

Quick-Freeze, Deep-Etch Visualization of the Cytoskeleton Beneath Surface Differentiations of Intestinal Epithelial Cells

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ABSTRACT The cytoskeleton that supports microvilli in intestinal epithelial cells was visualized by the quick-freeze, deep-etch, rotary-replication technique (Heuser and Salpeter. 1979. *J. Cell Biol.* 82: 150). Before quick freezing, cells were exposed to detergents or broken open physically to clear away the granular material in their cytoplasm that would otherwise obscure the view. After such extraction, cells still displayed a characteristic organization of cytoskeletal filaments in their interiors. Platinum replicas of these cytoskeletons had sufficient resolution to allow us to identify the filament types present, and to determine their characteristic patterns of interaction. The most important new finding was that the apical "terminal web" in these cells, which supports the microvilli via their core bundles of actin filaments, does not itself contain very much actin but instead is comprised largely of narrow strands that interconnect adjacent actin bundles with one another and with the underlying base of intermediate filaments. These strands are slightly thinner than actin, do not display actin's 53 Å periodicity, and do not decorate with myosin subfragment S₁. On the contrary, two lines of evidence suggested that these strands could include myosin molecules. First, other investigators have shown that myosin is present in the terminal web (Mooseker et al. 1978. *J. Cell Biol.* 79: 444-453), yet we could find no thick filaments in this area. Second, we found that the strands were removed completely in the process of decorating the core filament bundles with the myosin subfragment S₁, suggesting that they had been competitively displaced by exogenous myosin. We conclude that myosin may play a structural role in these cells, via its cross-linking distribution, in addition to whatever role it plays in microvillar motility.

In an earlier study, we used the quick-freeze, deep-etch, rotary-replication technique to visualize the cytoskeletons of detergent-extracted fibroblasts (10). This study illustrated the feasibility of using such a preparative procedure to determine the overall organization of cytoskeletal filament arrays, and to identify the filaments that comprise them. However, this study did not contribute toward understanding how cytoskeletal filaments could be linked together to form such arrays.

In the present report, we use this same technique to visualize the cytoskeleton inside differentiated epithelial cells of the vertebrate intestine. These cells are particularly interesting because they possess apical microvilli that have long been known to be supported by a distinct cytoplasmic specialization called the "terminal web" (5, 12, 20, 22). A large number of biochemical and immunocytochemical studies have focused on this region of the intestinal cell. These studies have defined the proteins that are present in the terminal web, but they have

not been able to demonstrate exactly how these proteins are put together (2, 3, 4, 6-9, 18). The micrographs presented here display certain advantages over traditional transmission electron micrographs of plastic-embedded and sectioned material, and thus help to reveal how the terminal web is organized.

MATERIALS AND METHODS

Male adult mice were sacrificed by decapitation and their small intestines were removed and cut into 2-cm lengths. These tubular segments were cleaned with calcium-free saline (155 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 0.5 mM NaH₂PO₄, 5 mM HEPES pH 7.0, and 2 mM EGTA) before further processing. Thereafter, two approaches were used to permeabilize the epithelial cells in these samples.

Permeabilizing Cells with Saponin

One approach was to incubate the samples in 0.1-0.2% Saponin (21) in a solution we termed artificial cytosol (100 mM KCl, 30 mM HEPES pH 7.0, 5 mM MgCl₂, and 2 mM EGTA). The incubation time was usually 30 min at 22°C,

after which the samples were fixed with 1% formaldehyde in the same artificial cytosol for 1.5 h.

Fragmenting Cells Mechanically

The other approach to permeabilizing cells was to disrupt them mechanically. To accomplish this, the tubular samples were split open longitudinally, placed in the artificial cytosol, and immediately scraped to obtain sheets of epithelial cells. These sheets were further dissociated by passing them back and forth through a 27-gauge needle a number of times, depending on how much comminution was desired. The resulting suspension of isolated cells and cell fragments was centrifuged at 1,000 *g* for 3 min, washed three times in fresh artificial cytosol, and then fixed for 1.5 h with 1% formaldehyde or a mixture of 1% formaldehyde and 1% glutaldehyde, in the same artificial cytosol. All procedures were done at 4°C.

For subfragment 1 (*S*₁) decoration, the fragmented epithelial cells were incubated for 2 h at 4°C in 3–4 mg/ml of myosin *S*₁ kindly provided by Dr. Roger Cooke, University of California in San Francisco. The *S*₁ was dissolved in the same artificial cytosol. After incubation, the cells were washed and fixed as described above.

Quick-Freezing, Freeze-Fracture, and Deep-Etching

Samples were placed on the surface of aldehyde-fixed slices of lung, which acted as spongy supports, and were then washed for ~15 s with distilled water containing 15% methanol (a volatile cryoprotectant). Then they were frozen by pressing them rapidly against a copper block cooled to 4°K with liquid helium, as described previously (11).

For freeze-fracture, the samples were transferred to Balzers 301 or 400 freeze-fractured devices (Balzers, Hudson, N.H.). The surfaces of the samples were barely cut at –196°C and then the samples were warmed to –95°C for 7 min of etching. Replicas were then made by rotary shadowing with a mixture of platinum and carbon, after the sample had been recooled to –105°C. The replicas were cleaned in Purex (Purex Corp. Lakewood, CA) household bleach for several

hours, and then picked up on formvar-carbon-coated 75-mesh grids. They were examined with JEOL 100B or 100CX electron microscopes operated at 100 kV, and photographed in stereo at ± 7° tilt.

RESULTS

Deep etching of intestinal cells that were quick frozen while they were still alive and whole exposed very little cytoplasmic structure, because their interior appeared to be filled with granular material (see Fig. 1). Cytoplasmic filaments could barely be discerned, and hence nothing could be learned about the terminal web in the apex of these cells. To visualize this structure, the granular material had to be removed.

This material turned out not to be just salts, because when cells were fixed in aldehydes and washed in distilled water for extended periods before freezing, their cytoplasm looked just as granular (Fig. 1). It could only be removed by permeabilizing the cells with detergents or by breaking them open physically before freezing (Fig. 2). Thus, we presume that the granular material must have represented cytoplasmic proteins.

The cytoskeleton appeared “cleaner” in cells that were broken open physically, as in Fig. 2, compared to those that were extracted with detergents (data not shown). Yet, surprisingly, physical rupture did not distort the overall organization of the cytoskeleton any more than did detergents. (Both approaches did result in some disruption of the detailed arrangement of the filaments involved. This is illustrated in Figs. 3, 4, and 5, which show the organization of actin filaments in individual microvilli before treatment (Fig. 3) versus after detergent ex-

