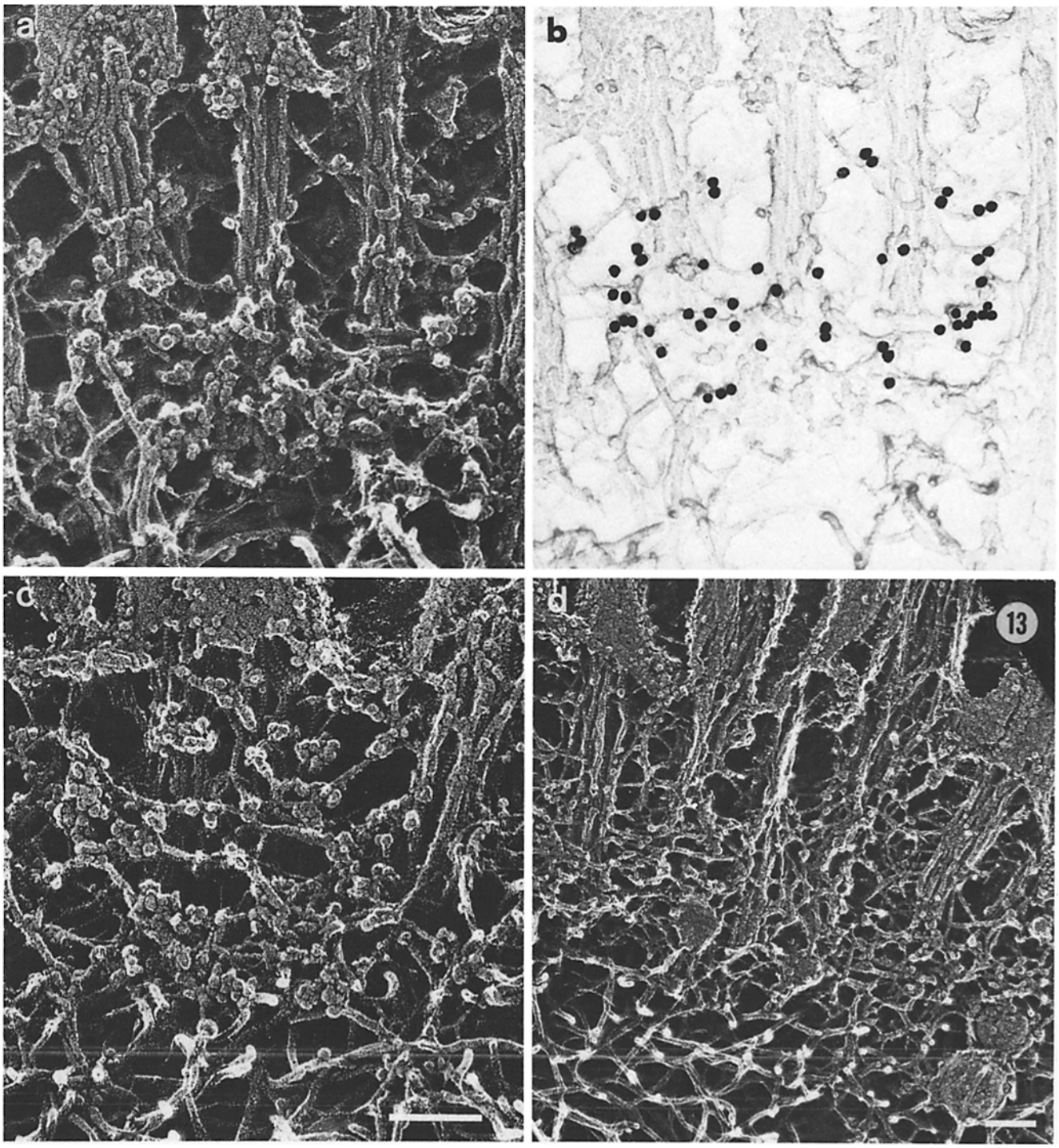


FIGURE 13 (*a*) Higher magnifications of the indirect immunocytochemical localization of myosin with ferritin-tagged antibodies shown in Fig. 12. Bar, 0.1 μm. × 160,000. (*b*) To aid the reader's identification of ferritin, we have included a lighter print of *a* on which we have blackened the regions of these micrographs occupied by ferritin. (*c*) This fracture passed directly behind several rootlet bundles thereby exposing in front of us the fine fibrils decorated with the ferritin tagged antibodies. Bar 0.1 μm. × 160,000. (*d*) A brush border incubated with preimmune serum (from the same rabbit which made the antimyosin antibodies) then washed and incubated with a ferritin labeled anti-rabbit IgG antibody. Notice that few ferritin particles are present in the terminal web. Bar, 0.1 μm. × 86,000.

DISCUSSION

Use of the Quick-freeze, Deep-etching Technique for Visualizing the Organization of Actomyosin Complexes in Nonmuscle Cells

As mentioned in the introductory section, what is needed for assessing the various models proposed to explain the in vitro movements of intestinal brush borders is a technique that bypasses osmium fixation, dehydration, and plastic embedding, yet which allows one to examine a potentially contractile system at high resolution and in three dimensions. As seen by the results, the quick-freezing technique has proved to be useful in this regard. It has provided an accurate determination of the