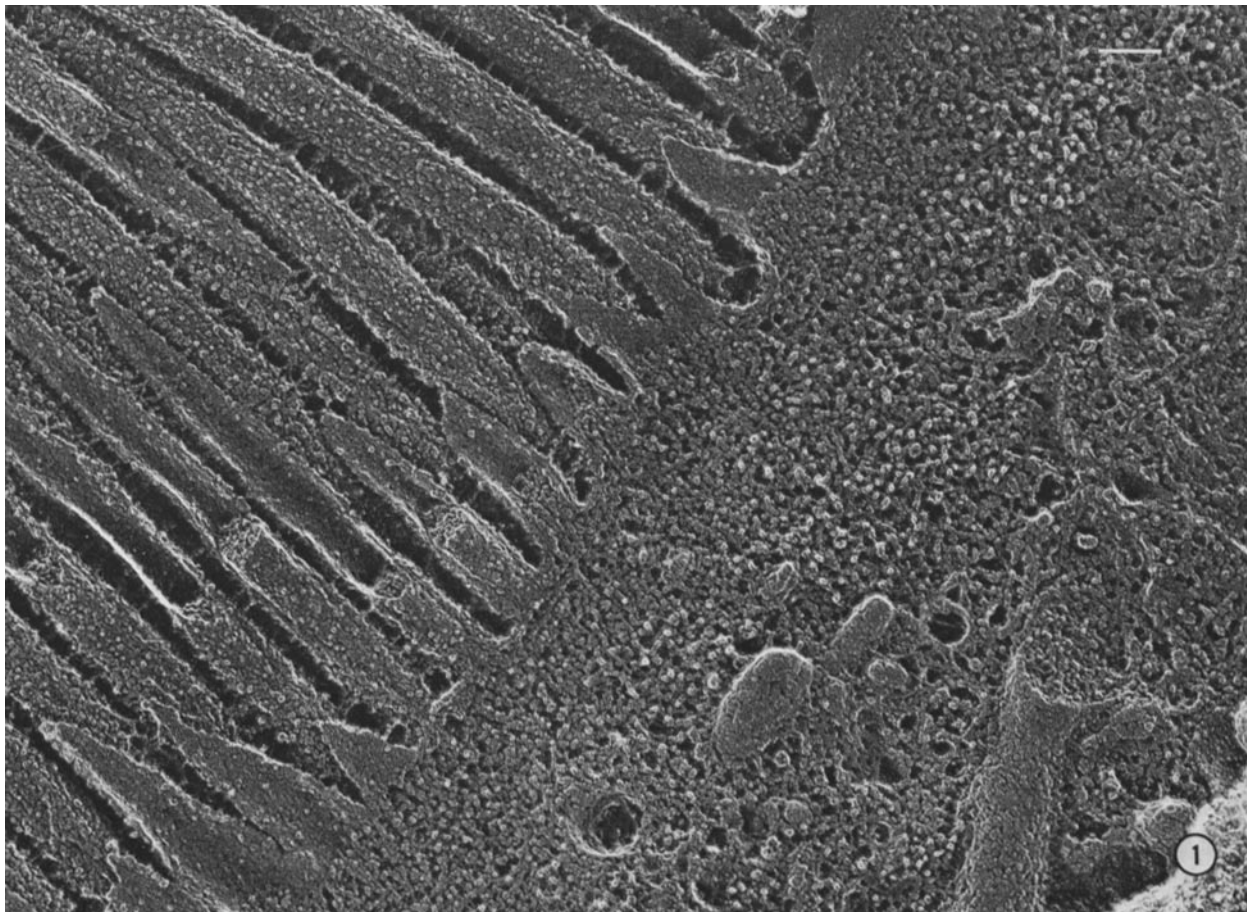


FIGURE 1 The cytoplasm in the apex of an intestinal epithelial cell fixed with aldehydes and frozen while whole is so filled with granular material that it is impossible to discern any cytoskeletal filaments. Bar, 0.1 μ m.

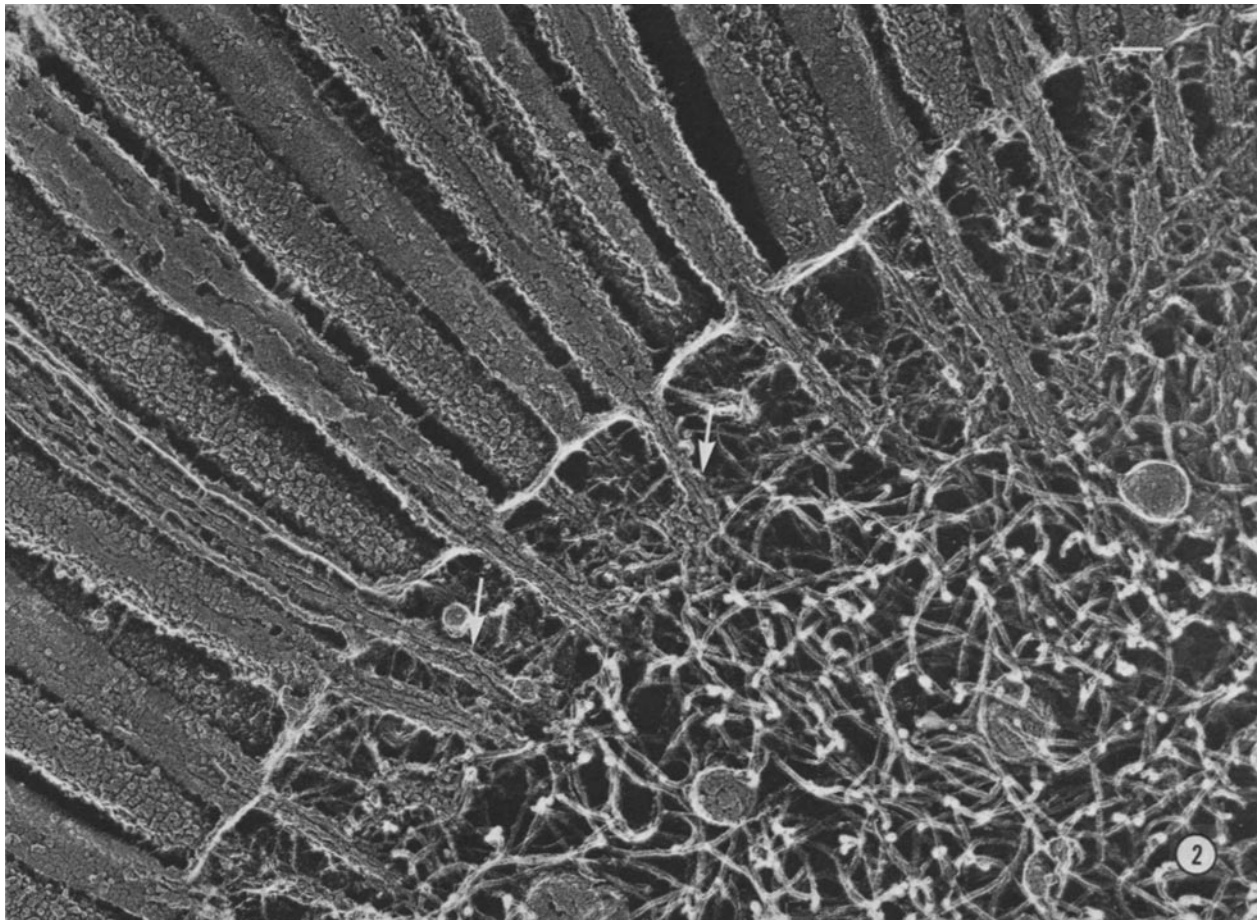


FIGURE 2 The granular material is extracted from cells that are sheared open before freezing. This reveals a filamentous cytoskeleton composed of a bed of intermediate filaments (lower right) upon which rest the bundles of microfilaments (arrows) that form the cores within the microvilli (upper left). Bar, 0.1 μm .

traction (Fig. 4) or after physical rupture (Fig. 5). After either treatment, the actin filament bundles were partly disorganized and the delicate "lateral arms" that normally connect these bundles to the plasma membrane (arrowheads in Fig. 3) were reduced in number. This level of disorganization had to be kept in mind when interpreting images of cytoskeletons in cell extracts.)

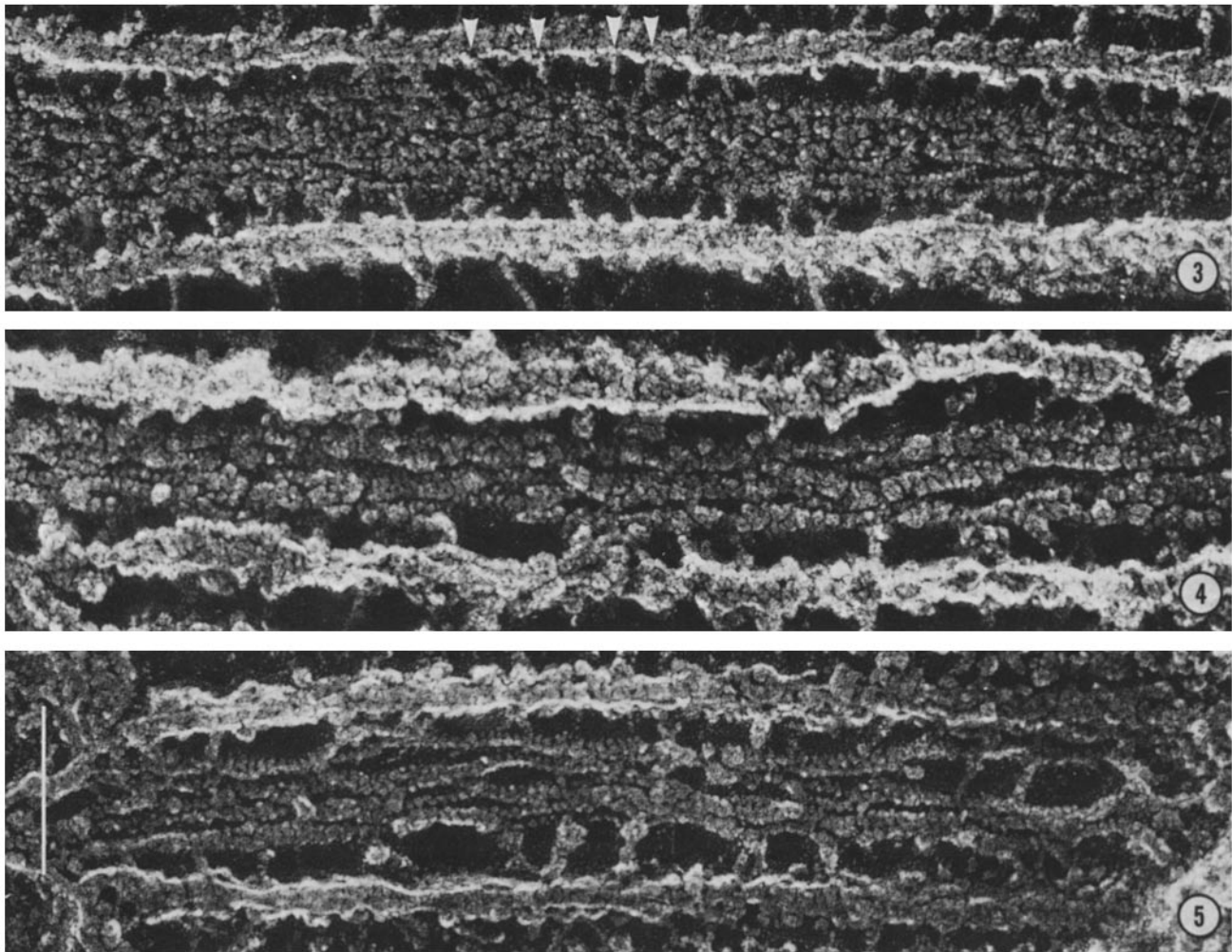
In any case, the cytoskeletons in extracted intestinal cells displayed a remarkable degree of order. Invariably, the cells possessed a rich tangle of intermediate filaments in the area just beneath the terminal web. These filaments could be identified as intermediate filaments by three criteria: (a) their thickness, which was greater than actin filaments by 20–30 \AA , (b) their smoothness and uniform caliber, which differed from the 53 \AA stripes seen on replicas of actin filaments (see reference 10), and (c) their failure to decorate with S_1 . These intermediate filaments were woven into a complex but loose fabric that filled the apices of these cells and contained in its interstices many membranous organelles (Fig. 6). Occasionally, this fabric displayed distinct granular plaques toward which the intermediate filaments appeared to converge and attach (Fig. 7). We suspect that these were desosomal plaques torn off the lateral edges of the cells during mechanical disruption. The bundles of actin filaments that formed the cores of microvilli reached down to this apical tangle of intermediate filaments but did not appear to penetrate very far into it (Fig. 2).

Higher magnifications of the apices of optimally fractured

cells illustrated the organization of the terminal web most clearly (Fig. 8). In such images, the bundles of actin filaments that formed the cores of microvilli lay meshed in a complex tangle of wisps that were variable in thickness and generally too thin to be actin themselves, and that appeared to branch and anastomose more than do the filaments found in the concentrated gels of actin such as are present in the lamellipodia or ruffles of other motile cells (10). The intermediate filaments that snake up from the cytoplasm beneath the terminal web appeared in regions to become entangled with these more delicate wisps.

Exposing broken cells to 4 mg/ml S_1 before freezing confirmed that the filaments in the cores of microvilli were actin (1, 13, 19), because they became thickened and helical after decoration (Figs. 9 and 10). (Elsewhere we have shown that S_1 -decorated actin does not look like a string of arrowheads in deep-etch replicas, the way it does in negative staining, but instead looks like a double helical rope [10]). Another interesting consequence of S_1 decoration, which could be appreciated by comparing images before and after decoration (Figs. 9 and 10), was that it caused the filament bundles to splay apart and also removed most of the interconnecting wisps. Presumably, this indicated that S_1 could displace the proteins that are normally involved in cross-linking the filaments to one another.

Incidental observations made during the course of this study included views of the true inside and outside surfaces of the microvillar membranes. These two surfaces appeared strikingly



FIGURES 3, 4, and 5 High-magnification views of the core bundles of filaments in individual microvilli, comparing their appearance in unextracted cells (Fig. 3) with saponin extraction (Fig. 4) and mechanical disruption (Fig. 5). In the intact villus, filaments are close-packed and attached to the plasma membrane by regularly repeating lateral arms (arrowheads). After either detergent treatment or physical rupture, the bundles are partly splayed apart and some lateral arms are missing. Bar, 0.1 μm .

different. The inside displayed a distinct geometrical pattern that consisted of small "washboards" with stripes spaced 4 nm apart. These washboards formed oriented plaques that intersected with one another at acute angles and almost completely covered the apical surface of the cell. Also, they appeared to extend part way out into the microvilli (Fig. 11, insert). In contrast, the outside surface of the microvillar membrane displayed a different texture. It was covered by a pavement of closely spaced 100–120 Å protrusions (Figs. 12 and 13). These protrusions were much larger and more numerous than the intramembrane particles that have been observed on microvillar membranes in previous freeze-fracture studies (20), and differed from intramembrane particles in their distribution, as