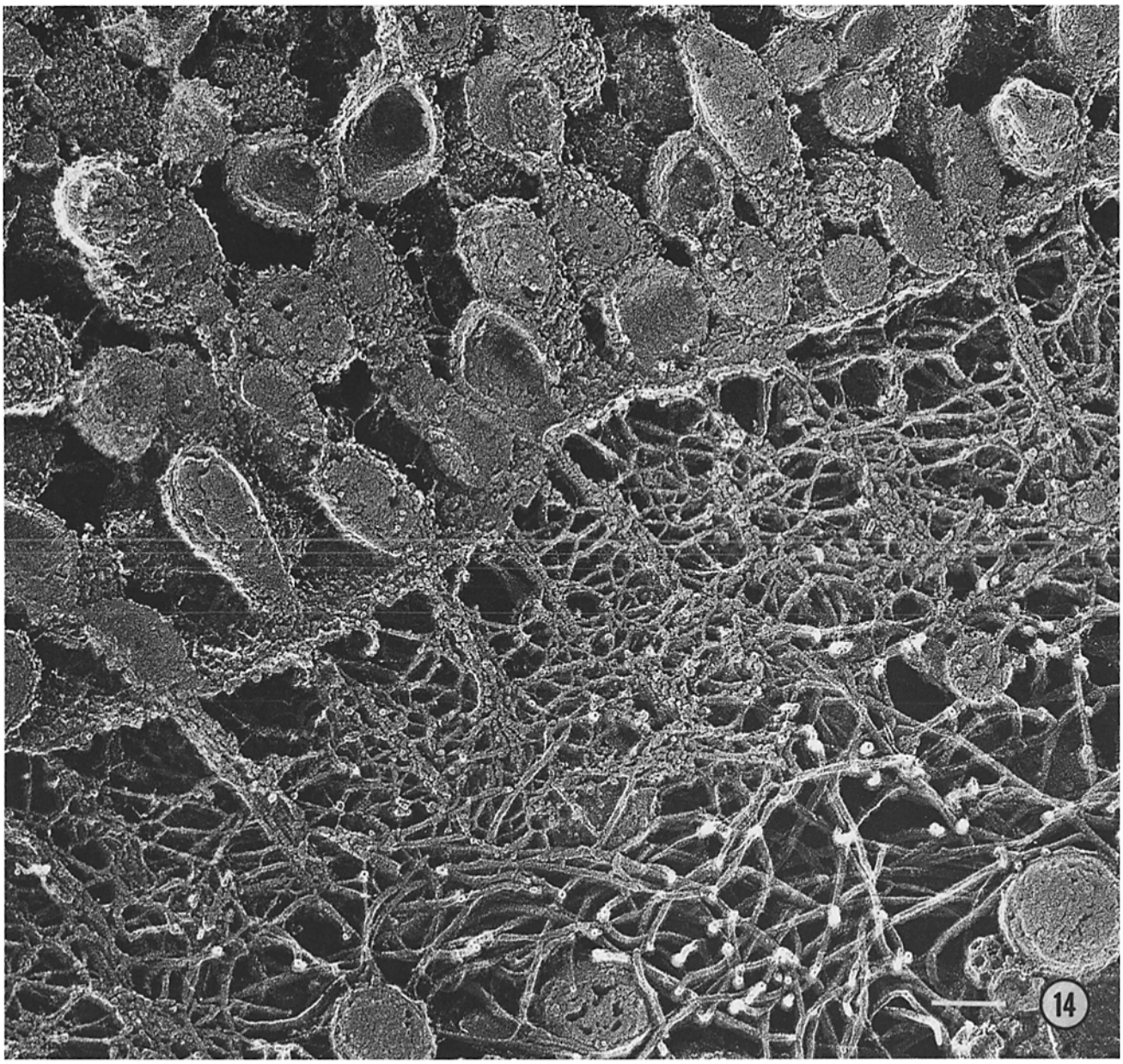


FIGURE 14 A deep-etch replica of a brush border incubated in 0.5 mM calcium chloride and 0.1 mM ATP. Our replicas confirm original reports of others, which demonstrated that this treatment results in complete disappearance of the actin bundles that are normally found inside of the microvilli. This apparently leads to extensive vesiculation of the microvillar membrane. Note, however, that the proximal ends of the microvillar actin bundles, the part which forms the rootlets, remain relatively intact. Also the fibrils which cross-link these rootlets remain unscathed. (Light microscope immunocytochemistry such as that shown in Fig. 7 confirm that such calcium chloride ATP treatment does not remove myosin.) Bar, 0.1 μm. × 109,000.

distribution of actin filaments in the terminal web, and new information on the form that myosin may take within this web. It has also shown that the intermediate filaments and the actin filaments are linked together by fine fibrils.

Myosin in the Brush Border

Recently there have been two reports (8, 11) demonstrating the distribution of myosin in the terminal web using antibodies to myosin. Both studies localized myosin at the electron microscope level and did so very convincingly. There shortcoming was that the fixation techniques they used were so severe that individual actin filaments could not be seen, nor could the myosin molecules be visualized directly. In contrast, the techniques employed in this study permitted recognition of individual actin filaments and possible myosin-containing structures, and helped to elucidate the mode of the interaction of these components.

Two lines of evidence were obtained which indicated that myosin molecules comprise at least in part the fine fibrils that interconnect rootlets. First, the brush borders extracted with ATP and high KCl lost these fine interconnecting fibrils and at the same time lost all immunocytochemically detectable myosin. Consistent with this, gel electrophoresis of the ATP-extracting solution demonstrated that the myosin had been released intact. (See below for further discussion of what else was released.) Second, incubation with antimyosin and ferritin-conjugated second antibodies resulted in the deposition of

ferritin mainly on the thin fibrils at the basal part of the rootlets.

Such localization of myosin to the fine fibrils that interconnect the rootlets fits well with the fact that the antibody we used was against the tail portion of the myosin molecule. A similar antibody raised against platelet myosin and conjugated directly to ferritin was used by Herman and Pollard (11), who found that the bulk of myosin in the terminal web is located around the rootlets. This was particularly evident in their unstained sections (see their Fig. 9 *b*). It is in this location that we found most of the thin fibrils which appear to be myosin.

A note of caution about potential artifacts in the technique used here was recently presented by Miller and Lassignal (25), who showed that when salts are present in a frozen sample, these can form artifactual "filaments" upon freeze-drying. However, we should stress that all the samples studied here were washed in distilled water after fixation, in order to remove all salts. Furthermore, they were not completely freeze-dried but were only etched briefly to expose the uppermost 0.25 μm of tissue. Under these conditions, salt is not a problem. Nonvolatile contaminants form continuous structures that look like filaments only when etching is prolonged to the point of very great removal of water, for example 1–2 h in the experiments of Miller and Lassignal (25). In contrast, the etching periods we used were usually 1–5 min. Thus there is no reason to suspect that the interconnecting fibrils that we observed are artifacts or residue.

Other Proteins in the Brush Border

The samples we extracted with ATP in high KCl also lost a 110-kdalton protein (cf. Fig. 7) which previous studies have also shown to be very easily solubilized by ATP (22). However, there are several reasons for why we do not believe that this 110-kdalton protein contributes to the delicate cross-linkers we see between rootlets. First, this protein is extracted by very low levels of ATP in isotonic buffers (22), while immunoreactive myosin and the cross-linkers in question are extracted efficiently by ATP in relatively hypertonic KCl. Second, previous low-ATP extraction experiments have shown that the lose of 110-kdalton protein is associated primarily with the loss of delicate "lateral arms" that connect the actin bundles within microvilli to the surrounding plasma membrane. These delicate 10 nm arms are structurally very different that the relatively stout 150 nm cross-bridges under consideration here. (We should add at this point that a report appeared during revision of this manuscript which showed by light and electron microscope immunocytochemistry that the 110-kdalton protein extends out of the microvilli and into the terminal web to some extent, and is also found beneath the plasma membrane in the lateral and basal portions of this cell type [7].) However, our results do not exclude a possibility that some other proteins, such as filamin (3), also contribute cross-linking fibrils between rootlets and between rootlets and plasma membrane, because several unidentified proteins were additionally released by high KCl and high KCl plus ATP (see Fig. 7), and because not necessarily all the cross-linkers were stained by antimyosin. Further detailed analysis will follow this report.

Physical State of Myosin in the Brush Border

By analogy with skeletal muscle (18, 19), one might imagine that the myosin in nonmuscle cells ought to be organized into bipolar filaments. In support of this is the observation that small bipolar filaments can be formed in vitro from platelet myosin (32, 34) and from brush border myosin (29). However, myosin filaments of comparable dimensions to those in skeletal muscle are not usually found in nonmuscle cells. Two prominent exceptions to this statement occur in *Protozoa*, namely in giant amoeba (33) and in the slime mold *Physarum* (1). (Actually, thick filaments have been reported in contracting platelets [29], but we doubt that these were actually myosin filaments. In the case of platelets, the cells had been treated with unusually high concentrations of calcium chloride which may have aggregated some other filament type.)

Three reasons have been offered to explain why thick myosin filaments are not observed in nonmuscle cells. One is that the concentration of myosin in such cells is so low that thick filaments would be overlooked in thin sections, particularly because they would usually be cut obliquely. Second is that fixation, dehydration, and embedding may alter their size or even dissolve them. Third is that myosin in nonmuscle cells may not be assembled into such thick filaments in vivo (11), but instead may occur as thinner bipolar filaments whose widths might be the same as actin or intermediate filaments (see references 9 and 27).

In this study we used a preparative technique that should not be subject to many of the limitations outlined above, and yet we still failed to find thick filaments in the brush border. Instead, we found that thinner cross-linking fibrils correlated with the presence of myosin. From the dimensions of these fibrils, we sought to learn whether myosin is present as monomers, dimers, or short oligomers. (This would be important to know, because even though the presence of myosin has been established in every cell where it has been sought, by either immunocytochemistry or biochemistry [3, 8, 10, 11, 29, 34, 36, 43, 45, 48), in none of these cases has its state of organization been determined.)