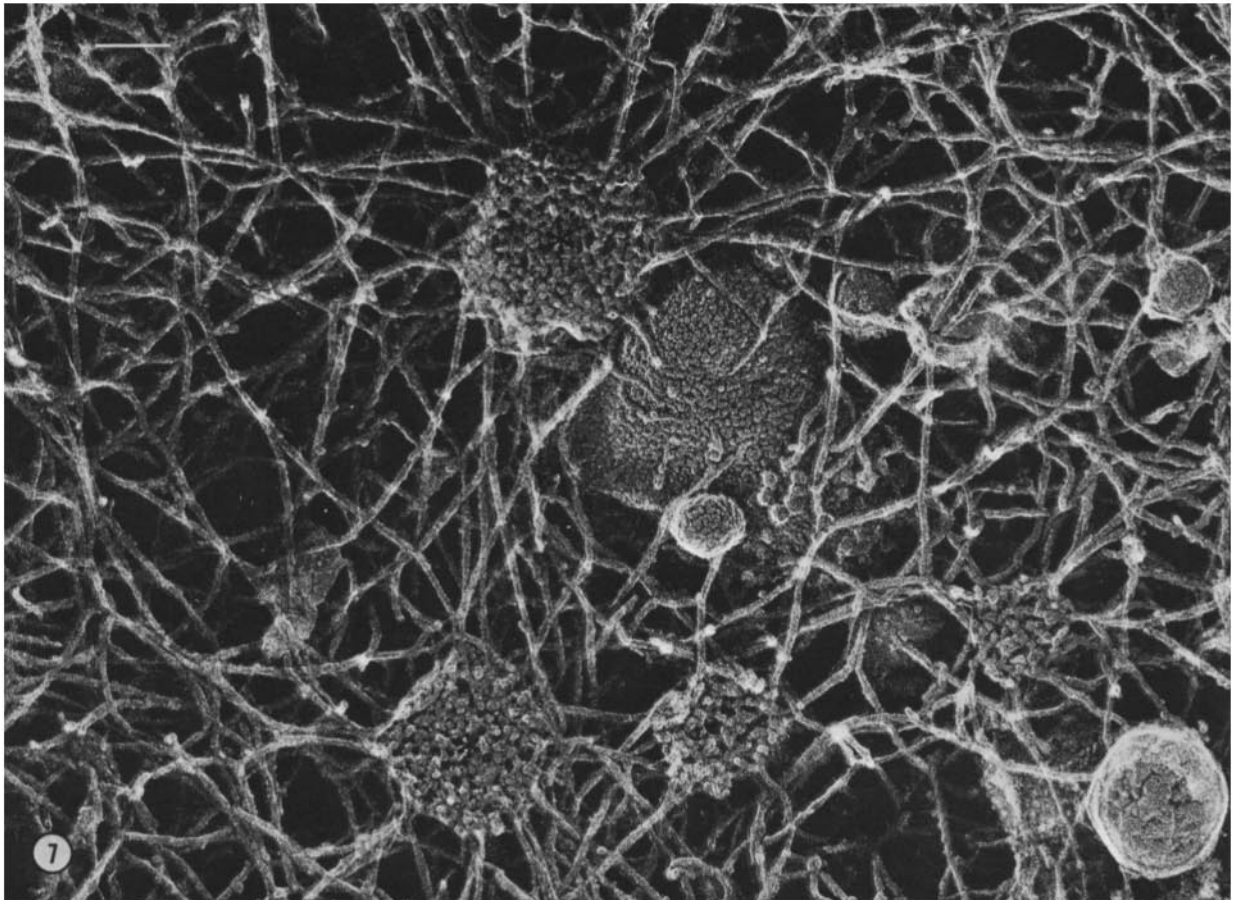
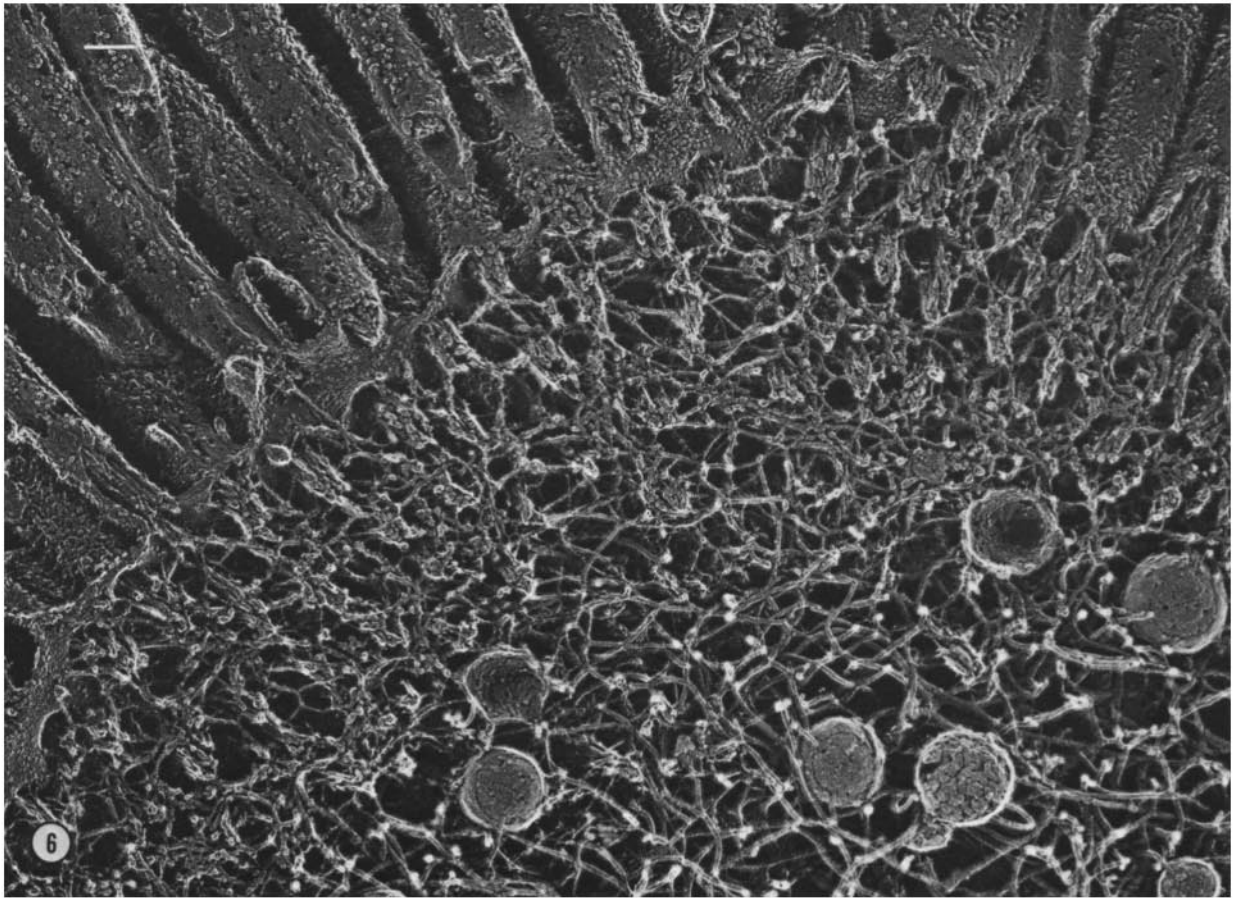
well, because they covered even the very tips of the microvilli where freeze-fracture particles are almost entirely absent (cf. Fig. 12).

The extracellular glycocalyx that coats intestinal cells could also be seen clearly after deep etching (Figs. 12 and 13). Its appearance in replicas fully confirmed earlier thin-section analyses of this mucopolysaccharide layer (14, 28), which described it as a mass of anastomotic strands. Indeed, it looked strikingly similar to deep-etch views of certain man-made polysaccharide gels such as Sephadex (data not shown).

Also apparent in deep-etch replicas was another sort of extracellular material that extended between adjacent cells at attachment zones and plaques. This material consisted of nar-

FIGURE 6 *En face* view of the terminal web of a broken open intestinal epithelial cell, from a vantage point inside the cell. At the very top and left-hand side of the field is a view of the inside of the apical plasma membrane, recognizable by the cross-fractured microvillar core bundles and their interconnecting filaments. Most of the field displays the extensive network of intermediate filaments that fills the apex of these cells. Bar, 0.1 μm .

FIGURE 7 Higher-magnification view of the complex fabric of intermediate filaments that is found in these cells. The granular plaques toward which these filaments converge (asterisks) are thought to be displaced desmosomal plaques that detached from the plasma membrane during cell rupture. Bar, 0.1 μm .



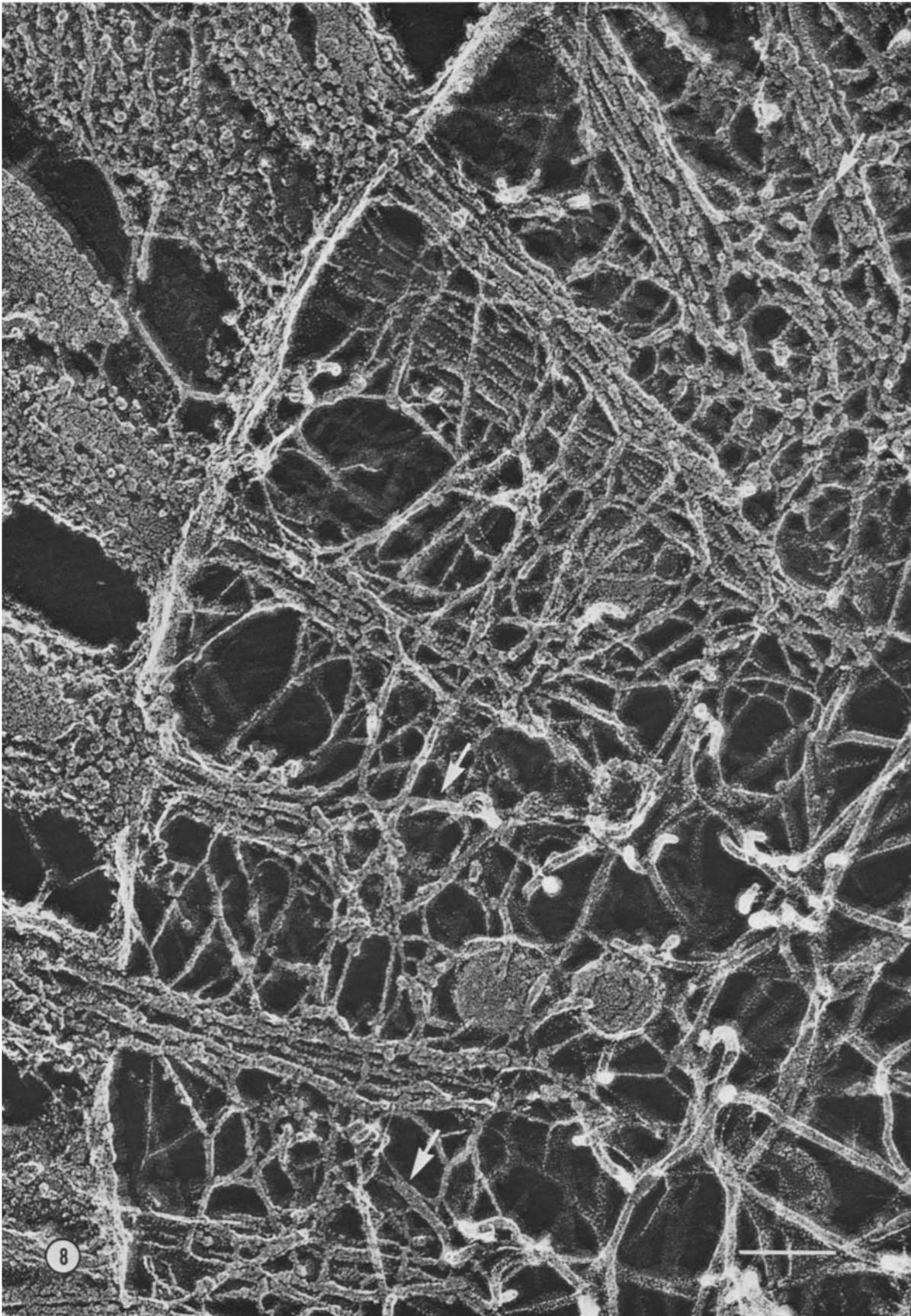
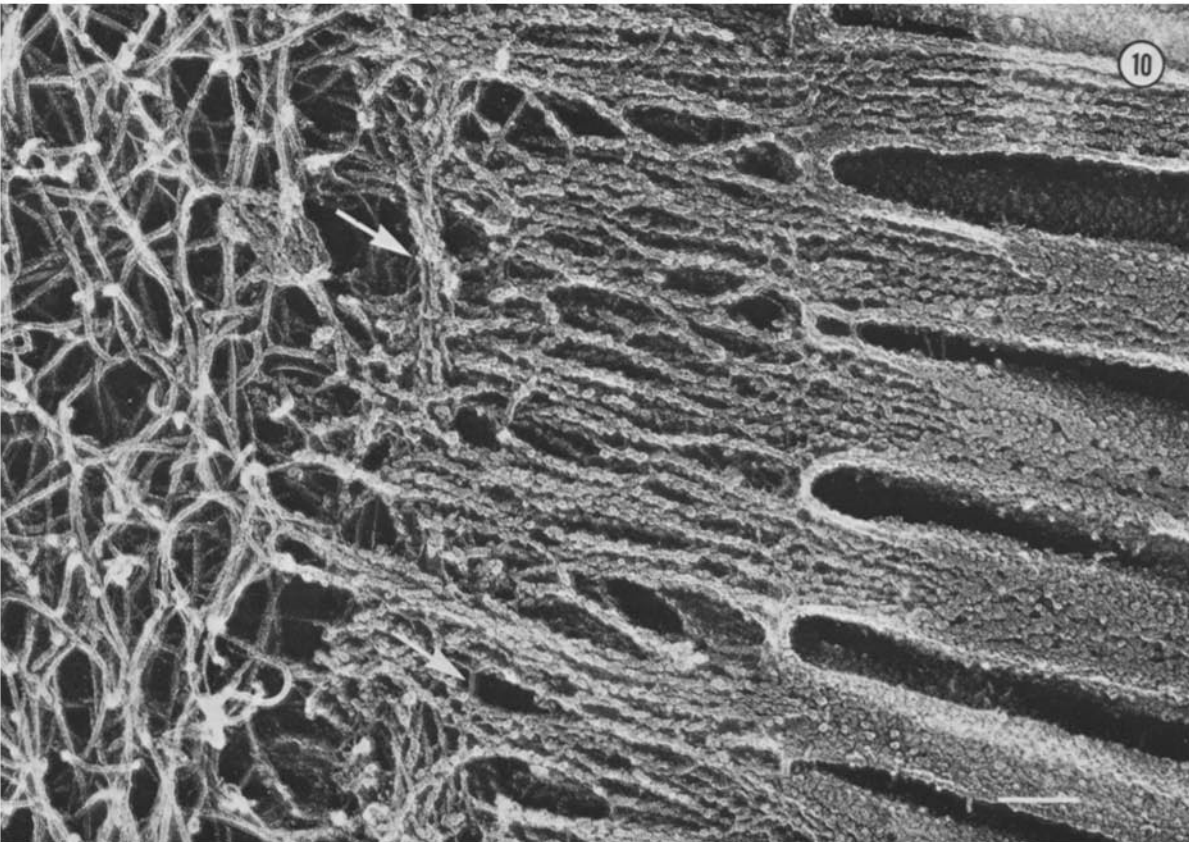
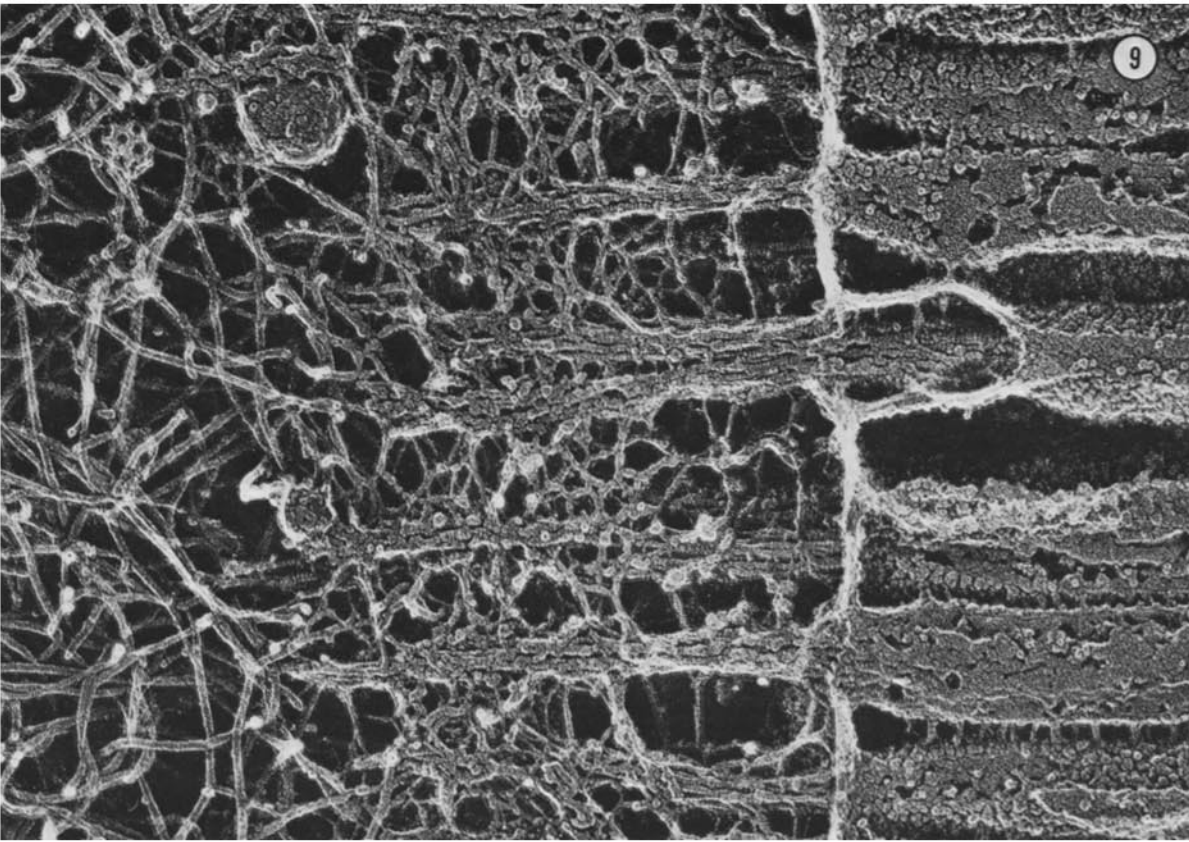