To do this, we compared the parameters of the fibrils observed *in situ* with synthetic myosin filaments prepared in vitro. Unfortunately, we were unable to form bipolar filaments as small as two to four myosin molecules. The best we could do was to make minifilaments composed of 16–18 myosin molecules and replicate these. We found that these minifilaments have bare zones in their centers of ~150 nm. (These bare zones are the regions in which myosin molecules are presumed to overlap [32] and thus are an indication of the length of the "rod" portion of the molecule.) The value of 150 nm is entirely consistent with previous measurements of this portion of the myosin molecule (9, 32). We presume that the fine fibrils observed in the brush border consist primarily of these "rod" portions of myosin. Consistent with this, we find that their lengths average 176 nm. (Those >150 nm may represent ones in which the myosin "heads" are also partially represented. These S_1 "heads" are reported to be ~15 nm long [26], so they would add some ~30 nm to a simple bimolecular fibril, bringing its total length to ~180 nm.) Thus from length measurements, we would suspect that the fine fibrils may be two to four opposed myosin molecules. The measured width of these fibrils (6 ± 2 nm) substantiates this idea. The rod portions of individual myosin molecules have been reported to be ~2 nm in diameter, both in platinum replicas (9) and in negative staining (32). Hence fibrils 6 nm in diameter could well be composed of two to four overlapping, opposed myosin molecules.

However, this conclusion would make it very hard to understand why more myosin was not extracted from the brush border simply with Mg-ATP. It ought to have been, if it were present as bipolar filaments that were attached only by their

opposed heads. But even though we varied dozens of different factors during the application of ATP, we could never extract the brush border myosin the way that Zigmond et al. (48), for example, removed myosin from fibroblasts. Only when we applied Mg-ATP in an unusually high concentration of KCl (0.3 M) could we remove it. We must conclude that myosin in the brush border is organized very differently from that in fibroblasts.

Interactions between Actin Filaments and Intermediate Filaments

A subsidiary finding in this study was that a different sort of cross-connection exists between actin filaments and intermediate filaments. These connections look very much like the fine fibrils that interconnect the rootlets, but they behave differently in that they are not removed by Mg-ATP in high salt. Indeed such connections could be seen in many preparations, but they were particularly noticeable after most of the cross-linking fibrils between rootlets had been extracted. On the other hand, this separate class of connections between actin and intermediate filaments could itself be removed by prolonged incubation in high concentrations of S_1. This treatment caused all such connections to disappear, and apparently freed the actin filaments from their underpinning of intermediate filaments, because the two filament types could no longer found together. We interpret this as indicating that S_1 can displace these fibrilar connections at their attachments to actin; but this need not imply that the fibrils are myosin. Prolonged decoration with S_1 also displaces the actin bundles from their attachments to the microvillar membranes and allows the bundles to splay apart, indicating that S_1 can also disrupt actin's attachments to several other structures. Not all of these associations are likely to be myosin mediated, yet S_1 evidently can compete off whatever protein is involved.

Relating Existing Models of Brush Border Motility to this New Structural Information

Three models have been proposed to explain the motion of the brush border in vitro; these are diagrammed in Fig. 15. The model that was initially suggested by Mooseker and Tilney (30) (Fig. 15*a*) and reiterated by Hull and Staehelin (17) assumed that some of the rootlet actin filaments splayed laterally and overlapped with the actin filaments from adjacent rootlets. This model proposed that short bipolar myosin filaments spanned these opposed actin filaments and when activated, pulled the actins together in a manner analogous to the contraction of skeletal muscle. This model further assumed that the overall deployment of actin somehow translated contraction into a shortening or movement of microvilli. Unfortunately, our present observations are inconsistent with this model. The actin filaments in the rootlets do not splay apart and do not bend enough to overlap with each other. Instead, the rootlet filaments are straight and are cross-linked to both their neighboring rootlets and to an underpinning of the intermediate filaments.