FIGURE 8 Our best image of the terminal web of a broken-open intestinal cell, to show (1) the internal extensions of the bundles of filaments that form microvillar cores, (2) the underlying foundation of intermediate filaments, which are clearly thicker and smoother than the actin filaments in the bundles, and (3) the anastomotic network of thin wisps that are found in between the above elements. These wisps appear to be neither actin nor intermediate filaments and may be the critical structural links in the terminal web. Intermediate filaments sometimes loop up and tangle with core bundles (arrows). Bar, 0.1 μm .



FIGURES 9 and 10 Comparison between the terminal webs of sheared-open intestinal cells before (Fig. 9) and after (Fig. 10) exposure to 4 mg/ml S_1 . After S_1 decoration, the core bundles of actin filaments are splayed apart and the wisps that normally crosslink adjacent bundles either are completely removed or at least are displaced toward the basal ends of the bundles (arrows). In addition, intermediate filaments curving up and associating with rootlets are displaced. Note that the intermediate filaments (on the left) remain undecorated. Bar, 0.1 μm .

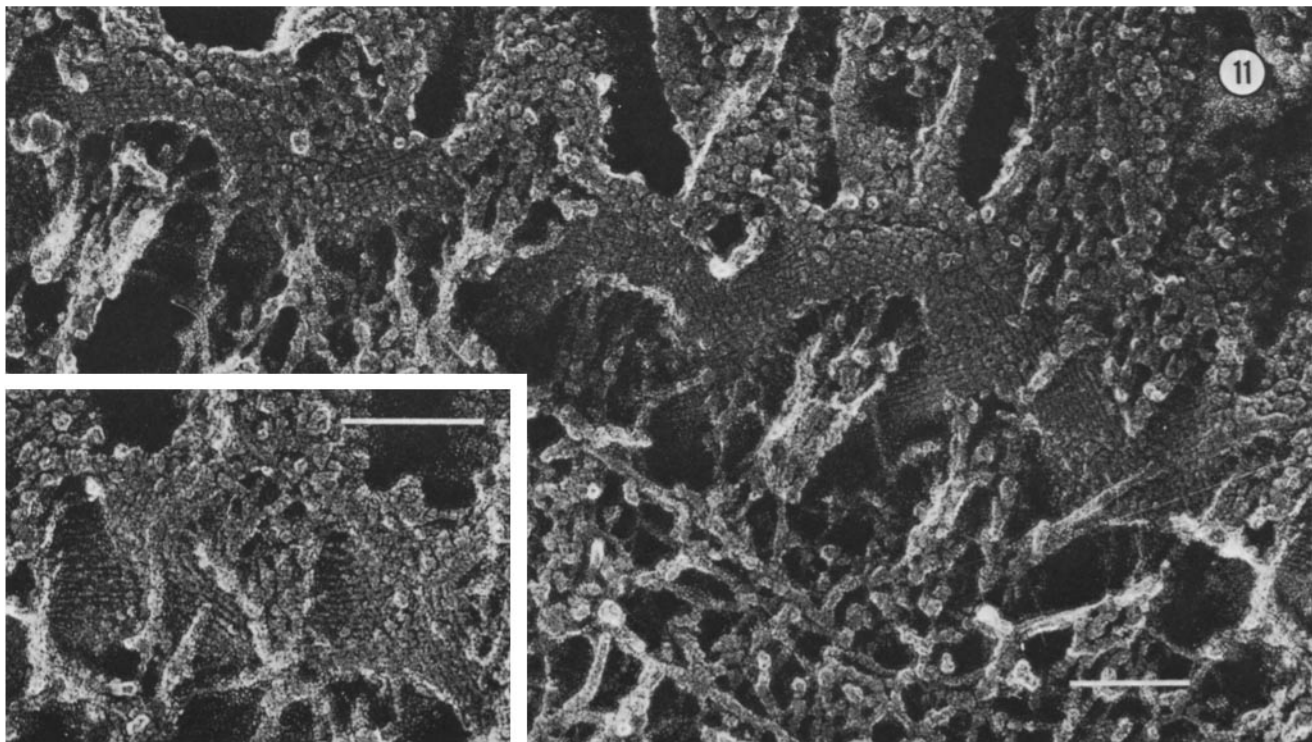


FIGURE 11 A view of the true inside surface of the apical plasma membrane, to illustrate that it displays a distinct geometrical pattern of regular stripes. The inset shows at higher magnification the regularity of these 4-nm stripes and their arrangement into small parallel arrays. Bar, 0.1 μm .

row strands that bridged the two opposed membranes. The strands were usually more concentrated at attachment plaques (desmosomes) than at attachment zones but otherwise looked the same at both sorts of contact (Figs. 14 and 15). Curiously, the desmosomal bridges (Fig. 15) did not display the central band of density that is one feature that has been used to distinguish this type of junction in thin-sectioned material.

DISCUSSION

It was initially disappointing to realize that whole cells were so filled with nonvolatile material that it would be impossible to visualize their cytoskeletons after deep-etching. This nonvolatile material looked granular in platinum replicas and seemed to fill every nook and cranny inside the cell. It was not just salt, because it was still present in cells that had been fixed with aldehydes and washed in distilled water for prolonged periods before quick freezing. It could only be removed by rupturing the cell membrane, either by detergents or by physical means, in the moments before freezing. Either of these approaches eliminated the granular material and left behind a distinct cytoskeleton composed of actin, intermediate filaments, and various cross-linking elements (as well as microtubules when suitable stabilizers like Taxol [25] were used).

An obvious implication of these results is that the cytoplasm of living cells may be composed of proteins in two different states: soluble and insoluble. The soluble ones appear granular and uniformly distributed in deep-etch replicas, whereas the insoluble ones are those that form the cytoskeleton.

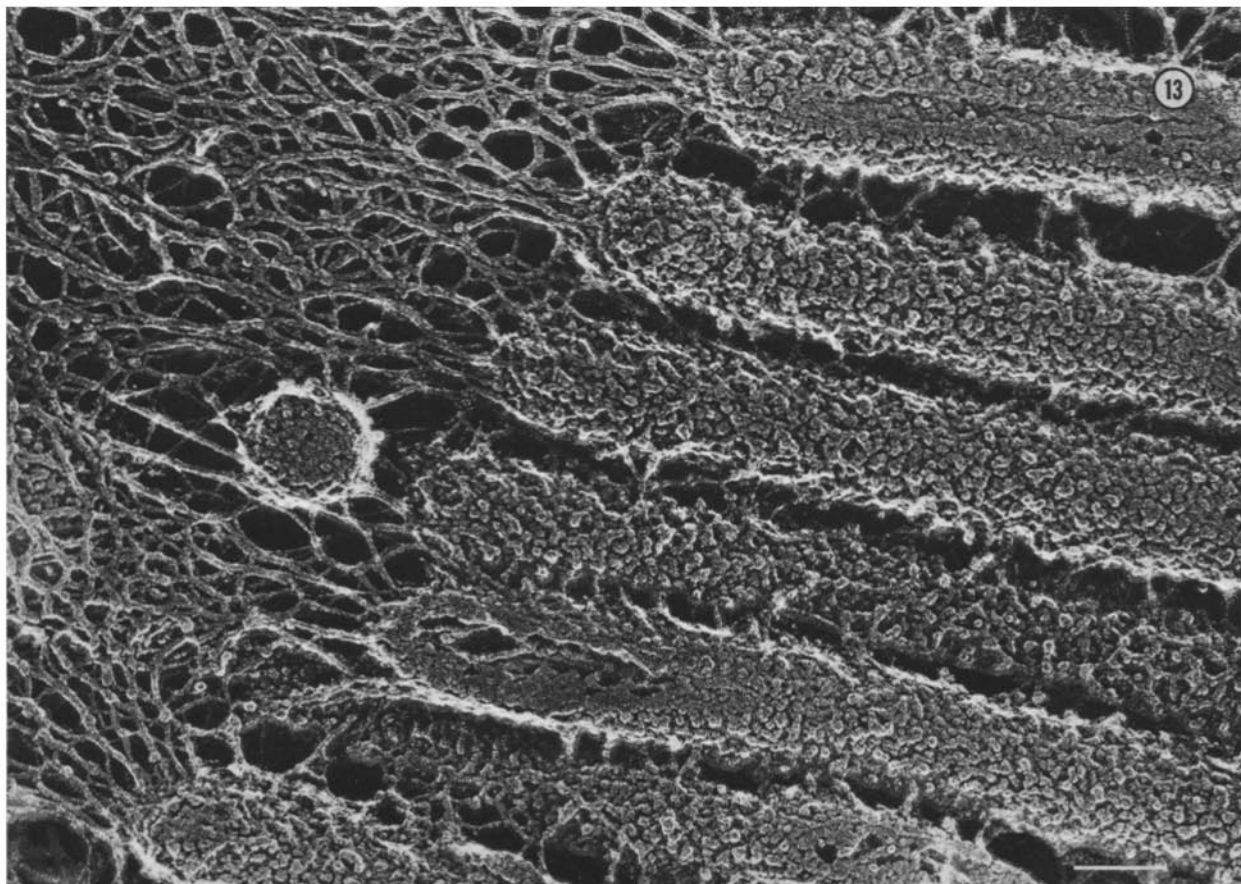
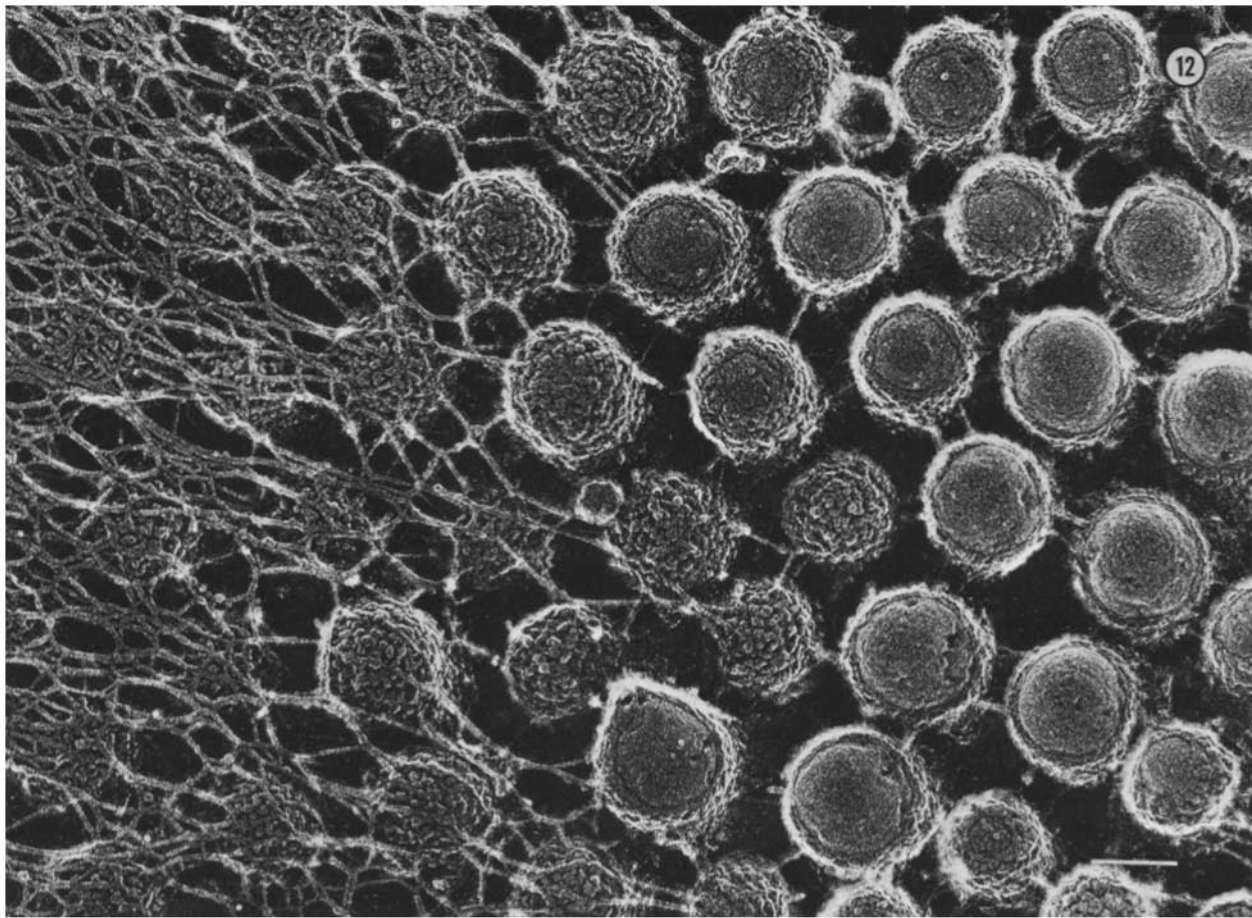
The Cytoskeleton of Intestinal Cells

The cytoplasmic differentiation found at the apex of intestinal cells, beneath their microvilli, has been called the terminal