A second model was proposed by Rodewald et al. (37) to explain the observation that when ATP is added to brush borders, they develop a circumferential constriction at the level of the zonula adherens. To explain this shape change, Rodewald et al. (37) postulated that rootlet filaments splay apart and interact not only with actin filaments in adjacent rootlets, but also with actin filaments which run horizontally from the zonula adherens. They imagined that small bipolar myosin oligomers span these two actin systems and thereby draw the cell margins inward during contraction. They further proposed that the constriction seen in vitro might be manifest as microvillar shortening in vivo, when adjacent cell margins would still be attached to each other. Thus the proposed mechanics of movement was essentially identical to the first model. Unfortunately, our data do not fit this model any better, because the actin filaments in the rootlets do not bend enough to overlap with each other or with the horizontally running actin filaments. However, we have observed a prominent circumferential band of actin filaments just inside the intermediate junction which may explain the constriction they observed. We would

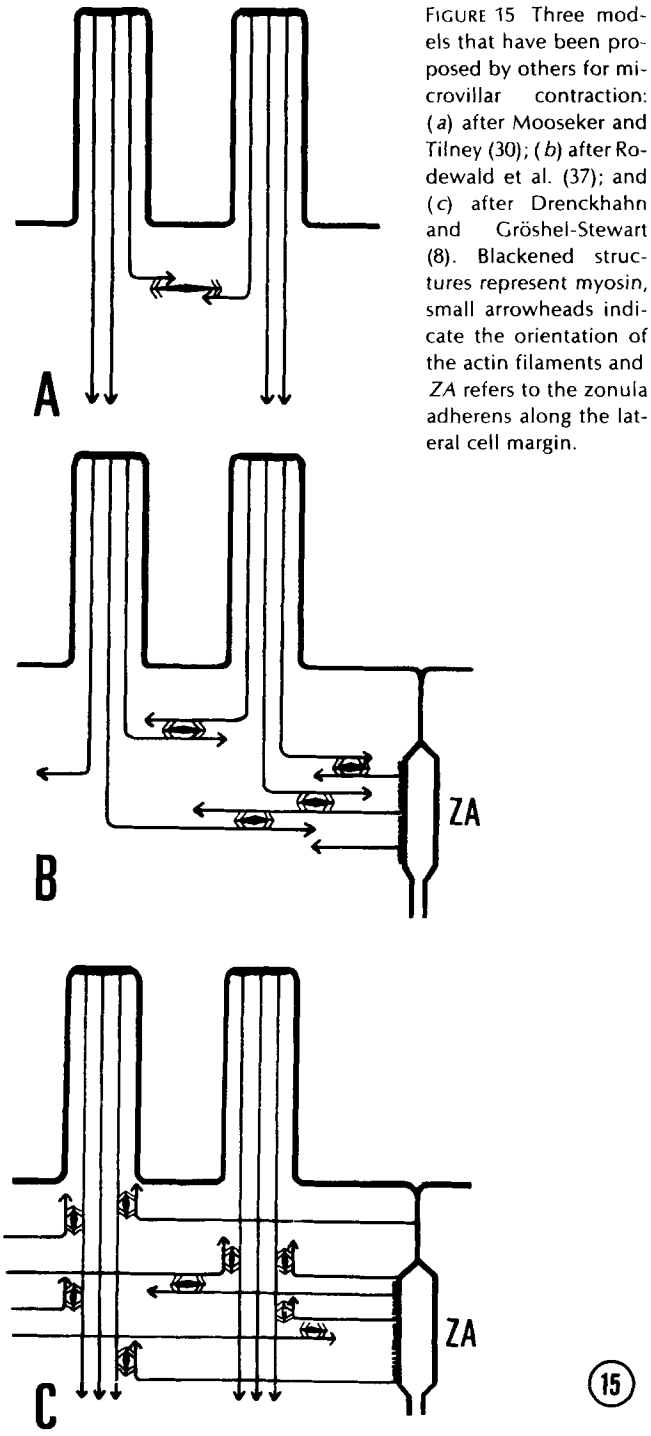


FIGURE 15 Three models that have been proposed by others for microvillar contraction: (*a*) after Mooseker and Tilney (30); (*b*) after Rodewald et al. (37); and (*c*) after Drenckhahn and Gröshel-Stewart (8). Blackened structures represent myosin, small arrowheads indicate the orientation of the actin filaments and *ZA* refers to the zonula adherens along the lateral cell margin.