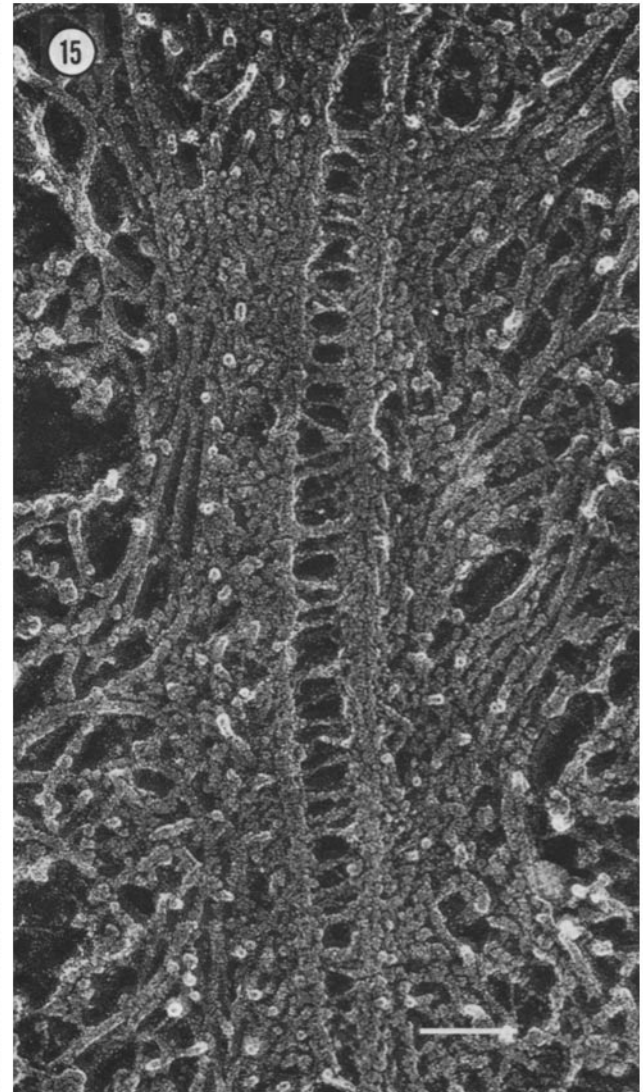
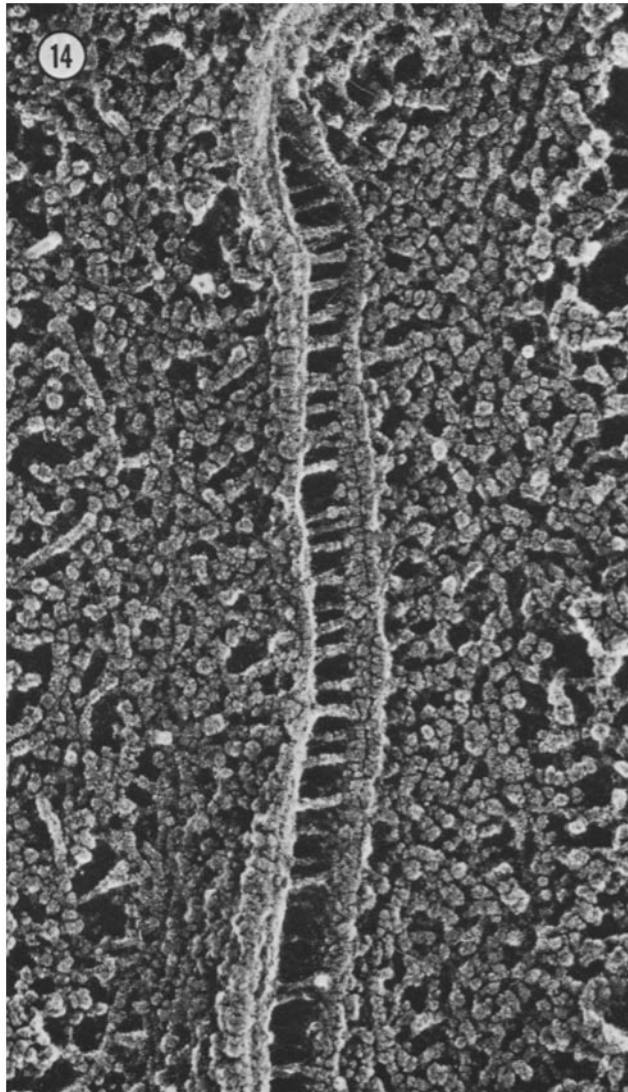
web ever since early electron microscopy (EM) revealed that it was composed of a delicate network of interwoven filaments (22). This network is apparently mechanically strong and stable, because it holds together when the intestinal cell is broken open. In addition, it may generate a type of cellular movement, if it is indeed true that microvilli are capable of moving during life (17, 19, 23, 24, 27).

Light and EM immunocytochemical studies to date (2, 7, 9, 18) have shown that the apices of intestinal cells contain the essential contractile proteins, including actin, myosin, tropomyosin, and α -actinin. EM has not revealed how all these proteins could fit together to provide rigid support and flexible contractility in an appropriate manner. To date, EM studies have shown that the core bundles of actin filaments in the microvilli extend down into the terminal web of the apex of the cell, and that intermediate filaments swoop up into the vicinity of this terminal web (5, 12, 20); but these studies have disagreed about whether the terminal web is composed of actin filaments that splay out from the core bundles (12, 16) or a separate set of actin filaments that run horizontally (12, 23), or whether it is mainly composed of a meshwork of distinctly thinner elements (19).

In the present study, deep-etch replicas have provided surface view of many of the components of the terminal web, views that are relatively easy to interpret. The terminal web turns out to be composed mostly of narrow wisps that interconnect adjacent actin bundles but which are thinner than actin and do not decorate with S_1 . In fact, S_1 treatment appears to remove these wisps or displace them to the bases of the core filament bundles, where they come to rest upon a platform of tangled intermediate filaments. The replicas make it clear that actin filaments rarely splay out from the core bundles and rarely bend far enough to run horizontal to the cell surface.



FIGURES 12 and 13 Views of intestinal microvilli exposed by deep etching, from vantage points either straight above (Fig. 12) or from the side (Fig. 13). The true external surface of each microvillus is covered with 10- to 12-nm bumps, except on the right of Fig. 12 where the fracture plane broke this surface away and exposed a smooth fracture face beneath. On the far left of both fields can be seen the complex glycocalyx that attaches to the apices of the microvilli. It is composed of a dense meshwork of anastomotic strands. Bar, 0.1 μm .



FIGURES 14 and 15 Deep-etch images of the bridging structures that can be seen to extend between adjacent cells at the attachment zone (Fig. 14) and the attachment plaque (Fig. 15). The cells in Fig. 14 were frozen while whole, so their cytoplasm appears granular, whereas the cells in Fig. 15 were extracted with Saponin before freezing, so cytoplasmic filaments can be seen running up to the inner surfaces of the interlinked membranes. Bar, 0.1 μ m.

Instead, the only place where a significant number of actin filaments are seen to course horizontal to the cell apex is in the girdle that circles the cell just inside the zonula adherens.

The chemical composition of the cross-linking wisps in the terminal web is not known. Efforts are underway to determine this, by using the viewing technique described here to analyze the results of experiments that are designed to produce selective extraction and selective decoration of different protein species. This viewing technique has already allowed us to resolve analogous cross-linkers, such as spectrin molecules interconnecting membrane proteins in erythrocytes (26), and HMM (heavy meromyosin) molecules interconnecting actin filaments in solution (Heuser and Cooke, manuscript submitted for publication.). It has also proven to have high enough resolution to discern antibody labeling directly (10), so it can be used to evaluate attempts to decorate such wisps with likely antibodies. Finally, this new technique also permits direct visualization of the effects of treatments that could extract such cross-linkers, treatments such as low ionic strength that removes α -actinin, detergents that remove the 95,000 and 68,000 mol wt actin

bundling proteins from microvillar cores (15), or magnesium ATP which removes the 110 K "lateral arm" protein from microvilli (15), and removes myosin from certain other cytoskeletal preparations (29). By analogy with what we have seen when HMM cross-links purified actin filaments (Heuser and Cooke, op cit), our working hypothesis is that many of the cross-linkers will turn out to be monomers or small oligomers of myosin.

We wish to thank Roger Cooke, University of California, San Francisco, for his kind gift of purified S_1 .

This investigation was supported in part by a U.S. Public Health Service International Research Fellowship and the George Meany M.D.A. Postdoctoral Fellowship to N. Hirokawa, and by grants to J. E. Heuser from the U. S. Public Health Service and the Muscular Dystrophy Association of America.

Received for publication 10 December 1980, and in revised form 9 July 1981.

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