suggest, as an alternative to their model, that this circumferential band may be analogous to the "contractile ring" of dividing animal cells (41). In support of this idea, we have determined the polarity of the actin filaments in a similar type of cricumferential band that occurs in the brush border in another type of epithelial cell; namely the hair cell of the ear (15). In that case, the actin filaments have mixed polarities, so they could act together with myosin to constrict the apical margin of the cell just as Rodewald et al. (37) observed.

Recently, a third model has been proposed by Drenckhahn and Gröschel-Stewart (8) which is a variant of the previous two. These observers conclude as we have that the rootlet filaments do not splay apart, but they still argue for microvillar shortening by proposing that the rootlet as a whole interacts via myosin with a broad network of horizontally oriented actin filaments. They further propose that the horizontal actin filaments are stabilized by association with the lateral cell margins at the level of the zonula occuludens and zonula adherens, such that the force generated between this system and the rigid actin rootlets is translated into a pistonlike sliding of the rootlets deeper into the cell. Regretably, our data do not seem to fit with this model either. It also would predict that a number of actin filaments should run horizontally to the rootlets and should be anchored to the zonula occuludens and zonula adherens. We see too few horizontally deployed actin filaments to provide the necessary stabilizing foundation for a myosin sleeve around each rootlet. Moreover, we find that the rootlets sit directly upon a dense network of intermediate filaments and are linked with it by many cross-connections, and regardless of whether ATP is present or not, the rootlets never appear to penetrate deeply into this intermediate filament network. Thus there doesn't seem to be any room for the core filament bundles to move deeper into the cell.

We are left with the disturbing impression that, even though the brush border contains all the proteins that would be needed to generate an actin-based movement (including myosin, a kinase for this myosin, tropomyosin, and calmodulin), the actin network appears to be so extensively and so stably cross-linked to itself and to underlying intermediate filaments that movement seems unlikely. Myosin appears to be one of the major cross-linkers in this system, yet we have not found conditions under which it can be made to alter the deployment of actin filaments.

The time is right for a definitive analysis at the light microscope level of whether or not the microvilli in this cell ever do move or ever do shorten. Only then will we know what sort of internal change to look for or have a clue about what in vitro condition would bring this movement about.

We wish to thank A. Weber, University of Pennsylvania, and Tom Pollard, Johns Hopkins Medical School, for their many needed and helpful suggestions. We also wish to thank A. Weber for her repeated gifts of S_1 and myosin and M. Mooseker, Yale University, for his great help with the SDS gel electrophoresis. Thanks also to J. Wuelling and M. Tilney for typing this manuscript, to M. Zimmerman for photographic works, and to the editors of this paper for their helpful comments which greatly improved it.

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Note Added in Proof: Recently we (Hirokawa and Cheney, unpublished observations) found that antifodrin (Fodrin: 240 and 250 kdaltons) also stains some of the cross-linking filaments in the terminal web. These filaments tend to be gone after high KCl and high KCl plus ATP treatments and relatively small amounts of high molecular weight proteins reacting with antifodrin are released in the supernatants of the high KCl and high KCl plus ATP treatments. We also found that the actin filaments in the circumferential band inside the intermediate junction of intestinal epithelial cells have mixed polarities. Because immunocytochemically myosin exists at this region, these actin rings could really work as "contractile rings."

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